Azospirillum brasilense Az39, a model rhizobacterium with AHL *quorum quenching* capacity

Journal:	*Applied Microbiology*
Manuscript ID	JAM-2018-1476.R1
Journal Name:	Journal of Applied Microbiology
Manuscript Type:	JAM - Original Article
Date Submitted by the Author:	12-Feb-2019
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Key Words:	Bioproducts, Mechanism of action, Microbial physiology, Quorum sensing, Genomics

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11

12 **Running head:** *Quorum quenching* in *A. brasilense* Az39

13

14 **Aims**

15 The aim of this research was to analyze the *Quorum sensing* (QS) and *Quorum quenching* (QQ)

16 mechanisms based on N-acyl-L-homoserine lactones (AHLs) in *A. brasilense* Az39, a strain with

17 remarkable capacity to benefit a wide range of crops under agronomic conditions.

18 **Methods and Results**

19 We performed an *in silico* and *in vitro* analysis of the quorum mechanisms in *A. brasilense* Az39.

20 The results obtained *in vitro* by the use of the reporter strains *C. violaceum* and *A. tumefaciens* and

21 Liquid Chromatography coupled to Mass-Mass Spectrometry (LC-MS/MS) analysis shown that

22 although Az39 does not produce molecules AHL, it is capable of degrading them by at least two

23 hypothetical enzymes identified by bioinformatics approach, associated to the bacterial cell. In

24 Az39 inoculated cultures incubated with 500 nmol l^{-1} of the C3 unsubstituted AHLs (C4, C6, C8,

25 C10, C12, C14), AHL levels were lower than non-inoculated LB media controls. Similar results

26 were observed upon addition of AHLs with hydroxy (OH-) and keto (oxo-) substitutions in carbon

27 3. These results not only demonstrate the ability of Az39 to degrade AHLs, but the wide spectrum

28 of molecules that can be degraded by this bacterium.

29 **Conclusions**

30 *A. brasilense* Az39 is a silent bacterium unable to produce AHL signals, but with the ability to
31 interrupt the communications between other bacteria and/or plants by a *quorum quenching* activity.

32 **Significance and Impact of Study**

33 This is the first report confirming by unequivocal methodology the ability of *A. brasilense*, one of
34 the most agriculturally used benefic bacteria around the world, to degrade AHLs by a *quorum*
35 *quenching* mechanism.

36

37 **Journal keywords:** Bioproducts, Mechanism of action, Microbial physiology, Quorum sensing,
38 Genomics

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40 **1. Introduction**

41 Microorganisms have the capacity to perceive population density by generating small signaling
42 molecules named autoinducers (Nealson 1977). As a result, at gene level a hierarchical response is
43 developed to coordinate social behavior. This process is called *quorum sensing* (QS) (Fuqua *et al.*
44 1994). The most studied QS system is undoubtedly the one that involves N-acyl homoserine
45 lactone or AHL-type signals, discovered for the first time in *Vibrio fischeri,* a seawater symbiont
46 bacterium (Nealson and Hastings 1979). In this bacterium, QS consists of a modulatory protein or
47 transcriptional regulator belonging to the LuxR family and its homologue LuxI, an enzyme that
48 produces the signal AHL molecule. Although a large number of bacteria possess the canonical
49 LuxR/LuxI QS system, it has been found almost exclusively in α, β and γ Proteobacteria (Williams
50 2007). In general, AHLs are small molecules composed of fatty acyl chain linked to a lactonized
51 homoserine through an amide bond. LuxI, more specifically, catalyzes the binding of S-
52 adenosylmethionine (SAM) to an acyl carrier protein (acyl-ACP). In other words, LuxI catalyzes
53 the binding between a homoserine lactone group derived from the metabolism of amino acids, and
54 an acyl lateral chain derived from fatty acid metabolism, which are the two structural components
55 of the resulting AHL (Fuqua *et al.* 2001). For their part, LuxR-like proteins (with approximately
56 250 amino acids) can be subdivided into two functional domains: the amino-terminal region that
57 contains the AHL-binding domain and the carboxyl-terminal region that contains the helix-turn-
58 helix of DNA (Whitehead *et al.* 2001). Once in contact with the AHLs, LuxR joins a palindrome 20

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59 bp sequence called the *lux* box, from the *luxI* promoter region, in the form of a LuxR-autoinducer
60 complex. This leads to transcriptional activation or repression, thus expressing a particular
61 phenotype.
62 On the other hand, some bacterial strains present quorum systems with a non-cognate LuxR protein
63 (i.e. they lack LuxI) and they thus respond to other signal molecules. These systems are called
64 LuxR orphans or LuxR *solos* (Patankar and Gonzalez 2009), and in some cases they act in concert
65 with the LuxR/LuxI canonical system. The appearance of LuxR-*solos* regulators indicates that
66 these protein families could be involved in intra-kingdom or inter-kingdom signaling systems
67 through the detection of different compounds produced by other prokaryotes or eukaryotes
68 organisms (Patankar and Gonzalez 2009, Patel et al. 2013).
69 In nature, there are also bacterial mechanisms that inactivate quorum signals called *Quorum*
70 *Quenching* (QQ) (Zhang 2003). These can generally act both at the level of signal generation and
71 reception. Although there are several QS mechanisms involving inhibitory proteins and/or AHL
72 antagonist molecules, the mechanisms that involve enzymes are widespread in different
73 environments. Three main enzymatic QQ mechanisms have been clearly described: (1) hydrolysis
74 of the lactone ring (AHL lactonase activity), (2) hydrolysis of the amide bound (AHL acylase
75 activity), and (3) modification of the acyl chain (AHL oxidase and reductase activity) (Uroz et al.
76 2009), but they have not been studied in depth in soil bacteria. As occurs in the QS system, QQ
77 mechanisms can serve in particular environments to modulate the interaction between a bacterial
78 community and eukaryotic organisms (Tait et al. 2009).
79 Soil bacteria living in the rhizosphere, or rhizobacteria, have the ability to associate with numerous
80 plant species. If this association is beneficial for plant growth or development, they are called Plant
81 Growth Promoting Rhizobacteria or PGPR (Kloepper et al. 1989). Among the most successful
82 associations and therefore the most studied in nature, are those related to the genus *Azospirillum* sp.
83 The ability of these rhizobacteria to promote plant growth depends mainly on the presence of one
84 or more mechanisms that might act individually or in synch on the physiology or metabolism of the
85 colonized plant (Bashan and de-Bashan 2010)
86 *A. brasilense* Az39 was isolated in 1982 from surface-sterilized wheat seedlings in Marcos Juarez,
87 Córdoba, Argentina, evaluated under agronomic conditions and selected based on its ability to

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88 increase crop yields of maize and wheat under agronomic conditions (Díaz-Zorita and Canigia
89 2009). *A. brasilense* Az39 has been widely used in agriculture in America during the last 40 years
90 (Cassán and Díaz Zorita 2016). The potential mechanisms responsible for growth promotion in this
91 strain have been partially unraveled (Perrig et al. 2007, Cassán et al. 2009). Despite its agro-
92 economic importance and the fact that several genomes from this genus have been sequenced, such
93 as those belonging to *Azospirillum* sp. B510, *A. lipoferum* 4B, *A. brasilense* Sp245, CBG497 and
94 Az39 (Kaneko et al. 2010, Wisniewski-Dyé et al. 2011, Wisniewski-Dyé et al. 2012, Rivera et al.
95 2014), there are few reports related to bacterial capacity to produce AHL-like molecules and/or
96 other phenomena associated with quorum mechanisms. Therefore, there is little understanding
97 about the *Azospirillum-Azospirillum, Azospirillum*-bacteria and *Azospirrillum*-plant interactions
98 mediated by quorum mechanisms, highlighting the need for a more exhaustive genomic-functional
99 analysis of these bacteria due their agricultural and economic interest. Considering this
100 background, the main objective of this work was to analyze both *in silico* and *in vitro* the *quorum*
101 *sensing* and *quorum quenching* phenomenon mediated by AHLs in the model strain *A. brasilense*
102 Az39.
103
104 **2. Material and Methods**
105 **2.1. Bacterial strains and growth conditions**
106 *A. brasilense* Az39 was obtained from the Bacterial Culture Collection at the INTA-IMYZA,
107 Castelar, Buenos Aires, Argentina (WDCM31). Pure cultures of *A. brasilense* Az39 were obtained
108 in Petri dishes containing Luria-Bertani medium (Miller 1972) modified by the addition of 15 ml l^{-1}
109 Congo Red (LB-RC) or MMAB minimal medium (Vanstockem et al. 1987). Typical colonies from
110 such media were used to inoculate LB liquid medium in 100 ml flasks and cultured at 37°C with
111 240 rpm shaking until late exponential growth phase was reached. *Chromobacterium violaceum*
112 CV026 (McClean et al. 1997) grew in LB medium supplemented with 25 μg ml^{-1} kanamycin (Km).
113 *Agrobacterium tumefaciens* NTL4/pZLR4 (Cha et al. 1998) was cultured in AT medium (Morton
114 and Fuqua, 2013) supplemented with 50 μg ml^{-1} gentamicin (Gm). These two strains were used as
115 reporter strains in the bioassays described below.
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117 **2.2. *In silico* analysis of quorum mechanisms in *A. brasilense* Az39**

118 We determined the presence of coding sequences for proteins involved in quorum sensing
119 mechanisms in the genome of *A. brasilense* Az39, and compared it with available sequences from
120 other strains belonging to the genus *Azospirillum*. For the analysis, the comparative tools KEGG
121 (Kanehisa et al. 2012), RAST (Aziz et al. 2008) and MaGe (Vallenet *et al.* 2006) were used, as
122 well as the bio-informatic tools UniProt (Apweiler et al. 2004) and InterPro (Mulder et al. 2005).
123 Our work focused on the identification of coding sequences related with: (1) enzymes and
124 transcriptional regulators involved in QS detection/response, (2) AHL synthases homologues, (3)
125 homologous LuxR-type regulatory proteins, (4) LuxR orphans or LuxR *solos* and (5) enzymes and
126 transcriptional regulators involved in QQ detection/response, including lactonases, acylases and
127 oxidoreductases. In order to predict sub cellular localization of a protein specific for Gram-
128 negative bacteria CELLO Web server (http://cello.life.nctu.edu.tw.) was used.

129

130 **2.3. *In vitro* analysis of quorum mechanisms in *A. brasilense* Az39**

131 **2.3.1. *Quorum sensing***

132 **2.3.1.1. Evaluation of production of AHLs by bioassays**

133 The presence of AHLs in Az39 cultures was validated by the use of the reporter strains *C.*
134 *violaceum* CV026 and *A. tumefaciens* NTL4/pZLR4 which are specific for AHLs with a short and
135 long acylic chains, respectively. A 500 µl aliquot of a *A. tumefaciens* NTL4/pZLR4 or *C.*
136 *violaceum* CV026 exponential cultures were individually transferred into a 10 ml capacity glass
137 tubes containing 4500 µl of semisolid AT medium 0.7 % (w/v agar), modified by addition of 50 µg
138 ml^{-1} X-gal at 45°C. The mixes were plated out on Petri dishes and solidified under aseptic
139 conditions. In both cases, small holes were made in the Petri dish containing AT or LB solidified
140 culture medium, using a 5 mm cylindrical punch. A 10 µl aliquot of filtered supernatants obtained
141 from 50 ml LB culture medium at 6, 12, 24 and 48 hours after inoculation with 50 µl of Az39 were
142 placed individually in one of the holes and evaluated. The plates were incubated at 30°C for 24 h to
143 reveal the presence of AHL by a colorimetric reaction. In addition, some experimental conditions
144 such as incubation temperature, pH and AHL concentration were previously evaluated to analyze

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145 the reproducibility of the methodology and the stability of the AHL molecules in the Petri dish
146 during incubation. Experiments were carried out in triplicate.
147
148 **2.3.1.2. Evaluation of production of AHLs by Az39 using Liquid Chromatography coupled to**
149 **Mass-Mass Spectrometry (LC-MS/MS) analysis**
150 **2.3.1.2.1. Extraction of AHLs from Az39 cultures**
151 Typically, *A. brasilense* AZ39 colonies grown on LBRC medium were used to inoculate 250 ml of
152 LB medium and incubated at 37°C, with shaking (200 rpm) until stationary growth phase had been
153 reached. Aliquots (100 ml) of centrifuged (5 min at 10000 rpm), and sterile filtered supernatant
154 (0.22 µm, Millipore Express PLUS) were acidified to pH 2 with the addition of HCl. Supernatant
155 samples were extracted three times by liquid-liquid extraction using an equal volume of acidified
156 ethyl acetate (1% (v/v) AcOH in EtOAc). Combined extracts were dried under vacuum and stored
157 at -80° C prior to analysis.
158
159 **2.3.1.2.2. LC-MS/MS analysis**
160 The LC-MS/MS analysis of extracted samples was conducted as previously described (Ortori et al.
161 2011) with minor modification. Dried extracts were re-dissolved in 50 µl of 0.1% (v/v) formic acid
162 in MeOH. The chromatography column used was a Phenomenex Gemini C18 (3.0 µm, 150 x 3.0
163 mm), and the mobile phases used were 0.1 % (v/v) formic acid and 0.1% (v/v) formic acid in
164 methanol. The analysis was conducted with the MS operating in multiple reaction monitoring
165 (MRM) mode, simultaneously screening the LC eluent for all specific AHLs, comparing the
166 retention time of detected analytes with authentic synthetic standards. For each detected
167 chromatographic peak a mean peak area was calculated from three biological replicates.
168
169 **2.3.2. *Quorum quenching***
170 **2.3.2.1. Evaluation of degradation of AHLs by Az39 by LC-MS/MS analysis**
171 A set of 9 glass flasks of 50 ml capacity containing 20 ml of LB medium was prepared. Only 6
172 were inoculated with 20 µl of Az39 culture obtained from liquid LB medium in late exponential

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173 growth phase (OD_{595} 1.0), and 3 remained without inoculation (controls). The 9 flasks were then
174 incubated overnight at 37°C with 200 rpm orbital shaking. After a 12 h incubation, the tubes
175 containing the Az39 cultures and the non-inoculated control tubes were modified by the exogenous
176 addition of 100 µl of a methanolic solution containing C4, C6, C8, C10, C12, C14, Oxo-C4, Oxo -
177 C6, Oxo-C8, Oxo-C10, Oxo-C12, Oxo-C14, OH-C4, OH-C6, OH-C8, OH-C10, OH-C12 and OH-
178 C14, each in a concentration of 100 µmol l^{-1}, which rendered a final concentration of 500 nmol l^{-1}
179 for each individually added AHL. A 100 µl methanol control treatment was used to evaluate
180 bacterial growth inhibition. The glass flasks were incubated for 6 h, and at 1, 3 and 6 h intervals 1
181 ml samples were taken and kept at -20° C until processing, extraction of the AHL and analysis by
182 liquid chromatography, mass spectrometry, as described above. The degradation of each AHL
183 across three timepoints was indicated by a significantly reduced chromatographic peak area from
184 cultures of Az39 with endogenously added AHLs compared with uninoculated control samples.
185

186 **2.3.2.2. Enzymatic activity associated with the AHLs degradation**

187 A 50 µl aliquot of *A. brasilense* Az39 exponential growth culture (OD_{595} 1.0) obtained in liquid LB
188 medium was used to inoculate 100 ml capacity glass flask containing 50 ml of MMAB medium.
189 When the cultures reached OD_{595} 0.8-1.0, corresponding to exponential growth phase, they were
190 fractionated into 5 ml portions, placed in sterile 10 ml tubes, and treated individually with 10 µmol
191 l^{-1} C6-HSL, hexanoyl-homoserine lactone or 10 µmol l^{-1} C10-HSL, decanoyl-homoserine lactone
192 (University of Nottingham, UK). Then, tubes were incubated for 12 h at 37°C with 240 rpm
193 shaking. After incubation, the presence of AHLs in the culture medium was evaluated by bioassays
194 using the reporter strains as described in section 2.3.1.1. In a second experiment under similar
195 conditions, a 1 ml aliquot of the AHL-treated Az39 culture was transferred to sterile micro-tubes
196 and heated at 100°C for 10 min with the aim of inactivating the bacterial cells and denaturing the
197 proteins in the culture. An additional tube without heat treatment was used as non-denaturing
198 control. Once heating finished, 10 µmol l^{-1} of C6-AHL or C10-AHL were individually added and
199 the tubes were incubated at 37°C with 240 rpm orbital shaking. After different incubation times
200 (0.5, 1, 3, 6, 12 and 24 h), 30 µl samples were taken to be analyzed in bioassays as described above.
201 To check the cellular localization of the putative enzyme (or enzymes) involved in this activity we

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202 performed a second analysis considering an induction stage according to Uroz et al. (2007). For
203 that, Az39 grew in MMAB medium supplemented by the exogenous addition of 10 µmol l^{-1}
204 individual AHL (C6-AHL or C10-AHL), and this was defined as a pre-induced Az39 culture
205 (Az39-pi). All the treatments performed after induction are detailed at follow: T1: Non-inoculated
206 LB supplemented with 10 µmol l^{-1} AHL (control); T2: Filtered supernatant of Az39-pi + 10 µmol l^{-}
207 ^{1} AHL; T3: Heated and filtered supernatant of Az39-pi + 10 µmol l^{-1} AHL; T4: Culture of Az39-pi
208 + 10 µmol l^{-1} AHL and T5: Heated culture of Az39-pi + 10 µmol l^{-1} AHL. The addition of
209 individual AHL to each treatment depended on the reporter strain used: C6-AHL for *C. violaceum*
210 and C10-AHL for *A. tumefaciens.*

211

212 **3. Results**

213 **3.1. *In silico* analysis**

214 **3.1.1. *Quorum sensing***

215 Different bioinformatic tools were used to identify putative proteins related to canonical and non-
216 canonical QS systems in these bacteria. When the genome of several strains belonging to the genus
217 *Azospirillum* was analyzed, the presence of a coding sequence for an AHL synthase (LuxI) (EC
218 2.3.1.184) could be confirmed in only 3 of them: *A. lipoferum* TVV3, *Azospirillum* sp. B510 and
219 *Azospirillum* sp. RU38E. This protein is formed by 2 typical domains defined as IPR001690
220 (autoinducer synthase) and IPR018311 (autoinducer synthesis, conserved site) according to Venturi
221 et al. (2018). The genes encoding the AHL synthases in these *Azospirillum* strains have been
222 annotated in the UniProt database as *alpI*, AZL_a05890, *luxI* AZA_90644,
223 SAMN05880556_102381 and SAMN05880556_11440 for *A. lipoferum* TVV3 (Q19U13_AZOLI),
224 *Azospirillum* sp. B510 (D3P0E1_AZOS), the only strain containing the domain IPR018311 and
225 *Azospirillum* sp. RU38E (A0A239I230) respectively. For *A. brasilense* Az39, no homologues of
226 LuxI or another AHL synthase (LuxS, CqsA , HdtS and LuxM) involved in QS were identified.

227

228 **3.1.2. *Quorum quenching***

229 Although N-acyl-homoserine lactonases (EC: 3.1.1.81) were not found in the genome of the
230 *Azospirillum* strains analyzed, there are several N-acyl-homoserine lactone acylases (EC: 3.5.1.97)

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231 annotated for this bacterial genus in the UniProt database: *A. brasilense* Sp7 (AMK58_19595), *A.*
232 *brasilense* Sp245 (AZOBR_p1130068), *Azospirillum sp.* B510 (AZL_013430), *A. lipoferum* 4B
233 (AZOLI_p40482) and *A. thiophilum* DSM 21654 (VY88_13715), and in particular for *A.*
234 *brasilense* Az39 (ABAZ39_22635). In the RAST server, a protein annotated as penicillin acylase
235 (fig 192.31.peg.4511) was identified in plasmid 1 of the Az39 genome (Figure S4, Supplementary
236 material). Its sequence has 100% identity and homology with the sequence identified through the
237 UniProt database. In addition to penicillin acylase, an aliphatic amidase AmiE (EC. 3.5.1.4) was
238 found in the genome of Az39 (fig 192.31.peg.3259) and both enzymes have been described as
239 AHL-acylases in some databases and literature (Ochiai et al. 2014). Results found through
240 BRENDA (http://www.brenda-enzymes.org) depended on the organism studied. In the case of
241 AmiE, there are 13 recorded entries, distributed in 4 cellular locations (cytoplasmic, extracellular,
242 lysosomal and in the membrane). On the other hand, 23 entries were registered for penicillin
243 acylase, associated with 5 cellular locations in different bacteria (cytosolic, extracellular,
244 intracellular, periplasmic and in the membrane). While it is evident that there are AHL-acylase
245 enzymes with different substrate specificities, there are records of an aculeacin-A acylase, a
246 putative N-acyl-homoserine lactone acylase with *quorum-quenching* activity (EC: 3.5.1.-) from the
247 Gram negative *Ralstonia solanacearum* with the same ability to Az39 to degrade AHLs (Chen et
248 al. 2009). A more detailed analysis of the aculeacin-A acylase using both UniProt and InterPro
249 revealed a structural organization of 786 amino acids distributed in 6 protein regions: signal
250 peptide, propeptide, aculeacin-A acylase itself, the small subunit of aculeacin-A acylase, peptide
251 spacer, and the large subunit of aculeacin-A acylase (Inokoshi et al. 1992). Subsequently, a
252 BLASTP analysis was made in block with these sequences against the Az39 genome, to determine
253 if all these regions were present. Interestingly, the absent region in Az39 is the signal peptide
254 responsible for releasing the enzyme into the extracellular space, in agreement with the analysis by
255 CELLO (http://cello.life.nctu.edu.tw/), which probabilistically locates this enzyme in the cytoplasm
256 or associated to the internal membrane and periplasmic space rather than to the extracellular space
257 or external membranes.
258

259 **3.1.3. Lux R transcriptional regulators**

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260 A total of 28 LuxR transcriptional regulators were found in *A. brasilense* Az39 genome (Table 1).
261 These sequences belong to the superfamily of LuxR regulators and share between them the InterPro
262 IPR000792, helix-turn-helix (HTH) binding to the DNA C-terminal domain that is characteristic of
263 this large superfamily. Although these proteins are annotated as LuxR regulators in *A. brasilense*
264 Az39, only one of them corresponds to a typical LuxR with an N-terminal domain binding to the
265 autoinducer and could be a putative LuxR *solo* since it lacks an AHL synthase cognate enzyme. It
266 is annotated as an uncharacterized protein ABAZ39_30865 under accession UniProtKB-
267 A0A060DZQ2 and as an autoinducer-binding transcriptional regulator of the LuxR family (fig
268 192.31.peg.6164) in the UniProt database and RAST server, respectively. *A. brasilense* Az39
269 genome contains also coding sequences associated with the biosynthesis of 8 GroEL/ES-type
270 chaperone proteins, which are fundamental for folding and stability in this type of receptors. Table
271 1 summarizes the findings of the *in silico* analysis of LuxR-type regulators from several strains
272 belonging to the genus *Azospirillum.*
273
274 **3.2. *In vitro* analysis**
275 **3.2.1. Evaluation of the biosynthesis of AHLs by Az39 using reporter strains**
276 The presence of AHL molecules in filtered supernatants of *A. brasilense* Az39 was evaluated in
277 bioassays using *C. violaceum* CV026 and *A. tumefaciens* NTL4/pZLR4, reporters for short- and
278 long-chain AHLs, respectively is summarized in Figure 1. The evaluation was performed at
279 different time points in the typical growth curve using two liquid culture media and synthetic AHLs
280 as control. According to the absence of an AHL synthase in the genome of Az39 renders the
281 bacteria unable to biosynthesize this type of molecules, something that was clearly evidenced in the
282 bioassays using *C. violaceum* CV026 (Fig. 1A) and *A. tumefaciens* NTL4/pZLR4 (Fig. 1B).
283 Additional extractions with organic solvents were made from larger volumes of culture medium in
284 order to increase the concentration of possible metabolites at different time points in the growth
285 curve. None of the analyzed samples presented reporter activity due to the presence of AHL-type
286 molecules (Figure S1, Supplementary material).
287
288 **3.2.2. Evaluation of AHL degradation by Az39 using reporter strains**

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289 The degradation of exogenous AHLs in cultures of *A. brasilense* Az39 was evaluated using the
290 bioassays system as described before. The evaluation was performed at different time points of the
291 typical growth curve using uninoculated liquid culture media modified by addition of synthetic
292 AHLs as control (Figures 2A and C). To determine whether the inactivation by Az39 was of
293 enzymatic origin, a simple experiment of induction and denaturation was carried out. Figures 2B
294 and D clearly shows that degradation of AHLs by Az39 has an enzymatic origin, because the
295 denaturation of the supernatant at 100°C revealed the presence of both short-chain and long-chain
296 AHLs in the supernatants respectively.

297

298 **3.2.3. Evaluation of AHL degradation by LC-MS/MS analysis**

299 In order to validate the results obtained by the use of reporter strains regarding the ability of *A.*
300 *brasilense* Az39 to produce or degrade AHLs (4 to 14 carbon atoms), a confirmation procedure was
301 performed by the use of Liquid Chromatography coupled to Mass-Mass Spectrometry (LC-
302 MS/MS). As seen in Figure 3, no AHLs were detected in the samples obtained from Az39 cultures
303 (Az39-AHL). In samples of Az39 cultures pre-incubated with unsubstituted AHLs in C3 (Az39 +
304 AHL), AHL levels were lower than in non-inoculated LB incubated with 500 nmol l^{-1} of each AHL
305 (LB + AHL) under similar experimental conditions. A similar behavior was observed in
306 experiments by addition of AHLs substituted with the hydroxy and keto (oxo-) groups in carbon 3
307 (Figure S2 and S3, Supplementary material). These results not only demonstrate the ability of Az39
308 to degrade AHLs, but the wide spectrum of molecules that can be degraded by this bacterium,
309 making this strain a putative regulator of bacterial quorum activity in the rhizosphere of higher
310 plants.

311

312 **3.2.4. *Quorum quenching* activity is associated with Az39 cells**

313 As seen in Figure 4, activity of reporter strain *C. violaceum* CV026 and synthetic short-chain AHLs
314 confirmed the influence of the denaturation process (100 °C) on the loss of degradation activity in
315 Az39 cultures. This phenomenon was visualized as a strong decrease in violacein production at
316 increasing incubation times (Fig 4. Treatment 5). Because the inactivation of AHLs was not
317 observed in the denaturized supernatants of Az39, we assume that quenching activity must be

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318 associated with the bacterial cell. In other words, the enzyme/s responsible/s for AHL degradation
319 is/are not secreted into the culture medium by *A. brasilense* Az39. Similar results were obtained in
320 the case of long-chain AHLs and *A. tumefacines* (data not shown). In summary, these results
321 support the notion that AHL degradation by Az39 is of enzymatic character and limited to a
322 specific cellular compartment, since the enzymes do not seem to be released into the external
323 environment, which suggests that the activity could be linked to the plasma membrane or
324 periplasm.
325
326 **4. Discussion**
327 Despite genomic information currently available about the genus *Azospirillum*, little is known
328 about the molecular mechanisms related to bacterium-bacterium and bacterium-plant
329 communication. Interestingly, some reports about mechanisms based on *quorum sensing* in some
330 strains of the genus *Azospirillum* agree with the *in silico* analysis presented in this paper. Vial et al.
331 (2006) used two biosensor strains to test AHL production in 40 strains belonging to six species of
332 *Azospirillum*, obtained or isolated from different geographic locations. They found that only 3
333 strains of *A. lipoferum* (TVV3, B52, B518) and a related strain (B510) were able to produce this
334 signal molecule. We also found that the genome of *Azospirillum* sp. RU38E presents two *luxI*
335 genes that are cognate to their respective *luxRs*. In the case of *A. brasilense*, other authors recently
336 investigated QS mechanisms in Ab-V5 and Ab-V6, the strains most commonly used for inoculant
337 formulation in Brazil (Fukami et al. 2017). They found no genes associated with an AHL synthase
338 but multiple LuxR *solos* in the genome, although their publication does not include a detailed
339 analysis. Similarly, in the case of *A. brasilense* Az39, there is no *luxI* gene associated with the
340 production of AHLs, something which was subsequently confirmed *in silico* and *in vitro* by both
341 the use of reporter strains *C. violaceum* CV026 and *A. tumefaciens* NTL4/pZLR4, and the LC-
342 MS/MS analysis. Several genes encoding putative proteins related to QS systems were identified in
343 this paper, but the absence of LuxI in all *A. brasilense* strains suggests that AHL production may
344 not be related to this bacteria species.
345 On the other hand, *A. brasilense* Az39 contains a luxR orphan or solo. An analysis of multiple
346 sequence alignment of this LuxR compared with LuxR cognates and LuxR solos already described

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347 in the literature allowed to show that some amino acid residues characteristic of the N-terminal
348 domain of binding to the autoinductor remain conserved, which classified them outside the family
349 of typical LuxR regulators (Data not shown). The conservation of amino acid residues present in
350 the LuxR of Az39 is a fact that could be associated with LuxRs that respond to exogenous AHLs
351 (by "eavesdropping") from bacteria with which they share niche and/or other molecules chemically
352 similar from their host plants (Patel et al., 2013, Venturi et al., 2018).
353 Signaling mediated by *quorum sensing* in bacteria can be interrupted by a wide variety of
354 phenomena collectively known as *quorum quenching*. The coding sequence for a N-acyl-
355 homoserine lactone acylase (EC: 3.5.1.97) was found in *A. brasilense* Az39, *A. brasilense* Sp7; *A.*
356 *brasilense* Sp245, *Azospirillum* sp. B510, *A. lipoferum* 4B and *A. thiophilum*. These findings
357 suggest that mechanisms of quorum signal interception prevail in different species of the genus,
358 regardless of whether they produce such molecules or not. In addition, the appearance of such
359 mechanisms in these strains, and especially in *A. brasilense* Az39, points towards the important
360 role this kind of regulation fulfils, not only in selecting the ecological niche and exchanging signals
361 with the host plant, but also in adapting to a lifestyle in the rhizospheric environment. We also
362 demonstrated, through the use of reporter strains, that the inactivation of synthetic AHLs by Az39
363 was related to an enzyme activity. In this sense, the capacity of this strain to degrade AHL was
364 confirmed *in vitro* and justified by the presence of two coding sequences for two putative AHL-
365 acylases. Considering the results, we obtained in this paper using reporter strains, the tentative
366 location of the putative AHL-acylase activity would be a cellular compartment, likely the plasmatic
367 membrane or the periplasmic space.
368 The ability of *A. brasilense* Az39 to degrade AHLs of different lengths (4 to 14 C) was confirmed
369 by the use of LC-MS/MS. According to the treatments proposed, the AHL levels in pure Az39
370 cultures incubated with unsubstituted AHLs and substituted at C3 were lower than in non-
371 inoculated LB medium. These results unequivocally indicate that although *A. brasilense* Az39 does
372 not produce AHLs, it is capable of degrading them in liquid culture conditions.
373 We compared the penicillin acylase (AHL-acylase) coding sequence in the genome of Az39 with
374 the *in silico* and *in vitro* characterization by Mukherji et al. (2014) of a Penicillin-G-acylase from
375 *Kluyvera citrophila*, an enzyme that also has the ability to cleave AHLs, and found them to have

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376 high similarity. This is an important biotechnological approach that represents a new positioning in
377 the large-scale production of biofunctional enzymes that govern the flow of chemical information
378 in the rhizosphere, where complex bacterial communication networks take place. In this sense,
379 several experiments have shown how plants respond to QS signals such as the AHLs produced by
380 Gram negative bacteria (Bauer and Mathesius, 2004, Von Rad et al. 2008). It is currently known
381 that plants, in addition to responding to AHLs, produce molecules that can mimic such QS signals
382 by somehow manipulating behavioral mechanisms associated with bacteria in the rhizosphere
383 (Teplitski et al. 2000; Corral-Lugo et al. 2016). On the other hand, Palmer et al. (2014) showed that
384 plants can produce AHL-acylase enzymes using L-homoserine for their own benefit. The
385 accumulation of L-homoserine has several effects on plant growth: it increases transpiration which
386 favors nutrient uptake by the roots, promotes defense responses mediated by Ca^{2+}, stimulates the
387 production of ethylene and promotes the synthesis of auxins. This last effect is correlated in the
388 rhizosphere with the capacity of *A. brasilense* Az39 to produce several phytohormones, auxins
389 among them (Cassán and Diaz Zorita 2016). This, coupled with its AHL *quorum quenching*
390 capacity, enhances the synergy of the interaction between Az39 and the plant.
391 The results obtained in this paper suggest that under the prevailing conditions in the rhizosphere,
392 Az39 is mute in the sense that it cannot speak the language mediated by AHLs, but it can to
393 interrupt conversations between other bacteria and plants by a *quorum quenching* mechanism. This
394 mechanism could regulate the capacity of the microbial populations interacting with plants and this
395 should be investigated in further experiments.
396

397 **5. Acknowledgments**

398 We thank Universidad Nacional de Río Cuarto (UNRC), Consejo Nacional de Investigaciones
399 Científicas y Tecnológicas (CONICET) and Fondo Nacional de Ciencia y Tecnología (FONCyT).
400 JG and AC are former PhD students at the UNRC granted by CONICET.
401

402 **6. Conflicts of interest**

403 The authors report no conflicts of interest.
404

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405 **7. References**

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523

524 **Table Headers**

525 **Table 1.** Details of the regulatory LuxR/I proteins present in the different *Azospirillum* strains

526

527 **Figure Legends**

528 **Figure 1.** Evaluation of violacein production and β-galactosidase activity induced by the presence
529 of AHLs in cultures of Az39. A: Bioassay using *C. violaceum* CV026. C (control): 10 µmol l^{-1} C6-
530 AHL. Treatments 1, 3, 4 and 5: filtered supernatants obtained from LB culture medium at 6 (OD_{595}
531 0.546); 12 (OD_{595} 1.186); 24 (OD_{595} 1.514) and 48 (OD_{595} 2.161) h after inoculation with Az39,
532 respectively. Treatment 2: non inoculated LB culture medium modified by addition of C6-AHL. B:
533 Bioassay using *A. tumefaciens* NTL4/pZLR4; C (control): 10 µmol l^{-1} C10-AHL. Treatments 2, 3,
534 4 and 5: filtered supernatants obtained from LB culture medium at 6 (OD_{595} 0.543); 12 (OD_{595}
535 0.923); 24 (OD_{595} 1.529) and 48 (OD_{595} 2.187) h after inoculation with Az39, respectively.
536 Treatment 1: non inoculated LB culture medium modified by addition of C10-AHL. The OD_{595}
537 values were obtained from average of 3 biological samples.

538

539 **Figure 2.** Evaluation of violacein production and β-galactosidase activity induced by the presence
540 of AHLs in cultures of Az39 using *C. violaceum* CV026 and *A. tumefaciens* NTL4/pZLR4 as
541 reporter strains. **A.** Left: Induction bioassay. Treatment 1: LB + 10 µmol l^{-1} of C6-HSL. Treatments
542 2 and 3: Az39 + 10 µmol l^{-1} of C6-AHL. **A.** Right: Denaturation bioassay. Treatment 1: LB + 10
543 µmol l^{-1} of C6-AHL. Treatments 2, 3, 4 and 5: Az39 + 10 µmol l^{-1} of C6-AHL after 30 min, 1, 3
544 and 6 h of incubation respectively. C (control): 10 µmol l^{-1} C6-AHL. **B.** Left: Induction bioassay.
545 Treatment 1: LB + 10 µmol l^{-1} C10-AHL; treatment 2: Az39 + 10 µmol l^{-1} of C10-AHL. Right:
546 Denaturation bioassay. Treatment 1: LB + 10 µmol l^{-1} of C10-AHL. Treatments 2, 3, 4 and 5: Az39
547 + 10 µmol l^{-1} of C10-AHL after 30 min, 1, 3 and 6 h of incubation respectively. C (control): 10
548 µmol l^{-1} C10-AHL.

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549

550 **Figure 3.** Identification and relative quantification of AHLs by liquid chromatography coupled to
551 mass-mass spectrometry (LC-MS/MS). In the experiments AHLs of 4 to 14 unsubstituted carbon
552 atoms were used at a final concentration of 500 nmol l^{-1}. The bars represent a mean peak area
553 calculated from three biological replicates of the following treatments: Az39 + AHLs, Az39 -
554 AHLs and non inoculated LB + AHLs after 1, 3 and 6 h of incubation time. Columns marked with
555 a different letter of the same group of treatments differ significantly by Tukey *post hoc* test at $p<$
556 0.05.

557

558 **Figure 4.** Evaluation of violacein production induced by the presence of AHLs in cultures of Az39
559 using *C. violaceum* CV026. C (control): 10 µmol l^{-1} C6-AHL. Treatment 1: LB modified with 10
560 µmol l^{-1} C6-AHL. Treatment 2: Filtered supernatant of Az39 (pi) + LB modified with 10 µmol l^{-1}
561 C6-AHL Treatment 3: Filtered supernatant of Az39 (pi) denaturized at 100°C + LB modified with
562 10 µmol l^{-1} C6-AHL. Treatment 4: Culture of Az39 (pi) denaturized at 100°C + LB modified with
563 10 µmol l^{-1} C6-AHL. Treatment 5: Culture of Az39 (pi) + LB modified with 10 µmol l^{-1} C6-AHL.
564 The bioassays were performed at 0, 6 and 16 h after addition of AHLs.

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565

566 **Supplementary material**

567 **Figure S1.** Evaluation of violacein production and β-galactosidase activity induced by the
568 presence of AHLs in cultures of Az39. Right: Bioassay using *C. violaceum* CV026. C (control): 10
569 µmol l^{-1} C6-AHL. Treatments 2 and 3: filtered supernatants obtained from different stages of Az39
570 growth curve at DO_{595} 0.823 and 1.654, respectively. Treatment 1: non inoculated LB culture
571 medium modified by addition of C6-AHL. Left: Bioassay using *A. tumefaciens* NTL4/pZLR4. C
572 (control): 10 µmol l^{-1} C10-AHL. Treatments 2, 3, 4 and 5: filtered supernatants obtained from Az39
573 growth curve at OD_{595} 0.621, 1.054 and 1.872 respectively. Treatment 1: non inoculated LB culture
574 medium modified by addition of C10-AHL.

575

576 **Figure S2.** Identification and relative quantification of AHLs by liquid chromatography coupled to
577 mass-mass spectrometry (LC-MS/MS). In the experiments AHLs of 4 to 14 carbon atoms

578 substituted at C3 with a hydroxyl group (-OH) were used at a final concentration of 500 nmol l^{-1}.
579 The bars represent a mean peak area calculated from three biological replicates of the following
580 treatments: Az39 + AHLs, Az39-AHLs and non inoculated LB + AHLs after 1, 3 and 6 h of
581 incubation time. Columns marked with a different letter of the same group of treatments differ
582 significantly by Tukey *post hoc* test at $p < 0.05$.

583

584 **Figure S3.** Identification and relative quantification of AHLs by liquid chromatography coupled to
585 mass-mass spectrometry (LC-MS/MS). In the experiments AHLs of 4 to 14 carbon atoms
586 substituted at C3 with an oxo group (-oxo) were used in a final concentration of 500 nmol l^{-1}. The
587 bars represent a mean peak area calculated from three biological replicates of the following
588 treatments: Az39 + AHLs, Az39-AHLs and non inoculated LB + AHLs after 1, 3 and 6 h of
589 incubation time. Columns marked with a different letter of the same group of treatments differ
590 significantly by Tukey *post hoc* test at $p < 0.05$.

591

592 **Figure S4.** Structural organization of the Az39 genome at the level of the putative Penicillin
593 acylase.

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Figure 1

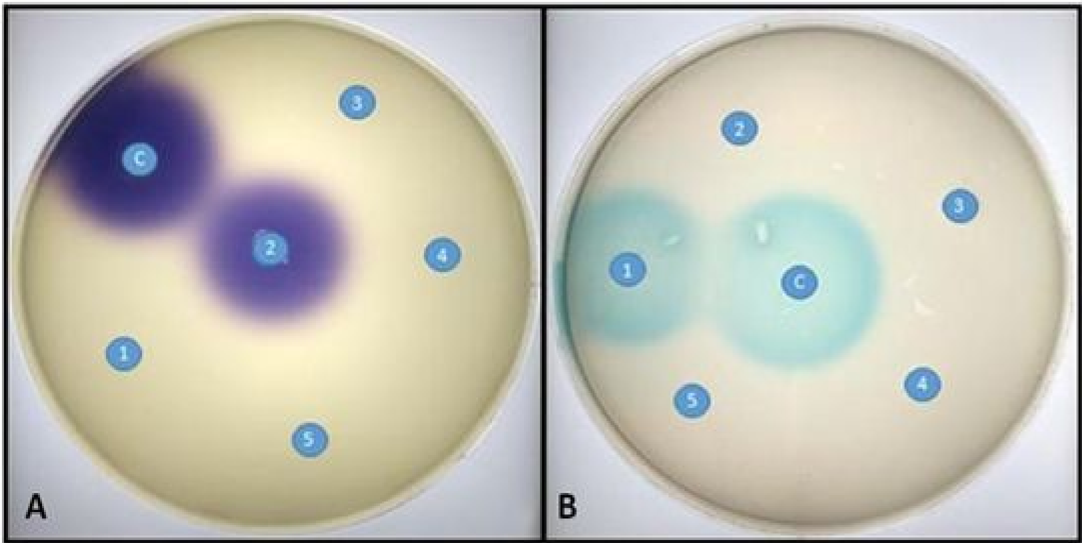

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Figure 2

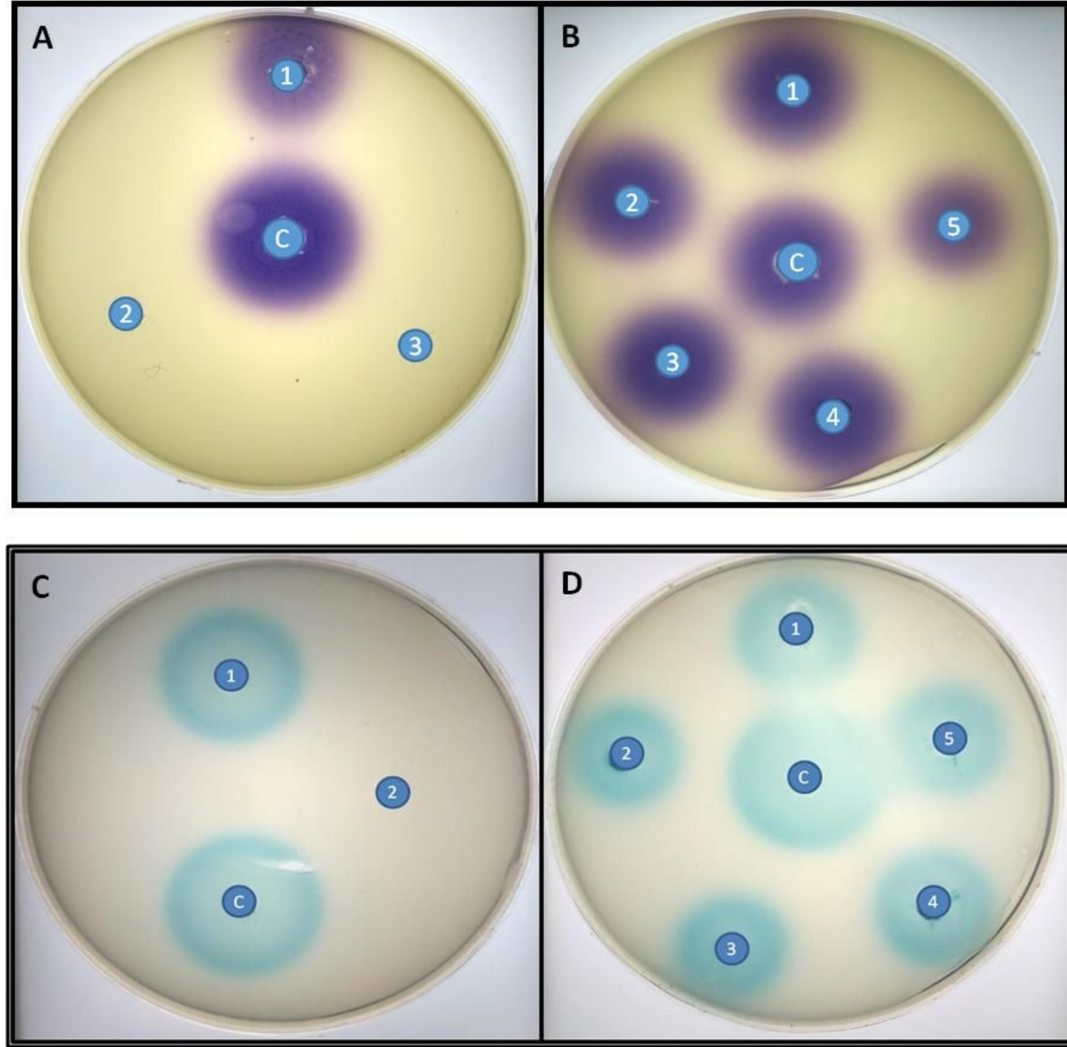

Figure 3

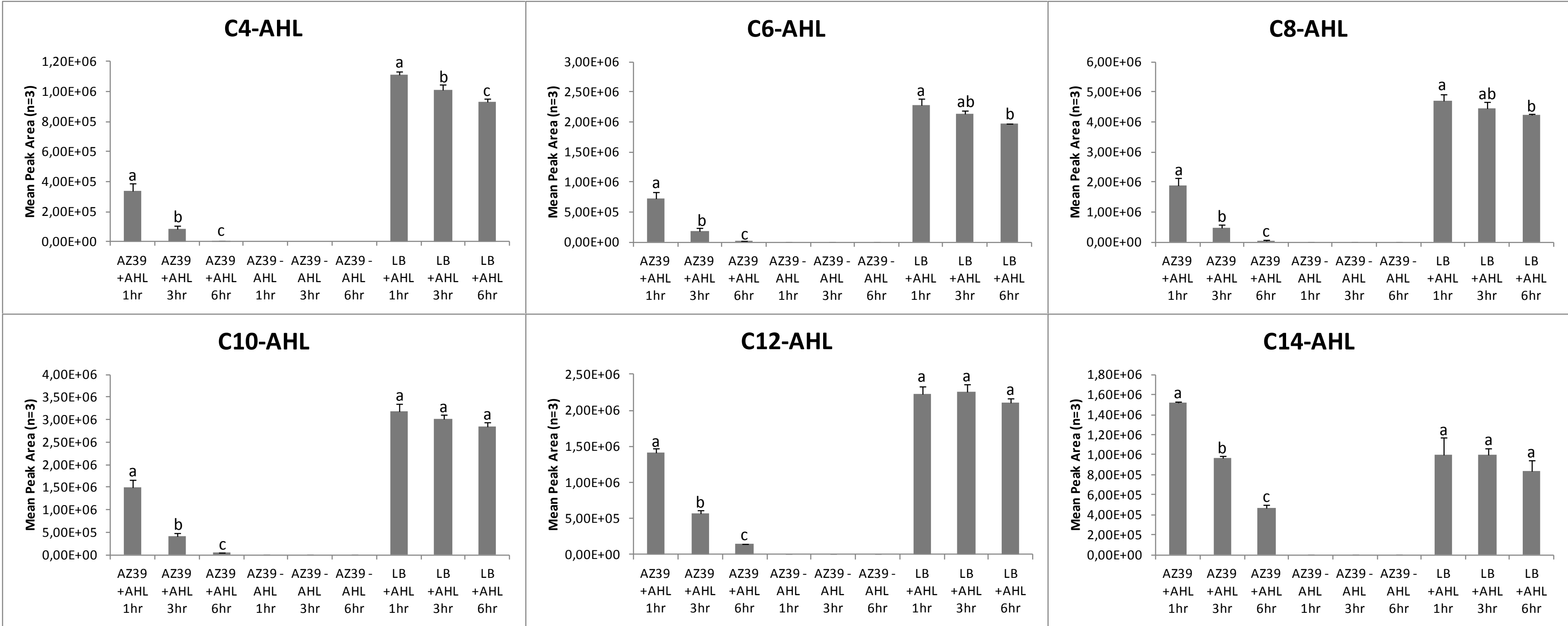

Figure 4

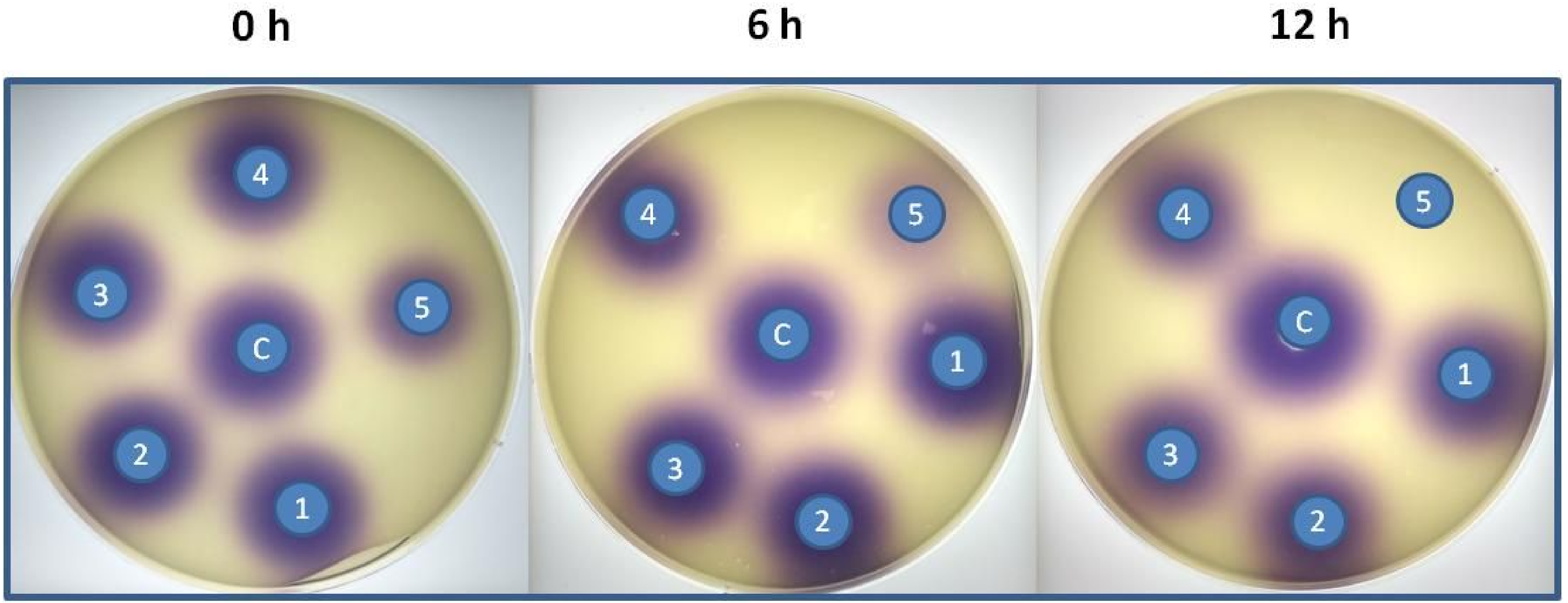

Table 1.

Bacterial Strains*	P-LuxR	P-LuxI	LuxR-C	LuxR-SP
Az39	28	-	-	1
SgZ-5	2	-	-	1
Sp245	25	-	-	-
B510	27	1	1	-
4B	27	-	-	-
TVV3	1	1	1	-
RU38E	2	2	2	-
DSM 21654	61	-	-	-
CAG:260	2	-	-	-
CAG:239	2	-	-	-
Cd	2	-	-	-

*Az39, Sp245 y Cd (*A. brasilense*); B510, CAG:239, CAG:260 y RU38E (*Azospirillum* sp.); B4 y TVV3 (*A. lipoferum*); SgZ-5 (*A. humicireducens*); DSM 21654 (*A. thiophilum*). P-LuxR: LuxR-type proteins; P-LuxI: LuxI-type proteins; LuxR-C: Cognate LuxR homologs; LuxR-SP: Putative-orphan LuxR homologs

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Figure S1

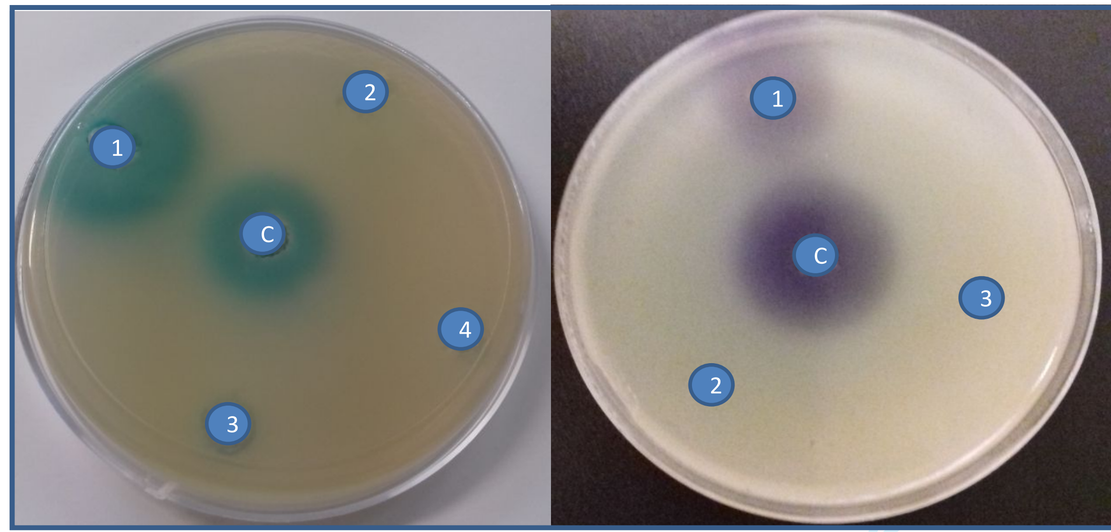


Figure S2

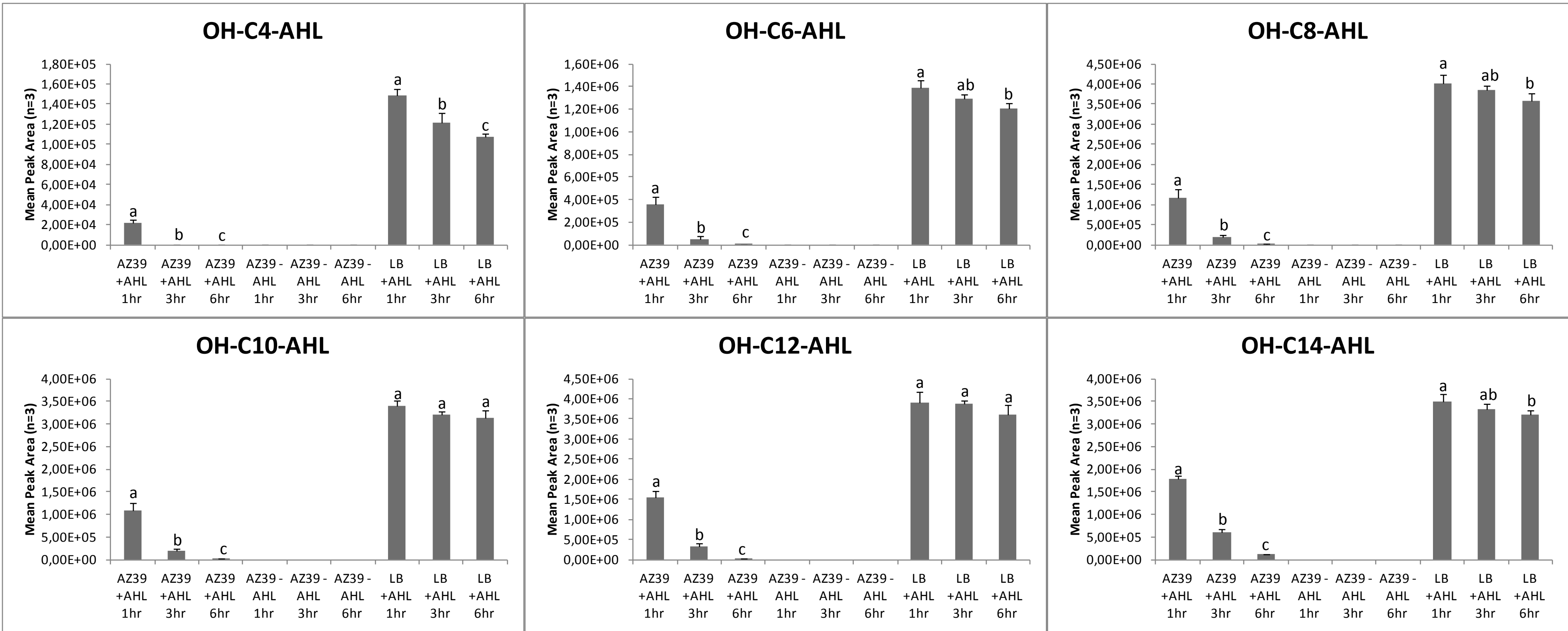


Figure S3

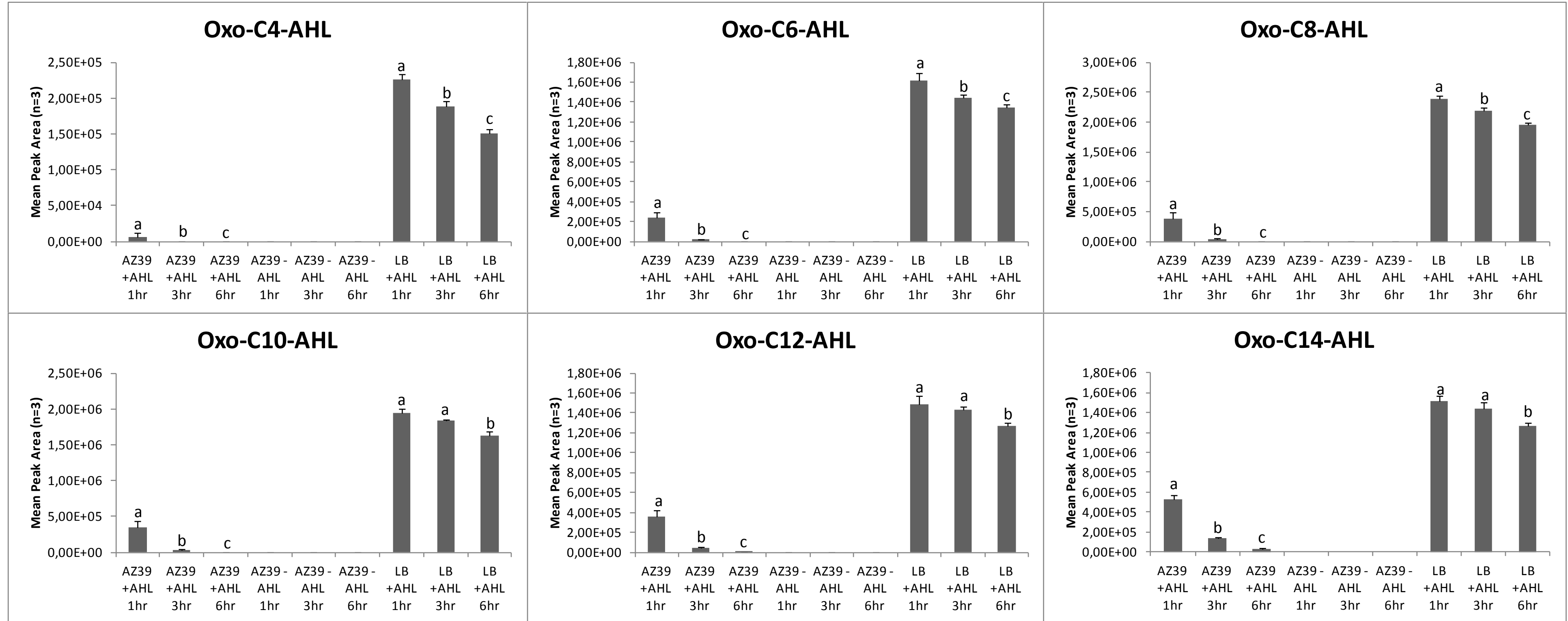
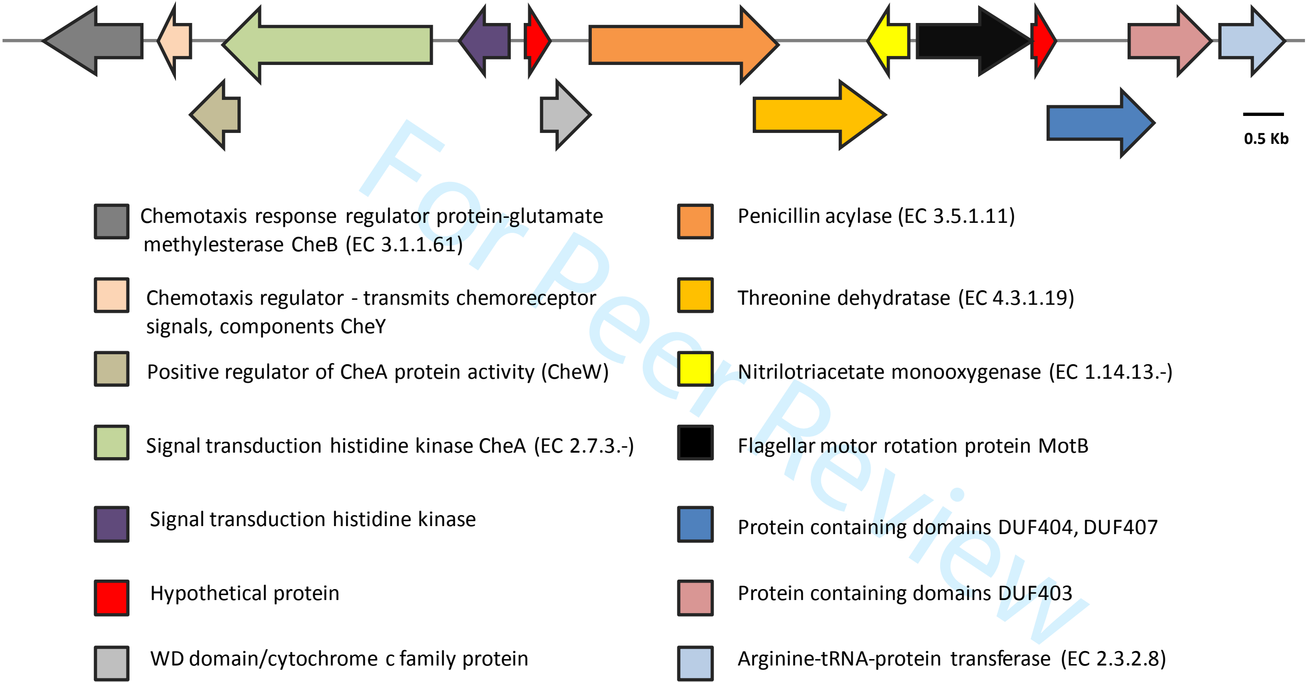


Figure S4



For Peer Review