The Irr and RirA proteins participate in a complex regulatory circuit and act in concert to modulate bacterioferritin expression in *Ensifer meliloti* 1021

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Running Head: Bfr function and regulation in *E. meliloti*

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Abstract

In this work we found that the *bfr* gene of the rhizobial species *Ensifer meliloti*, encoding a bacterioferritin iron storage protein, is involved in iron homeostasis and oxidative stress response. This gene is located downstream and overlapping the *smc03787* ORF. No well predicted RirA or Irr boxes were found in the region immediately upstream the *bfr* gene although two presumptive RirA boxes and one presumptive Irr box were present in the putative promoter of *smc03787*. We demonstrate that *bfr* gene expression is enhanced under iron sufficient condition and that Irr and RirA modulate this expression. The pattern of *bfr* gene expression as well as the response to Irr and RirA, are inversely correlated to that of *smc03787*. Moreover, our results suggest that the small RNA SmelC759 participates in RirA- and Irr-mediated regulation of *bfr*-expression, and that additional unknown factors are involved in iron-dependent regulation

Importance

E. meliloti belongs to the Alphaproteobacteria, a group of bacteria that includes several relevant species able to associate with eukaryotic hosts, from mammals to plants, in a symbiotic or pathogenic manner. Regulation of iron homeostasis in this group of bacteria differs from that found in the well-studied Gammaproteobacteria. In this work we analyzed the effect of *rirA* and *irr* mutations in *bfr* gene expression. We demonstrate the effect of an *irr* mutation in iron homeostasis in this bacterial genus. Moreover, results obtained indicate a complex regulatory circuit where multiple regulators, including RirA, Irr, the small RNA SmelC759 and still unknown factors, act in concert to balance *bfr* gene expression.

40 **Introduction**

41 *Ensifer meliloti* (formerly *Sinorhizobium meliloti*) 1021 is an Alphaproteobacterium able to
42 establish a symbiotic association with the leguminous plant *Medicago sativa* and to fix
43 nitrogen as bacteroids within root nodules. Iron is a pivotal component in the symbiotic
44 nitrogen fixation process as it forms part of the catalytic sites of the nitrogenase and the
45 plant synthetized leghemoglobin (1). Additionally, iron is an essential nutrient and
46 appropriate levels should be tuned in cells in order to sustain cell life avoiding iron toxicity.
47 To maintain iron homeostasis, bacteria have evolved a plethora of high-affinity iron
48 acquisition, utilization, export and storage systems. Iron storage proteins are ubiquitous
49 factors able to sequester intracellular ferrous ions and store them in a non-reactive form.
50 This allows cells to be protected from iron-induced formation of reactive oxygen species,
51 and provide a nutritional iron source in case of starvation (2, 3).
52 Three types of iron storage proteins have been identified in bacteria: a) the non-heme
53 classical ferritin, composed of 24 subunits; b) the DNA binding protein from starved cells
54 (Dps) and Dps-like proteins, which are present only in prokaryotes and are composed of 12
55 subunits; and c) the heme-containing bacterioferritin, consisting of 24 subunits and 12 Fe-
56 protoporphyrin IX groups (4-6). Iron homeostasis is mainly achieved by strict regulation of
57 the dedicated systems.
58 Expression of iron storage proteins has been found to be positively regulated by iron in
59 *Escherichia coli* (7, 8), *Pseudomonas aeruginosa* (9) and *Bradyrhizobium japonicum* (10),
60 but not in the cyanobacterium *Synechosystis* (11, 12). In *E. coli* and *Pseudomonas*, Fur
61 (*F*erric *u*ptake *r*egulator) is the major iron-response regulator, and is involved in the
62 regulation of iron-storage proteins expression (8, 9, 13). In some Alphaproteobacteria the
63 Fur homologue has been described mainly as a manganese responsive regulator and has

been renamed as Mur (14-16). In this bacterial group, iron homeostasis is handled mainly by RirA and/or Irr regulatory proteins (1). RirA, standing for *R*hizobial *i*ron *r*egulator, is an Fe-S protein that belongs to the Rrf2 family of putative regulators. It has been identified in *Rhizobium leguminosarum* but homologues have also been found in other bacterial genera belonging to the Rhizobiales order such as *Ensifer*, *Mesorhizobium*, *Agrobacterium*, *Brucella* and *Bartonella* (17). The mechanism of action of RirA has not been entirely elucidated, although an iron-responsive operator (IRO) motif (TGA-N9-TCA) has been described as a putative DNA-binding site for RirA (18, 19). In Bradyrhizobia and some Alphaproteobacteria, Irr has been identified as the major regulatory protein responsible for iron homeostasis. The Irr iron responsive regulator belongs to the Fur family of proteins; however, Irr and Fur mechanisms of action are different. While Fur directly perceives intracellular Fe^{+2} levels, in the *Bradyrhizobium* genus Irr senses iron through heme biosynthetic levels, by means of a heme regulatory motif (17). Despite an *irr* homolog gene and putative Irr boxes have been identified in *E. meliloti* genome, the role of Irr has not yet been determined in this bacterium.

In this study, we analyzed the role of Bfr in iron homeostasis and oxidative stress response in *E. meliloti* 1021, and we provide evidences that Bfr affects rhizobia infectivity of alfalfa plants. To gain insight into the regulation of *bfr* gene expression, we studied its transcriptional pattern under iron limiting and sufficient conditions. Moreover we analyzed the role that RirA, Irr and the small RNA SmelC759 have on *bfr* expression. Results obtained lead us to propose a hypothetical model of *bfr* regulation in *E. meliloti* that is further discussed in this paper.

88 **Results**

89 ***E. meliloti* bacterioferritin participates in iron homeostasis.** In order to determine the
90 role of bacterioferritin in *E. meliloti* 1021, total iron content was assessed by atomic
91 absorption spectroscopy in the parental and the *bfr* mutant strains under iron sufficient
92 condition. Iron content in the parental strain was 4.82 ± 0.06 µg Fe/mg of total cell
93 proteins, while in the *bfr* mutant strain the content was 4.09 ± 0.05 µg of Fe/mg total cell
94 proteins. Considering that according to our results 1 ml of a *E. meliloti* culture with an
95 OD_{620} =1 contains about 10^9 cells, then *E. meliloti* 1021 contains about $0.9x10^7$ iron atoms
96 per cell. Provided that a Bfr mutation results in a 15% decrease of total iron cell content,
97 such difference represents a reduction of ca. $1.3x10^6$ iron atoms per cell. When cells were
98 grown in medium supplemented with 100 µM EDDHA, cellular iron content was reduced
99 more than ten times but no significant differences were found between the parental and the
100 mutant strains (data not shown).
101 To determine if iron-loaded bacterioferritin could be used as a nutritional iron source under
102 iron depleted conditions, cells of *E. meliloti* 1021 parental and *bfr* mutant strains grown
103 under iron sufficiency, were transferred to either iron-limited or iron-sufficient medium and
104 their growth was evaluated. As shown in Fig. 1 and Fig. S1the growth of the *bfr* mutant
105 was slightly impaired under iron sufficient conditions compared to that of the parental
106 strain but, under iron depleted conditions the *bfr* mutant growth was severely affected. To
107 assess if growth impairment was a consequence of a reduced rhizobactin 1021 production
108 we quantified siderophore accumulation in supernatants of the parental and the *bfr* mutant
109 strains grown under iron depleted conditions. No significant differences were detected
110 among rhizobactin 1021 production by both strains (data not shown).
111

112 **Bfr is not involved in protection against iron or hemin toxicity but affects oxidative**
113 **stress response against H_2O_2**. As previously mentioned, some iron storage proteins are not
114 only involved in providing iron as a nutritional source, but also they may participate in
115 protection against oxidative stress. In order to test the role of Bfr on the response to
116 iron/hemin toxicity, the growth of parental and *bfr* mutant strains was compared in media
117 containing 1 to 5 mM $FeCl_3$, 25 mM or 50 mM hemin. Colony sizes were significantly
118 reduced with 5 mM $FeCl_3$ or 50 mM hemin, but no significant differences were observed
119 between the parental and the *bfr* mutant strains, suggesting that Bfr is not relevant for
120 iron/hemin toxicity response (data not shown). We cannot rule out the possibility that
121 systems responsible of maintaining iron homeostasis are robust enough to prevent
122 intracellular iron concentration to reach lethal levels. When the bacterial response to
123 exogenous H_2O_2 was evaluated, the data obtained showed that the *bfr* mutant was more
124 tolerant to H_2O_2 than the parental strain (Fig. 2). Moreover, differences were more striking
125 under iron sufficient conditions.
126
127 **Symbiotic phenotype of the *bfr* mutant**. Alfalfa plants inoculated with the parental or the
128 *bfr* mutant strains developed effective nodules and no detectable differences in aerial plant
129 dry weights were obtained between plants inoculated with each strain (Fig. S2). However,
130 when the nodulation kinetics of alfalfa plants was compared, nodule formation was induced
131 earlier in plants inoculated with the *bfr* mutant strain (Fig. S3). These results indicate that
132 the absence of Bfr affects positively the early events of *E. meliloti* 1021 plant infection.
133
134 **The *bfr* gene expression is positively regulated by iron**. To assess iron responsiveness of
135 *bfr* gene expression, a chromosomally integrated *bfr::lacZ-accC1* transcriptional fusion was

136 evaluated in *E. meliloti* cells grown under iron-sufficient and iron-limited conditions. As
137 shown in Fig. 3, expression of the *bfr::lacZ-accC1* fusion was detected in both conditions
138 of iron availability; nevertheless, the activity was almost two-fold higher in cultures grown
139 in medium supplemented with 37 µM $FeCl_3$, indicating that *bfr* gene expression is
140 positively regulated by iron.
141
142 **Irr is involved in the regulation of *bfr* gene expression**.
143 In silico analysis indicates that the promoter region of Smc03787, the gene upstream of *bfr*,
144 contains an Irr box. Therefore, we tested whether Irr is involved in *bfr* expression. As
145 shown in Fig. 3, *bfr* expression was up-regulated in the *irr* defective mutant under iron
146 sufficient conditions, whereas the opposite was observed under iron depleted conditions,
147 pointing to a role of Irr in *bfr* gene regulation. Data obtained by qRT-PCR under iron
148 sufficient conditions are consistent with these results, as *bfr* transcript levels were
149 significantly higher in the *irr* mutant (Fig. 5-A). According to these results, it could be
150 hypothesized that Irr acts as a repressor of *bfr* gene under iron sufficient conditions, and as
151 an activator under iron depleted conditions. These findings provide experimental evidence
152 for a role of Irr in the modulation of *E. meliloti* gene expression. Irr seems not to be the
153 only factor involved in regulation as *bfr* gene expression still respond to iron in the *irr*
154 mutant harboring the *bfr::lacZ-accC1* construction (Fig. 3) reduced when iron was limiting.
155 **RirA is involved in regulation of *bfr* gene expression**. The RirA protein has been
156 described in *E. meliloti* as a repressor of iron regulated genes under iron sufficient
157 conditions (15, 20). To test if this is also the case for the *bfr* gene, we evaluated *bfr*
158 expression by qRT-PCR in a *rirA* knock-out mutant. Contrary to our expectations, the *bfr*

159 transcript level was slightly lower in the *rirA* mutant in comparison with the parental strain
160 (Fig 5A), suggesting that RirA activates *bfr* expression in either a direct or indirect manner.
161 These findings prompted us to determine whether the observed RirA activation is a
162 consequence of alleviating the repressive effect of Irr under iron sufficiency. If this was the
163 case, we would expect that in the absence of both proteins in the *irr/rirA* double mutant, *bfr*
164 gene expression should be similar to that of the *irr* mutant. As shown in Fig.5A, no
165 significant increase in *bfr* transcript levels could be detected in the *irr/rirA* double mutant
166 compared to the *irr* mutant, indicating that the observed RirA mediated activation of *bfr*
167 gene expression is not through the alleviation of Irr repression or, at least, that this is not
168 the only mechanism involved.
169
170 **Regulation of *smc03787* gene expression is inversely correlated to that of *bfr***. *In silico*
171 studies revealed a presumptive *bfr* promoter sequence upstream the *smc03787-bfr* gene
172 tandem, suggesting that *smc03787* and *bfr* could be part of an operon. Moreover, Rodionov
173 et al. (18) described putative RirA and Irr boxes in a region upstream of *smc03787*. More
174 recently, Schlüter et al. (21) identified the location of *smc03787* and *bfr* transcription start
175 sites by using RNAseq. To assess if the region immediately upstream of *bfr* could contain a
176 promoter, a 160 bp region (bfr; Fig. 4) was cloned into the pOT1 vector, upstream a
177 promoterless GFP reporter gene and expression was evaluated under low and high iron
178 conditions. However, GFP expression could not be detected, indicating that this region has
179 no promoter activity in the assayed conditions (data not shown).
180 Based on these results, a 230 bp region upstream of the *smc03786-bfr* gene cluster (P3787)
181 was cloned into the pOT1 vector, and GFP production was assessed. As expected,
182 negligible mean values of relative fluorescence were obtained for *E. meliloti* 1021 control

183 strain carrying the empty vector pOT1 (data not shown). As shown in Fig. 6A, *E. meliloti*
184 1021 (p3787) cells grown in iron sufficient medium showed a 5-fold reduction of promoter
185 activity when compared with cells grown under iron deficient conditions, indicating that
186 *smc03787* is repressed by iron. This response was opposite to that obtained for *bfr* gene
187 expression (Fig. 3 vs Fig. 6A). Moreover, when we examined the effect of single or
188 combined *irr* and *rirA* deletions by qRT-PCR, we observed that the general response of
189 *smc03787* expression was opposite to that obtained for *bfr* expression (Fig 5A vs Fig 5B).
190 A similar pattern was observed when we compared P3787 promoter activity data (Fig 6A
191 and Fig. S4) and *bfr* gene expression (Fig. 3) or *bfr* transcript levels (Fig 5A). These
192 findings strongly indicate that under iron sufficient conditions, Irr is not a repressor of
193 *smc03787* gene expression, while *rirA* deletion enhances transcript levels of this gene, as
194 expected for it classical role as an iron responsive repressor. Interestingly, when both
195 regulators were absent, *smc03787* gene expression was still observed but in a less extent
196 than in the *rirA* mutant indicating the Irr activity is not only through the alleviation of RirA
197 repression (Fig. 5B).
198 When *smc03787* gene expression was evaluated in the *irr/ rirA* double mutant by qRT-
199 PCR, values obtained were lower than expected according to the analysis of promoter
200 activity in the *E. meliloti* 1021 (p3787) strain (Fig. 6A), suggesting the existence of
201 additional regulatory factors, not properly reflected with the GFP-reporter fusion approach.
202
203 **The small RNA SmelC759 participates in the regulation of *smc03787* gene expression.**
204 Schlüter et al. (21) had previously reported the presence of a putative 224 bp cis-encoded
205 mRNA leader, named SmelC759, partially overlaping the *smc03787* gene (Fig.4). To
206 explore whether this non-coding RNA is linked to the expression of *smc03787* and *bfr*

genes, we evaluated the *smelC759*/*smc03787* transcript levels under iron sufficient conditions (i.e. when *bfr* gene expression is activated). With this purpose, specific primers able to amplify the -23 to +72 bp region relative to the *smc03787* ORF were designed (Fig. 4). As shown in Fig. 5C, the relative expression of *smc03787* and *smelC759* were similar as expected if they are part of the same transcriptional unit. Moreover, we found that the regulatory patterns of *smc03787/smelC759* transcripts were alike and opposite to that obtained for *bfr*. Subsequently, we analyzed the promoter activity of an extended 428 bp region (P3787T) covering the 230 bp region of the presumptive *smc03787* promoter plus the entire SmelC759 sRNA sequence. With this construction we wanted to evaluate the effect of additional copies of the small RNA. As shown in Fig. 6B, the presence of an extended promoter region results in an overall reduction of promoter activity when compared to that displayed by the P3787 region (Fig. 6A). This could mean either that the length of the promoter region affects RNA polymerase processivity or that there is a post-transcriptional regulation that negatively affects *smc03787* transcript. Interestingly, when promoter activity was analyzed in the *irr*/rirA (p3787T) double mutant, containing extra copies of the SmelC579 non-coding RNA, the expression was significantly reduced compared to that obtained with cell harboring only P3787. This observation suggests that presence of SmelC579 is required for the concerted action of RirA and Irr.

Discussion

In this work we demonstrate that the *E. meliloti* 1021 bacterioferritin modulates cell iron content under iron sufficient conditions and that it can be used as a nutritional iron source when iron became scarce. Although bacterioferritins are iron storage proteins, its role is not the same for all bacteria. For instance, in *E. coli* iron content was found to be altered in the

231 ferritin *ftn* mutant, being less abundant than in the parental strain (3). Meanwhile, cellular
232 iron levels were not affected in a *bfr* mutant in this bacterium (22). In *Brucella abortus* for
233 which bacterioferritin represents the main protein responsive of iron storage, Bfr accounts
234 for 70% of the intracellular iron pool (23). As *E. meliloti* 1021 genome reveals no other
235 genes with homology to iron storage proteins, it was conceivable that a defect in Bfr would
236 cause a defect in iron intracellular pool. Our data demonstrate that the *bfr* mutant has
237 impaired growth compared with the parental strain, mainly when iron became scarce (Fig. 2
238 and Fig S1) and that *bfr* gene expression is up-regulated under iron sufficiency (Fig.4) .
239 These results are consistent with the following model: when cells are faced to a surplus of
240 iron, bacterioferritin abundance increases in order to store the metal in an intracellular form
241 that can be available when the cell requires coping iron starvation.
242 According to the Fenton chemistry, in the presence of iron H_2O_2 is able to produce reactive
243 oxygen species with highly deleterious to cells and it could be expected that the iron
244 storage protein Bfr may confer protection against oxidative stress (23-25). Nonetheless, our
245 results show that the *bfr* mutant is more resistant to H_2O_2 than the parental strain. A similar
246 unusual phenotype was reported for *Helicobacter pylori*, in which the iron storage ferritin
247 Pfr does not protect the cell from the superoxide radical generator paraquat (26). We
248 speculate that in the *bfr* mutant a reduced iron cellular content together with a putative
249 functional iron export system cooperate to protect the cell against ROS. During the first
250 events of the rhizobial infection process it has been demonstrated that the leguminous plant
251 responds by releasing H_2O_2 and ROS (27). In this work, we found that the *bfr* mutant
252 displayed an early nodulation phenotype in *M. sativa* plants compared to the parental strain.
253 These results reinforce the observation that the absence of bacterioferritin protects the cell,
254 probably indirectly, against H_2O_2 toxicity.

255 Bacteria have developed multiple regulatory systems and mechanisms to sense iron and
256 adapt to environments with changing iron availability conditions. In this regard, the RirA
257 iron responsive repressor (20) as well as the HmuP and RhrA transcriptional activators
258 (28), have been identified as being involved in regulation of high-affinity iron acquisition
259 systems in *E. meliloti* 1021. Here, we aimed to determine for *bfr* gene expression and the
260 regulatory factors involved in this strain. Our results indicate that *bfr* gene expression is
261 positively regulated by iron (Fig. 4). According to Rodionov et al. (18) both *smc03787* and
262 *bfr* genes seem to be part of the same transcriptional unit designated as *bfd-bfr,* which
263 comprises a promoter region upstream *smc03787* containing the canonical -10 and -35
264 promoter sequences plus two RirA boxes and one Irr box (Fig. 1). Nonetheless, Schlüter et
265 al. (21) reported the presence of four transcriptional start sites associated with mRNAs for
266 this region: two of them (SMc_TSS09935 and SMc_TSS09936) located upstream
267 SMc03787 and the other two (SMc_TSS09932 and SMc_TSS09933), located immediately
268 upstream the *bfr* ORF. With this information in mind, we analyzed the promoter activity of
269 the P3787 and the Pbfr regions (Fig. 1). In the assayed conditions, no promoter activity was
270 detected in *E. meliloti* 1021 strain containing the plasmid construction harboring the Pbfr
271 region. Moreover, despite we demonstrated participation of Irr and RirA proteins in *bfr*
272 gene regulation, no well predicted RirA or Irr boxes were found in the region immediately
273 upstream of the *bfr* gene or inside the *bfr* coding sequence. Currently, we do not have data
274 to explain this behavior but our working model is that transcription from SMc_TSS09932
275 or SMc_TSS09933 requires additional factors, as it will be further discussed.

276 We demonstrated that Irr is involved in *bfr* gene regulation in *E. meliloti,* thus representing
277 experimental proof of a biological role for the Irr protein in this bacterium. According to

our results in *E. meliloti* under iron-sufficient conditions, Irr represses *bfr* gene expression whereas it activates *smc03787* (Figs. 5A vs Fig. 5B). Concerning the role of RirA, we found that RirA activates *bfr* gene expression under iron sufficient conditions while a classical role of RirA as an iron-responsive repressor was observed (Fig. 5B and Fig. 6A) for *smc03787*/*smelC579* expression. This result agrees with the enhanced expression of *smc03787* under iron sufficient conditions in a *E. meliloti rirA* mutant reported by Chao et al. (15). Two RirA boxes were found in the promoter region of *smc03787*/*smelC579*. One RirA box covers the -108 to -88 bp relative to SMc_TSS09936, immediately upstream the Irr box, suggesting that RirA could interfere with Irr binding and *vice versa*. The second RirA box is located between the -41 and -14 bp relative to SMc_TSS09936, and overlapping the -35 to -10 region, thus suggesting that RirA may interfere with the RNA polymerase binding and therefore with *smc03787*/*smelC579* transcription initiation from this transcription start site.

According to the data obtained through the different approaches used in this work and to previously reported results (18, 21), we propose a hypothetical model for the regulation of *smc03787* and *bfr* gene expression (Fig. 7). We suggest the presence of at least three different transcripts. Transcript T1 starts at SMc_TSS09935 or SMc_TSS09936 giving rise to the bicistronic *smc03787*/*smelC579* -*bfr* mRNA, allowing *bfr* gene expression. Based on our *in silico* analysis we expect the presence of a "leaky terminator" (5′-GCGGCCGTTGCTGC-3′) located at +156 downstream SMc_TSS09936. Therefore, the *bfr* gene could be transcribed when this "leaky terminator" allows transcription to proceed. Transcript T2 starts at SMc_TSS09932 or SMc_TSS09933 and allows *bfr* gene expression but not of *smc03787*/*smelC579*. Finally, we can also expect a third transcript (T3) which

301 also starts at SMc_TSS09935 or SMc_TSS09936, but unlike T1 it does not allow
302 expression of *smc03787/smelC579* neither of *bfr* gene. According to our hypothetical
303 model of regulation, the occupancy of the RirA box located between the -41 and -14 bp
304 relative to SMc_TSS099362 prevents *smc03787/smelC759* expression. The absence of the
305 non-coding SmelC759 transcript allows the expression of an unknown factor facilitating T2
306 transcription and consequently *bfr* gene expression. This hypothesis is based on data
307 showing that expression of *smelC759* is inversely correlated with *bfr* expression (Fig. 5A
308 vs 5B and 5C). Moreover, differences observed when we compared expression in cells
309 harboring p3787T or p3787 (the former with extra copies of SmelC759 provided from the
310 plasmid) let us speculate that this small RNA would be involved in this regulation (Figs 6A
311 vs 6B and S4A vs S4B). In this scenario Irr would be an indirect iron responsive repressor
312 and RirA an indirect iron responsive activator of *bfr* gene expression. It is worth noting that
313 the participation of the RNA chaperone Hfq, a major factor involved in activity and
314 stability of sRNAs and mRNAs (29), has been previously reported as involved in *bfr* gene
315 expression in *E. meliloti* (30-32). Intriguingly *bfr* expression is upregulated by Hfq in *E.*
316 *meliloti* 1021 (30), while the opposite was found in the *E. meliloti* 2011 derivative strain
317 (31). The reasons for this discrepancy are still unknown. Moreover Torres-Quesada et al.
318 (32) found that Hfq could bind, directly or indirectly, to the *bfr* transcript. However, to our
319 knowledge, SmelC759 has not been identified as an Hfq dependent sRNA. Altogether,
320 these observations indicate that in addition to RirA, Irr, Hfq and the small RNA SmelC759,
321 other factors may be involved in the regulation of *bfr* gene expression. Under iron
322 limitation, *bfr* expression is not completely repressed. Therefore, we presume that in this
323 situation the T1 transcript is present and also the small RNA *SmelC759* is present, thus
324 allowing repression, probably indirectly, of *bfr* expression from T2 (Fig. 7). Certainly more

information is required to validate our hypothetical model of regulation, for instance site-directed mutagenesis over the putative Irr and RirA binding sites of the *smc03787/bfr* promoter could provide evidences for the molecular bases of *smc03787/bfr* regulation.

In conclusion, in this study we demonstrated that in *E. meliloti* 1021, Bfr influence cellular iron content and that it could be used as a nutritional iron source when iron became scarce. Results presented here provide novel data on the regulation of *bfr* expression and enabled us to propose a comprehensive hypothetical model of regulation. We demonstrate that: a) regulation of *bfr* gene is inversely correlated with that of *smc03787/smelC759*, b) that *bfr* gene expression responds to a complex mechanism of regulation; and c) that at least RirA, Irr and the small RNA, SmelC759, are involved in the regulation. Moreover, our data indicate the existence of still unknown actors involved in controlling *bfr* gene expression in *E. meliloti*.

Materials and methods

Bacteria, plasmids and growth conditions. Bacterial strains and plasmids are listed in Table 1. *Escherichia coli* strains were grown at 37 °C in Luria-Bertani (LB) medium (33). *E. meliloti* strains were grown at 30 °C in tryptone-yeast extract medium (TY) (34), or in defined minimal medium M9 (35) plus 6 mM glutamate, 200 µM methionine and 1 µM biotin (M9S) or in M3 medium (36). Low-available iron conditions were obtained by supplementation with 150 µM or 300 µM ethylendiamine-di-o-hydroxy-phenylacetic acid (EDDHA), whereas iron sufficient conditions were obtained by addition of 37 µM $FeCl_3$.

When required, 50 µg ml/l kanamycin (Km), 100 µg ml/l neomycin (Nm), 100 µg ml/l streptomycin (Str), or 5 µg ml/l gentamycin (Gm), were added to the media.

Construction of a *bfr* mutant strain. In order to generate a *bfr* mutant containing a transcriptional reporter fusion, almost the entire *bfr* gene was replaced by *lacZ-accC.* With this purpose, a 2560 bp DNA fragment containing the *bfr* gene and flanking regions was amplified from *E. meliloti* 1021 genome using primers 5´- GGCGCACCCCGTTTCCTTC-3´ and 5´- AGCCGCAATGCCGTCCTG -3´. The amplicon was digested with *Eco*RV and cloned into pBlueScriptSK (pBSK) (Stratagene) to generate plasmid pBSKbfr12. A subsequent amplification was performed using pBSKbfr12 DNA as template and primers 5'- CAGAGCGTTGCGTATGATGGACAC-3' and 5'- CAAGGCAGAGCGGCGTGT-3'. The 890 bp amplicon was digested using *Eco*RV and cloned into pBSK obtaining pBSKbfr34. By an inverse amplification using the *Pfu* enzyme, a region containing 220 bp (from +66 to +286) of *bfr* coding region was deleted from pBSKbfr34. The PCR product was ligated with the *lacZ-accC1* cassette obtained from pAB2001 (Becker et al. 1995) and cloned in the +66 position of the *bfr* ORF, generating pBSK-*bfr::lacZ-accC1*. The *bfr::lacZ-accC1* fragment was sub-cloned as a *Bam*HI/*Hind*III insert into pK18*mobsacB* (pK18) (Schäfer et al. 1994) to obtain the plasmid pK18-*bfr*::*lacZ-accC1,* which was then mobilized into *E. meliloti* 1021 by triparental mating using *E. coli* DH5α-pRK2013 (37) as a helper strain. Str^r and Gm^r colonies able to grow in 15 % (w/v) sucrose (Sac) were selected and the mutation was confirmed by Southern blot hybridization.

Cellular iron content. Cultures grown in M3 (36) minimal medium supplemented with 37 µM $FeCl_3$ were washed 3 times with 0.1 M sodium phosphate buffer, pH 7 and cells were

freeze-dried. Iron content was determined by flame atomic absorption spectrometry in the Analytical Chemistry Laboratory of the Uruguayan Chemistry School of UdelaR. Total protein content was evaluated with the bicinchoninic acid assay according the Sigma´s kit specification.

Cellular growth under different conditions of iron availability. In order to determine the effect of iron availability on cell growth, *E. meliloti* 1021 and *bfr* mutant strains were grown in iron sufficient minimal medium until stationary phase, washed, and subsequently subcultured in M3 or Ty medium supplemented with 37 µM $FeCl_3$, 150 µM EDDHA or 300 µM EDDHA as indicated. OD_{620nm} of cultures grown in M3, M9S or TY media supplemented with either EDDHA or $FeCl_3$, was monitored with a Varioskan Flash® (Thermo Scientific).

Rhizobactin production. Presence of the rhizobactin 1021 siderophore in supernatants of *E. meliloti* 1021 and *bfr* mutant grown in M3 minimal medium supplemented with 150 µM EDDHA, was evaluated with the iron perchlorate method according to Carson et al. (38).

Iron and hemin sensitivity assay. *E. meliloti* 1021 and *bfr* mutant strains were grown in M3 medium supplemented with 37 µM $FeCl_3$ until stationary phase. Subsequently, cells were washed and M3 medium was added until obtaining a cell suspension of an OD_{620nm}=1. Afterwards, serial dilutions were performed and 10 µM droplets were placed onto M3 solid medium supplemented with 1 mM, 2 mM, 4 mM or 5 mM $FeCl_3$, and on medium supplemented with 25 mM or 50 mM hemin. Cells were incubated at 30 ºC until colonies were visible.

395

396 **Hydrogen peroxide sensitivity assay**. *E. meliloti* 1021 and *bfr* mutant strains were grown
397 in M3 minimal medium supplemented with 37 µM $FeCl_3$ until stationary phase.
398 Subsequently, cells were washed and resuspended in M3 medium. In order to obtain
399 confluent growth, $1x10^5$ cfu were plated on top of M3 solid medium supplemented with
400 either 37 µM $FeCl_3$ or 100 µM EDDHA. Sterile filter paper disks were placed onto the
401 solid medium and 10 µl droplets of 0.9 M H_2O_2 were added to the paper disks. After 72 hs
402 of incubation at 30 ºC, inhibition zones around the disks were measured.

403

404 **Plant growth promotion assays**. With the aim to determine Bfr role in the establishment
405 of plant-rhizobium symbiosis, nodule kinetic and plant growth promotion assays were
406 performed. *Medicago sativa* seeds were surface-sterilized with 0.2 M $HgCl_2$ in HCl 0.5%
407 (v/v), as described by Battistoni *et al.* (39). The sterilized seeds were incubated 1 h in
408 sterile water at 30 ºC with shaking, and germinated on agar-water 0.8 % (w/v) in petri
409 dishes. Seedlings were transferred aseptically to plant-tubes containing 20 ml semisolid
410 Jensen media (40) plus 0.8% (w/v) agar, without nitrogen supplementation. Seven days
411 after, plants were inoculated with about $1x10^6$ cfu of *E. meliloti* parental or *bfr* mutant
412 strains. Nitrogen fertilization positive controls with 1 ml of 1% (w/v) KNO_3 and negative
413 controls with 1 ml of sterile water were performed. A total of 10 tubes per treatment, with
414 two plants per tube were used. Plants were grown at 21 ± 4 ºC with a 16 hs light period. For
415 nodulation kinetics assays, emergence of the first nodule in each plant was registered along
416 the time. For plant growth promotion assays, approximately three months after inoculation,
417 plants were harvested, the aerial part of each plant was heat-dried for 72 hs at 60 ºC and
418 weighted. Dry weights of plants were compared by Tukey analysis with a p-value of 0.05.

Construction of the *irr* mutant, the *irr/rirA* double mutant and the *irr/bfr*::*lacZ-accC1* double mutant. The *irr* mutant strain was obtained using a similar strategy to that used for the construction of the *fur* mutant (14). The almost entire *irr* gene, except for 20 and 40 bp at 5′ and 3′ end respectively, was replaced by an Ω45 cassette obtained from digestion of plasmid pΩ45 (41) with *Sma*I. Genomic DNAs of mutants were isolated to check gene replacement by PCR. In order to obtain the *irr/rirA* double mutant, deletion of *irr* gene was transduced with the phage ϕM12 (42) to the *rirA* mutant strain (15). Transduction was done as previously described (42, 43).The *irr/bfr*::*lacZ-accC1* double mutant was constructed similarly to the *bfr*::*lacZ-accC* mutant but using the *irr* simple mutant as parental strain.

Beta-galactosidase assays. *E. meliloti* 1021 *bfr::lacZ-accC1* strain, and the *irr/bfr::lacZ-accC1* double mutant, were grown 48 hs in TY medium supplemented with 37 µM $FeCl_3$ or with 100 µM EDDHA. Beta-galactosidase assays were performed according to Miller (44) with modifications described by Pool *et al.* (45). β-galactosidase units were recorded as Miller units.

Promoter transcriptional fusions. A 230 bp fragment (P3787, Fig. 1) containing the presumptive promoter of *smc*03787-*bfr* genes was obtained by PCR amplification using primers P3787F (5'-TTGAAGCTTGGGCGCCCCTGTGTA-3') and P3787R (5'-TCCTCTAGATCGTCGTGTCCATCATACG-3'). An extended 428 bp long region (P3787T) containing the presumptive promoter plus the putative *smelC759* gene (Fig. 1) was obtained using primers P3787F and P3787TradR (5'-

442 TACTCTAGACGGGCGTGATATTGC-3'). A presumptive promoter immediately
443 upstream of the *bfr* gene was obtained by using PbfrF (5'-GTCAAGCTTATGGAAAAA
444 CGCGGCC-3') and Pbfr R (5'- ATCTCTAGACGCCCTCCGGTT TTCTTCA-3') primers.
445 Recognition sequences for restriction enzymes added in each primer are underlined. DNA
446 fragments were purified, digested with *Hind*III/*Xba*I and cloned in *Hind*III/*Xba*I digested
447 pOT1 vector (46), generating plasmids pBfr, p3787 and p3787T. Cloning was confirmed by
448 restriction mapping and sequencing. Plasmids obtained were subsequently mobilized to *E.*
449 *meliloti* 1021 strain and the isogenic *irr*, *rirA* and *irr/rirA* mutants by triparental mating
450 using *E. coli* DH5α (pRK2013) as a helper strain. Presence of transcriptional fusions in the
451 transconjugant strains was confirmed by PCR using pOT-forward and pOT-reverse primers
452 (46).
453
454 **Expression of *smc03787* and *bfr***. Two different approaches were used to study regulation
455 of *bfr* expression: i) quantification of transcriptional promoter fusions to GFP-UV reporter
456 gene; ii) quantitative real-time PCR assessment of transcript abundance.
457 For the GFP-UV reporter fusions, the promoter activity was determined by measuring
458 emitted GFP fluorescence by spectrofluorometry or by flow cytometry in M9 broth under
459 iron sufficient and iron starved conditions. Briefly, cultures of *E. meliloti* 1021 strain and
460 *irr*, *rirA*, *irr/rirA* mutants harboring either pOT, p3787, p3787T or pBfr plasmids, were
461 grown in iron sufficient conditions until early stationary phase, washed and diluted 100-
462 fold in M9 supplemented with either 37 µM $FeCl_3$ or 100 µM EDDHA.
463 For spectrofluorometric determinations, expression of green fluorescence (λ exc=395nm, λ
464 em=509 nm) and OD_{620nm} were evaluated during cell growth in liquid media in a Varioskan
465 Flash® (Thermo Scientific). Relative fluorescence (RF) was determined according to the

466 method of Allaway et *al*. (14), and expressed as fluorescence emission at 509nm
467 normalized to OD_{620nm} in cultures at early stationary phase (*ca.* 48 hs)RF produced by
468 strains harboring fusion plasmids in each genomic context was normalized with the RF of
469 pOT plasmid in the same genomic context. Non-parametric Kruskal-Wallis statistic test
470 was applied in order to compare the different groups of samples.

471 For flow cytometry fluorescence determinations, freshly harvested cells were washed with
472 sterile sodium phosphate buffer 0.1 M, pH 7. Cell suspensions containing about $1x10^6$ cells
473 per ml were injected in a FACSVantage (BD, USA) flow cytometer, set to emit at 488nm
474 and 100mW. For quantitative real-time PCR, the *E. meliloti* 1021 parental strain, the *irr*
475 and *rirA* mutants and the *irr,rirA* double mutant were grown in M3 medium supplemented
476 with 37 µM $FeCl_3$. RNA was purified using PureLink® RNA Mini Kit (Ambion) following
477 manufacturer recommendations. Remainder DNA was digested using 2 units of DNase I
478 (Ambion) per µg of extracted RNA. RNA concentration was determined with a NanoDrop
479 (Thermo Scientific). One hundred nanograms of RNA were used for cDNA synthesis using
480 iScript™ Select cDNA Synthesis Kit (Biorad) and random primers, according to
481 manufacturer's instructions. Expression of different regions inside *smc*03787-*bfr* operon
482 was quantified by qPCR using iQ SYBR Green Supermix (Biorad) on a CFX96 Touch™
483 Real-Time PCR Detection System (Biorad). Expression of an internal *smelC759* region was
484 quantified using primers srnaF (5' –TTGCGTATGATGGACACGAC- 3') and srnaR (5'-
485 ACGATCAACTGCCAACAGTC-3'); expression of an internal region of the *smc03787*
486 gene was quantified using bfdF (5'-GGACGCCGATATATTTGATTT C-3') and bfdR (5'-
487 CGTCGCCTTTCAAGATCC-3'); and expression of an internal region of *bfr* gene was
488 quantified using bfrF (5'-CGATTACGTCTCGATGAAGC-3') and bfrR (5'-

489 CTGGCCGTATTTTTCCTCAC-3'). PCR conditions were: 5 min at 95 ºC, 40 cycles of 10
490 sec at 95 ºC and 30 sec at 60 ºC. A dissociation curve (65 to 95 ºC in 0.5ºC increments)
491 was carried out for all reactions.

492

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497 **TABLE 1 Bacterial strains and plasmids used in this work**

Strain/Plasmid	Remarkable characteristics	References
Strain		
E. coli DH5α	*sup*E44Δ*lac*U169(ϕ80*lacZ*ΔM15)*hsd*R17 *rec*A1*end*A1 *gyr*A96 *thi*-1 *rel*A1	(47)
Ensifer meliloti		
1021	Streptomycin derivative of wild-type strain SU47 (Str^r)	(48)
irr	1021 *irr::*Ω, (Str^r, Spc^r)	This work
rirA	1021 *rirAΔ* (Str^r)	(15)
irr/rirA	1021 *rirAΔ irr::*Ω (Str^r, Spc^r)	This work
bfr	1021 *bfr::lacZ-accC1* (Str^r, Gm^r)	This work
bfr/irr	1021 *bfr::lacZ-accC1 irr::*Ω (Str^r, Gm^r, Spc^r)	This work
1021(pOT)	1021 containing plasmid pOT1	(49)
irr (pOT)	1021 *irr* mutant containing plasmid pOT1 (Str^r, Gm^r)	This work
rirA (pOT)	1021 *rirA* mutant containing plasmid pOT1 (Str^r, Gm^r)	This work
irr/rirA (pOT)	1021 *irr/rirA* double mutant containing plasmid pOT1 (Str^r, Gm^r)	This work
1021 (p3787)	1021 containing plasmid p3787 (Str^r, Gm^r)	This work
irr (p3787)	1021 *irr* mutant containing plasmid p3787 (Str^r, Gm^r)	This work

rirA (p3787)	1021 *rirA* mutant containing plasmid p3787 (Str^r, Gm^r)	This work
irr/rirA (p3787)	1021 *irr/rirA* double mutant containing plasmid p3787 (Str^r, Gm^r)	This work
1021 (p3787T)	1021 containing plasmid p3787T (Str^r, Gm^r)	This work
irr (p3787T)	1021 *irr* mutant containing plasmid p3787T (Str^r, Gm^r)	This work
rirA (p3787T)	1021 *rirA* mutant containing plasmid p3787T (Str^r, Gm^r)	This work
irr/rirA (p3787T)	1021 *irr/rirA* double mutant containing plasmid p3787T (Str^r, Gm^r)	This work
Plasmid		
pBlueScript SK	Cloning vector (Ap^r)	Stratagene
pAB2001	Carrying lacZ-Gm R promoter-probe cassette (Gm^r, Ap^r)	(50)
pΩ45	Plasmid containing Ω Sp^{r}Sr^r cassette	(41)
pRK2013	ColE1 replicon with RK2 tra genes. Used for mobilizing incP and incQ plasmids (Km^r)	(37)
pOT1	Wide host range gfp-UV promoter-probe plasmid, derivative of pBBR1 (Gm^r)	This work
pBfr	pOT1 containing a putative Bfr promoter	This work
p3787	pOT1 containing the P3787 promoter region	This work
p3787T	pOT1 containing the P3787T promoter region	This work

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FIG 1. Growth of the *bfr* mutant strain was severely impaired under iron-limited conditions. *E. meliloti* 1021 (circles) and the *bfr::lacZ-accC1* mutant strain (triangles) were grown in M9 medium supplemented with 37 µM $FeCl_3$ (closed symbols) or 100 µM EDDHA (open symbols). Error bars on each point indicate standard error, for a set of 3 to 5 replicates.

FIG 2. Bfr affects oxidative stress response against H_2O_2. Disc diffusion assay was performed on M3 solid medium with either 37 µM $FeCl_3$ or 100 µM EDDHA and inoculated with *E. meliloti* 1021 or the *bfr* mutant strain. Zone of growth inhibition around paper disks containing 10 µl of 0.892 M H_2O_2 was determined by measuring the diameter of the clear zone around the disc. Grey hyphens correspond to medians of 18 replicates per strain per condition. A non-parametric Kruskal Wallis test was applied. Significative differences, with a p-value < 0.01, were found between *E. meliloti* 1021 and the *bfr* mutant strains grown in iron sufficient medium (***), significative difference with a p-value < 0.05 (**) were found , were found while (###) indicates a significative difference with a p-value < 0.01 between the *bfr* mutant strain grown under different iron conditions.

FIG. 3. *In vivo* effect of *irr* mutation on *bfr::lacZ-accC1* activity. β-Galactosidase activity in the *E. meliloti* 1021*bfr*::*lacZ-accC1* strain and in the *E. meliloti irr*/ *bfr::lacZ* mutant. Cultures were grown in TY medium supplemented with 37 µM $FeCl_3$ (solid bars) or 100 µM EDDHA (open bars). β-galactosidase activity is expressed as Miller units. Error bars, 1SD. In both strains β-galactosidase activity was significantly higher under iron-

670 sufficient conditions. A clear effect of *irr* deletion was detected indicating an Irr role in *bfr*
671 gene expression.

672

673 **FIG 4. Physical mal of the SMc03787-*bfr* region.** The *bfr* gene is located on the (-) strand
674 of *E. meliloti* 1021 chromosome, from position 3,451,466 to 3,451,951, partially
675 overlapped with SMc03787, a gene that codifies a hypothetical conserved protein. The
676 small RNA *SmelC759* by Schlüter et al. (21) as partially overlapping with SMc03787.
677 Position of RirA boxes are shown in blue and the Irr box in pink. Position of transcriptional
678 start sites (TSS) identified by Schlüter et al. (21) are indicated as purple arrows . Double-
679 headed arrows indicate the positions of the amplified fragments P3787, P3787T, sRNA, bfd
680 and bfr, and their position are indicated under the arrows. Numbers of positions are referred
681 to SMc03787 TSS.

682

683 **FIG 5. Transcription of *bfr* and of *SMc03787/SmelC759* respond to Irr and RirA.**
684 Relative transcripts levels, determined by qRT-PCR, under iron sufficient conditions of *bfr*
685 (A), *SMc0378* (B) or *SmelC759* (C) (see Fig. 1) in different genomic context: parental
686 strain, *irr* mutant, *rirA* mutant and *irr*,*rirA* double mutant. White hyphens inside boxes
687 correspond to the medians, while the upper and lower limits of the boxes represent first and
688 third quartiles respectively.

689

690 **FIG 6. Effect of Irr, RirA and SmelC759 on *smc03787/smelC759* promoter activity**.
691 Panel shows the relative GFP fluorescence in cells carrying pP3787 (upper panel) or cells

carrying pP3787T (lower panel). *Ensifer meliloti* 1021 (1021), *irr* mutant, *rirA* mutant or *irr,rirA* double mutant were grown in M9 medium supplemented with either 37 μM $FeCl_3$ (solid bars) or 100 μM EDDHA (open bars). Relative fluorescence (RF) was expressed as fluorescence emission at 509nm normalized to OD620nm in cultures at early stationary phase (ca. 48 hs). Measurements were done in cultures grown in 96-well plates and, fluorescence emission and OD620nm values were determined simultaneously with a Varioskan equipment. Error bars indicate standard error for five replicates. The asterisks above bars indicate a statistical significative difference relative to the parental strain under similar conditions of iron sufficiency, while hash symbol (#) indicate a significative difference relative to the same strain in different iron conditions, according to a Kruskal Wallis test.

FIG. 7. Hypothetical model of the regulation of *bfr* gene expression when *E. meliloti* 1021 cells are grown under iron sufficient (A) or iron-depleted (B) conditions. We postulate that under iron-sufficient condition, transcripts T2 is produced from TSS09932 or TSS09933 (A) while under iron-depleted conditions, transcript T1 is produced from TSS09935 or TSS09936. According our results we propose that under iron sufficient conditions, iron-loaded RirA is able to bind to the RirA box located between the -41 and -14 bp relative to SMc_TSS099362 and repress *smelC759/smc03787* transcription while Irr interacts with the Irr box activating the expression. Balance between RirA repression and Irr activation, decrease *smelC759* concentration into the cell and this effect allows production of T2 (Fig.7A). Under iron-depleted conditions, iron-unloaded RirA is not able to bind the RirA box located between the -41 and -14 bp relative to SMc_TSS099362 but it

715 is able to interact with the RirA box located between the -108 and -88 bp relative to
716 SMc_TSS09936. In this condition RNA polymerase is able to bind to the
717 *smelC759/smc03787* promoter allowing T1, or T3, production according the effect of the
718 leaky terminator located at the +156 position from SMc_TSS09936.