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

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

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

**Aims**

The aim of this research was to analyze the *Quorum sensing* (QS) and *Quorum quenching* (QQ) mechanisms based on N-acyl-L-homoserine lactones (AHLs) in *A. brasilense* Az39, a strain with remarkable capacity to benefit a wide range of crops under agronomic conditions.

**Methods and Results**

We performed an *in silico* and *in vitro* analysis of the quorum mechanisms in *A. brasilense* Az39. The results obtained *in vitro* by the use of the reporter strains *C. violaceum* and *A. tumefaciens* and Liquid Chromatography coupled to Mass-Mass Spectrometry (LC-MS/MS) analysis shown that although Az39 does not produce molecules AHL, it is capable of degrading them by at least two hypothetical enzymes identified by bioinformatics approach, associated to the bacterial cell. In Az39 inoculated cultures incubated with 500 nmol $l^{-1}$ of the C3 unsubstituted AHLs (C4, C6, C8, C10, C12, C14), AHL levels were lower than non-inoculated LB media controls. Similar results were observed upon addition of AHLs with hydroxy (OH-) and keto (oxo-) substitutions in carbon 3. These results not only demonstrate the ability of Az39 to degrade AHLs, but the wide spectrum of molecules that can be degraded by this bacterium.

**Conclusions**

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

**Significance and Impact of Study**

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

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

## 1. Introduction

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

bp sequence called the *lux* box, from the *luxI* promoter region, in the form of a LuxR-autoinducer complex. This leads to transcriptional activation or repression, thus expressing a particular phenotype.

On the other hand, some bacterial strains present quorum systems with a non-cognate LuxR protein (i.e. they lack LuxI) and they thus respond to other signal molecules. These systems are called LuxR orphans or LuxR *solos* (Patankar and Gonzalez 2009), and in some cases they act in concert with the LuxR/LuxI canonical system. The appearance of LuxR-*solos* regulators indicates that these protein families could be involved in intra-kingdom or inter-kingdom signaling systems through the detection of different compounds produced by other prokaryotes or eukaryotes organisms (Patankar and Gonzalez 2009, Patel et al. 2013).

In nature, there are also bacterial mechanisms that inactivate quorum signals called *Quorum Quenching* (QQ) (Zhang 2003). These can generally act both at the level of signal generation and reception. Although there are several QS mechanisms involving inhibitory proteins and/or AHL antagonist molecules, the mechanisms that involve enzymes are widespread in different environments. Three main enzymatic QQ mechanisms have been clearly described: (1) hydrolysis of the lactone ring (AHL lactonase activity), (2) hydrolysis of the amide bound (AHL acylase activity), and (3) modification of the acyl chain (AHL oxidase and reductase activity) (Uroz et al. 2009), but they have not been studied in depth in soil bacteria. As occurs in the QS system, QQ mechanisms can serve in particular environments to modulate the interaction between a bacterial community and eukaryotic organisms (Tait et al. 2009).

Soil bacteria living in the rhizosphere, or rhizobacteria, have the ability to associate with numerous plant species. If this association is beneficial for plant growth or development, they are called Plant Growth Promoting Rhizobacteria or PGPR (Kloepper et al. 1989). Among the most successful associations and therefore the most studied in nature, are those related to the genus *Azospirillum* sp. The ability of these rhizobacteria to promote plant growth depends mainly on the presence of one or more mechanisms that might act individually or in synch on the physiology or metabolism of the colonized plant (Bashan and de-Bashan 2010)

*A. brasilense* Az39 was isolated in 1982 from surface-sterilized wheat seedlings in Marcos Juarez, Córdoba, Argentina, evaluated under agronomic conditions and selected based on its ability to

increase crop yields of maize and wheat under agronomic conditions (Díaz-Zorita and Canigia 2009). *A. brasilense* Az39 has been widely used in agriculture in America during the last 40 years (Cassán and Díaz Zorita 2016). The potential mechanisms responsible for growth promotion in this strain have been partially unraveled (Perrig et al. 2007, Cassán et al. 2009). Despite its agro-economic importance and the fact that several genomes from this genus have been sequenced, such as those belonging to *Azospirillum* sp. B510, *A. lipoferum* 4B, *A. brasilense* Sp245, CBG497 and Az39 (Kaneko et al. 2010, Wisniewski-Dyé et al. 2011, Wisniewski-Dyé et al. 2012, Rivera et al. 2014), there are few reports related to bacterial capacity to produce AHL-like molecules and/or other phenomena associated with quorum mechanisms. Therefore, there is little understanding about the *Azospirillum-Azospirillum, Azospirillum*-bacteria and *Azospirrillum*-plant interactions mediated by quorum mechanisms, highlighting the need for a more exhaustive genomic-functional analysis of these bacteria due their agricultural and economic interest. Considering this background, the main objective of this work was to analyze both *in silico* and *in vitro* the *quorum sensing* and *quorum quenching* phenomenon mediated by AHLs in the model strain *A. brasilense* Az39.

## 2. Material and Methods

### 2.1. Bacterial strains and growth conditions

*A. brasilense* Az39 was obtained from the Bacterial Culture Collection at the INTA-IMYZA, Castelar, Buenos Aires, Argentina (WDCM31). Pure cultures of *A. brasilense* Az39 were obtained in Petri dishes containing Luria-Bertani medium (Miller 1972) modified by the addition of 15 ml $l^{-1}$ Congo Red (LB-RC) or MMAB minimal medium (Vanstockem et al. 1987). Typical colonies from such media were used to inoculate LB liquid medium in 100 ml flasks and cultured at 37°C with 240 rpm shaking until late exponential growth phase was reached. *Chromobacterium violaceum* CV026 (McClean et al. 1997) grew in LB medium supplemented with 25 µg $ml^{-1}$ kanamycin (Km). *Agrobacterium tumefaciens* NTL4/pZLR4 (Cha et al. 1998) was cultured in AT medium (Morton and Fuqua, 2013) supplemented with 50 µg $ml^{-1}$ gentamicin (Gm). These two strains were used as reporter strains in the bioassays described below.

## 2.2. *In silico* analysis of quorum mechanisms in *A. brasilense* Az39

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

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

### 2.3.1. *Quorum sensing*

#### 2.3.1.1. Evaluation of production of AHLs by bioassays

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

the reproducibility of the methodology and the stability of the AHL molecules in the Petri dish during incubation. Experiments were carried out in triplicate.

**2.3.1.2. Evaluation of production of AHLs by Az39 using Liquid Chromatography coupled to Mass-Mass Spectrometry (LC-MS/MS) analysis**

**2.3.1.2.1. Extraction of AHLs from Az39 cultures**

Typically, *A. brasilense* AZ39 colonies grown on LBRC medium were used to inoculate 250 ml of LB medium and incubated at 37°C, with shaking (200 rpm) until stationary growth phase had been reached. Aliquots (100 ml) of centrifuged (5 min at 10000 rpm), and sterile filtered supernatant (0.22 µm, Millipore Express PLUS) were acidified to pH 2 with the addition of HCl. Supernatant samples were extracted three times by liquid-liquid extraction using an equal volume of acidified ethyl acetate (1% (v/v) AcOH in EtOAc). Combined extracts were dried under vacuum and stored at -80° C prior to analysis.

**2.3.1.2.2. LC-MS/MS analysis**

The LC-MS/MS analysis of extracted samples was conducted as previously described (Ortori et al. 2011) with minor modification. Dried extracts were re-dissolved in 50 µl of 0.1% (v/v) formic acid in MeOH. The chromatography column used was a Phenomenex Gemini C18 (3.0 µm, 150 x 3.0 mm), and the mobile phases used were 0.1 % (v/v) formic acid and 0.1% (v/v) formic acid in methanol. The analysis was conducted with the MS operating in multiple reaction monitoring (MRM) mode, simultaneously screening the LC eluent for all specific AHLs, comparing the retention time of detected analytes with authentic synthetic standards. For each detected chromatographic peak a mean peak area was calculated from three biological replicates.

**2.3.2. *Quorum quenching***

**2.3.2.1. Evaluation of degradation of AHLs by Az39 by LC-MS/MS analysis**

A set of 9 glass flasks of 50 ml capacity containing 20 ml of LB medium was prepared. Only 6 were inoculated with 20 µl of Az39 culture obtained from liquid LB medium in late exponential

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

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

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

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

## 3. Results

### 3.1. *In silico* analysis

#### 3.1.1. *Quorum sensing*

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

#### 3.1.2. *Quorum quenching*

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

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

**3.1.3. Lux R transcriptional regulators**

A total of 28 LuxR transcriptional regulators were found in *A. brasilense* Az39 genome (Table 1). These sequences belong to the superfamily of LuxR regulators and share between them the InterPro IPR000792, helix-turn-helix (HTH) binding to the DNA C-terminal domain that is characteristic of this large superfamily. Although these proteins are annotated as LuxR regulators in *A. brasilense* Az39, only one of them corresponds to a typical LuxR with an N-terminal domain binding to the autoinducer and could be a putative LuxR *solo* since it lacks an AHL synthase cognate enzyme. It is annotated as an uncharacterized protein ABAZ39_30865 under accession UniProtKB-A0A060DZQ2 and as an autoinducer-binding transcriptional regulator of the LuxR family (fig 192.31.peg.6164) in the UniProt database and RAST server, respectively. *A. brasilense* Az39 genome contains also coding sequences associated with the biosynthesis of 8 GroEL/ES-type chaperone proteins, which are fundamental for folding and stability in this type of receptors. Table 1 summarizes the findings of the *in silico* analysis of LuxR-type regulators from several strains belonging to the genus *Azospirillum.*

### 3.2. *In vitro* analysis

#### 3.2.1. Evaluation of the biosynthesis of AHLs by Az39 using reporter strains

The presence of AHL molecules in filtered supernatants of *A. brasilense* Az39 was evaluated in bioassays using *C. violaceum* CV026 and *A. tumefaciens* NTL4/pZLR4, reporters for short- and long-chain AHLs, respectively is summarized in Figure 1. The evaluation was performed at different time points in the typical growth curve using two liquid culture media and synthetic AHLs as control. According to the absence of an AHL synthase in the genome of Az39 renders the bacteria unable to biosynthesize this type of molecules, something that was clearly evidenced in the bioassays using *C. violaceum* CV026 (Fig. 1A) and *A. tumefaciens* NTL4/pZLR4 (Fig. 1B). Additional extractions with organic solvents were made from larger volumes of culture medium in order to increase the concentration of possible metabolites at different time points in the growth curve. None of the analyzed samples presented reporter activity due to the presence of AHL-type molecules (Figure S1, Supplementary material).

#### 3.2.2. Evaluation of AHL degradation by Az39 using reporter strains

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

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

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

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

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

associated with the bacterial cell. In other words, the enzyme/s responsible/s for AHL degradation is/are not secreted into the culture medium by *A. brasilense* Az39. Similar results were obtained in the case of long-chain AHLs and *A. tumefacines* (data not shown). In summary, these results support the notion that AHL degradation by Az39 is of enzymatic character and limited to a specific cellular compartment, since the enzymes do not seem to be released into the external environment, which suggests that the activity could be linked to the plasma membrane or periplasm.

## 4. Discussion

Despite genomic information currently available about the genus *Azospirillum*, little is known about the molecular mechanisms related to bacterium-bacterium and bacterium-plant communication. Interestingly, some reports about mechanisms based on *quorum sensing* in some strains of the genus *Azospirillum* agree with the *in silico* analysis presented in this paper. Vial et al. (2006) used two biosensor strains to test AHL production in 40 strains belonging to six species of *Azospirillum*, obtained or isolated from different geographic locations. They found that only 3 strains of *A. lipoferum* (TVV3, B52, B518) and a related strain (B510) were able to produce this signal molecule. We also found that the genome of *Azospirillum* sp. RU38E presents two *luxI* genes that are cognate to their respective *luxRs*. In the case of *A. brasilense*, other authors recently investigated QS mechanisms in Ab-V5 and Ab-V6, the strains most commonly used for inoculant formulation in Brazil (Fukami et al. 2017). They found no genes associated with an AHL synthase but multiple LuxR *solos* in the genome, although their publication does not include a detailed analysis. Similarly, in the case of *A. brasilense* Az39, there is no *luxI* gene associated with the production of AHLs, something which was subsequently confirmed *in silico* and *in vitro* by both the use of reporter strains *C. violaceum* CV026 and *A. tumefaciens* NTL4/pZLR4, and the LC-MS/MS analysis. Several genes encoding putative proteins related to QS systems were identified in this paper, but the absence of LuxI in all *A. brasilense* strains suggests that AHL production may not be related to this bacteria species.

On the other hand, *A. brasilense* Az39 contains a luxR orphan or solo. An analysis of multiple sequence alignment of this LuxR compared with LuxR cognates and LuxR solos already described

in the literature allowed to show that some amino acid residues characteristic of the N-terminal domain of binding to the autoinductor remain conserved, which classified them outside the family of typical LuxR regulators (Data not shown). The conservation of amino acid residues present in the LuxR of Az39 is a fact that could be associated with LuxRs that respond to exogenous AHLs (by "eavesdropping") from bacteria with which they share niche and/or other molecules chemically similar from their host plants (Patel et al., 2013, Venturi et al., 2018).

Signaling mediated by *quorum sensing* in bacteria can be interrupted by a wide variety of phenomena collectively known as *quorum quenching*. The coding sequence for a N-acyl-homoserine lactone acylase (EC: 3.5.1.97) was found in *A. brasilense* Az39, *A. brasilense* Sp7; *A. brasilense* Sp245, *Azospirillum* sp. B510, *A. lipoferum* 4B and *A. thiophilum*. These findings suggest that mechanisms of quorum signal interception prevail in different species of the genus, regardless of whether they produce such molecules or not. In addition, the appearance of such mechanisms in these strains, and especially in *A. brasilense* Az39, points towards the important role this kind of regulation fulfils, not only in selecting the ecological niche and exchanging signals with the host plant, but also in adapting to a lifestyle in the rhizospheric environment. We also demonstrated, through the use of reporter strains, that the inactivation of synthetic AHLs by Az39 was related to an enzyme activity. In this sense, the capacity of this strain to degrade AHL was confirmed *in vitro* and justified by the presence of two coding sequences for two putative AHL-acylases. Considering the results, we obtained in this paper using reporter strains, the tentative location of the putative AHL-acylase activity would be a cellular compartment, likely the plasmatic membrane or the periplasmic space.

The ability of *A. brasilense* Az39 to degrade AHLs of different lengths (4 to 14 C) was confirmed by the use of LC-MS/MS. According to the treatments proposed, the AHL levels in pure Az39 cultures incubated with unsubstituted AHLs and substituted at C3 were lower than in non-inoculated LB medium. These results unequivocally indicate that although *A. brasilense* Az39 does not produce AHLs, it is capable of degrading them in liquid culture conditions.

We compared the penicillin acylase (AHL-acylase) coding sequence in the genome of Az39 with the *in silico* and *in vitro* characterization by Mukherji et al. (2014) of a Penicillin-G-acylase from *Kluyvera citrophila*, an enzyme that also has the ability to cleave AHLs, and found them to have

high similarity. This is an important biotechnological approach that represents a new positioning in the large-scale production of biofunctional enzymes that govern the flow of chemical information in the rhizosphere, where complex bacterial communication networks take place. In this sense, several experiments have shown how plants respond to QS signals such as the AHLs produced by Gram negative bacteria (Bauer and Mathesius, 2004, Von Rad et al. 2008). It is currently known that plants, in addition to responding to AHLs, produce molecules that can mimic such QS signals by somehow manipulating behavioral mechanisms associated with bacteria in the rhizosphere (Teplitski et al. 2000; Corral-Lugo et al. 2016). On the other hand, Palmer et al. (2014) showed that plants can produce AHL-acylase enzymes using L-homoserine for their own benefit. The accumulation of L-homoserine has several effects on plant growth: it increases transpiration which favors nutrient uptake by the roots, promotes defense responses mediated by $Ca^{2+}$, stimulates the production of ethylene and promotes the synthesis of auxins. This last effect is correlated in the rhizosphere with the capacity of *A. brasilense* Az39 to produce several phytohormones, auxins among them (Cassán and Diaz Zorita 2016). This, coupled with its AHL *quorum quenching* capacity, enhances the synergy of the interaction between Az39 and the plant.

The results obtained in this paper suggest that under the prevailing conditions in the rhizosphere, Az39 is mute in the sense that it cannot speak the language mediated by AHLs, but it can to interrupt conversations between other bacteria and plants by a *quorum quenching* mechanism. This mechanism could regulate the capacity of the microbial populations interacting with plants and this should be investigated in further experiments.

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## 6. Conflicts of interest

The authors report no conflicts of interest.

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**Table Headers**

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

**Figure Legends**

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

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

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.

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

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

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

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

Figure 1

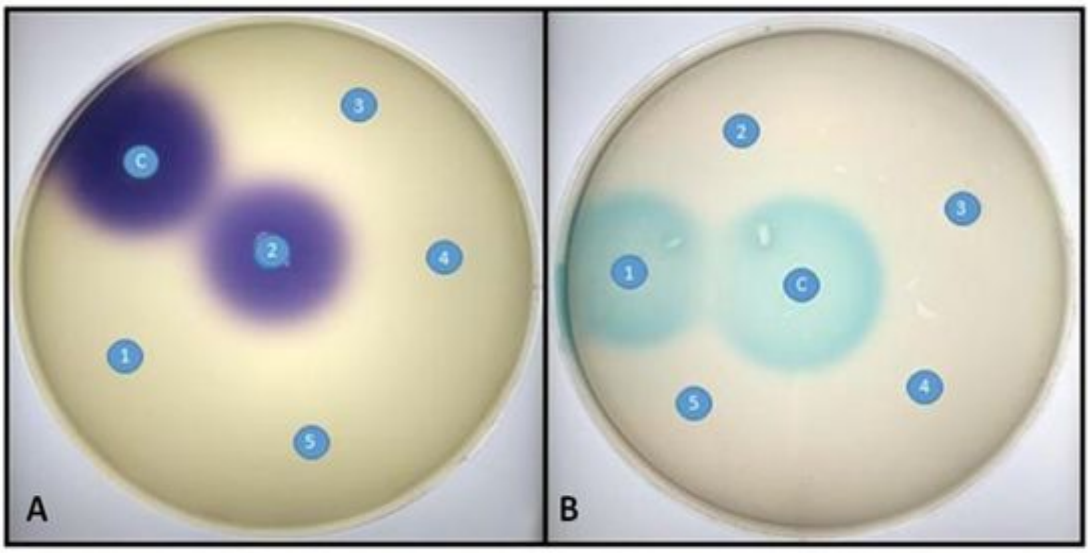


Figure 2

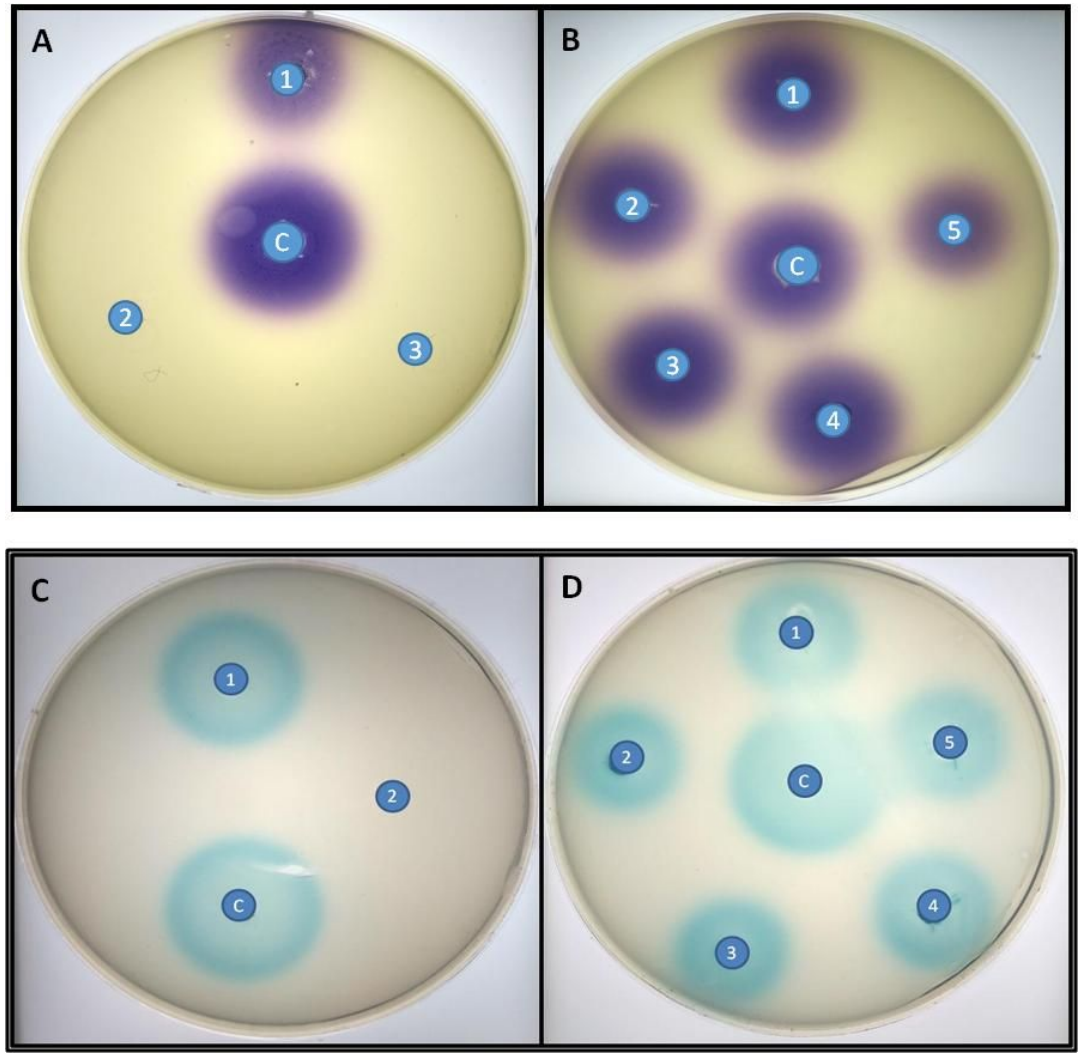
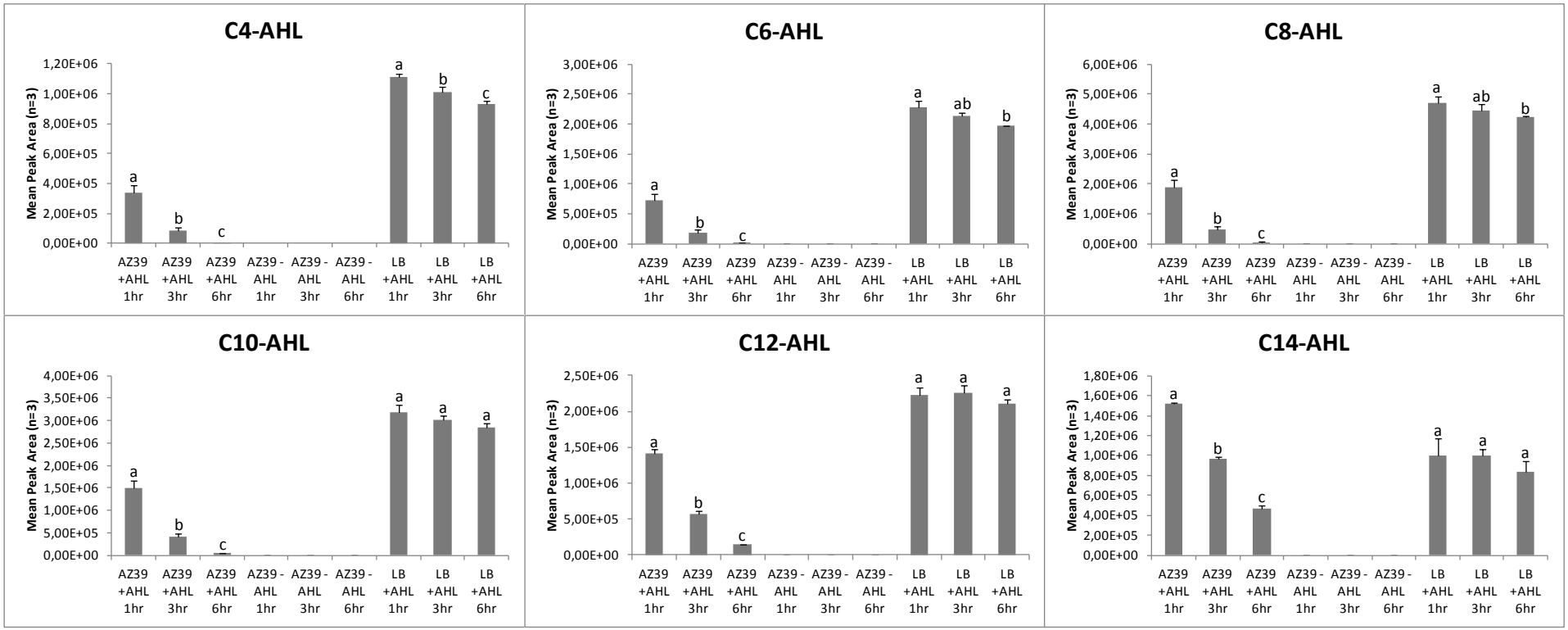


Figure 3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0,00E+00
a
b
c
a
b
c
AZ39 +AHL 1hr
AZ39 +AHL 3hr
AZ39 +AHL 6hr
AZ39 - AHL 1hr
AZ39 - AHL 3hr
AZ39 - AHL 6hr
LB +AHL 1hr
LB +AHL 3hr
LB +AHL 6hr
C6-AHL
Mean Peak Area (n=3)
3,00E+06
2,50E+06
2,00E+06
1,50E+06
1,00E+06
5,00E+05
0,00E+00
a
b
c
a
ab
b
C8-AHL
Mean Peak Area (n=3)
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5,00E+06
4,00E+06
3,00E+06
2,00E+06
1,00E+06
0,00E+00
a
b
c
a
ab
b
C10-AHL
Mean Peak Area (n=3)
4,00E+06
3,50E+06
3,00E+06
2,50E+06
2,00E+06
1,50E+06
1,00E+06
5,00E+05
0,00E+00
a
b
c
a
a
a
C12-AHL
Mean Peak Area (n=3)
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2,00E+06
1,50E+06
1,00E+06
5,00E+05
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a
b
c
a
a
a
C14-AHL
Mean Peak Area (n=3)
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a
a
a

Figure 4

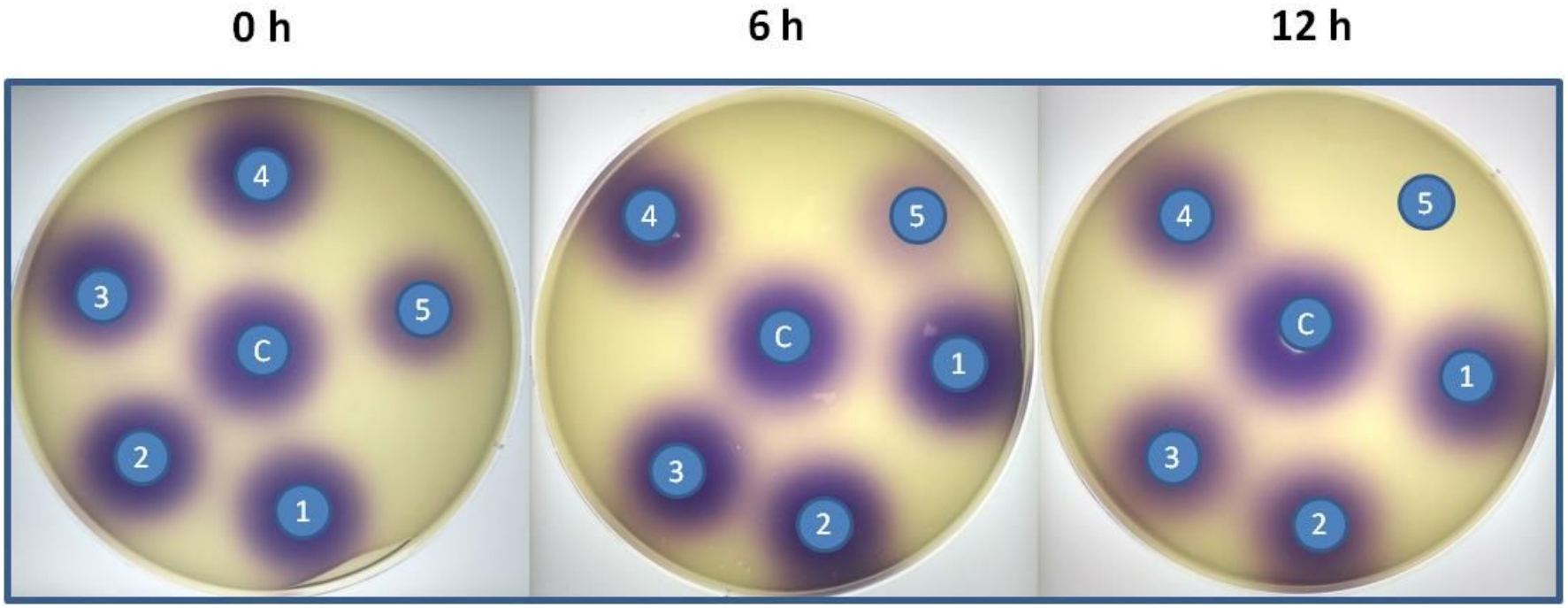


**Table 1.**

| 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

Figure S1

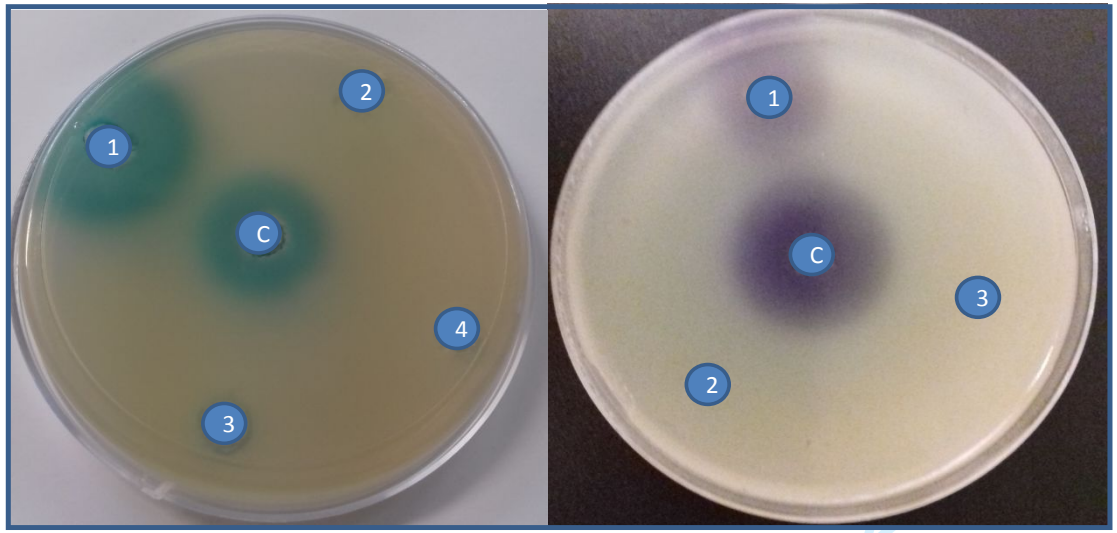
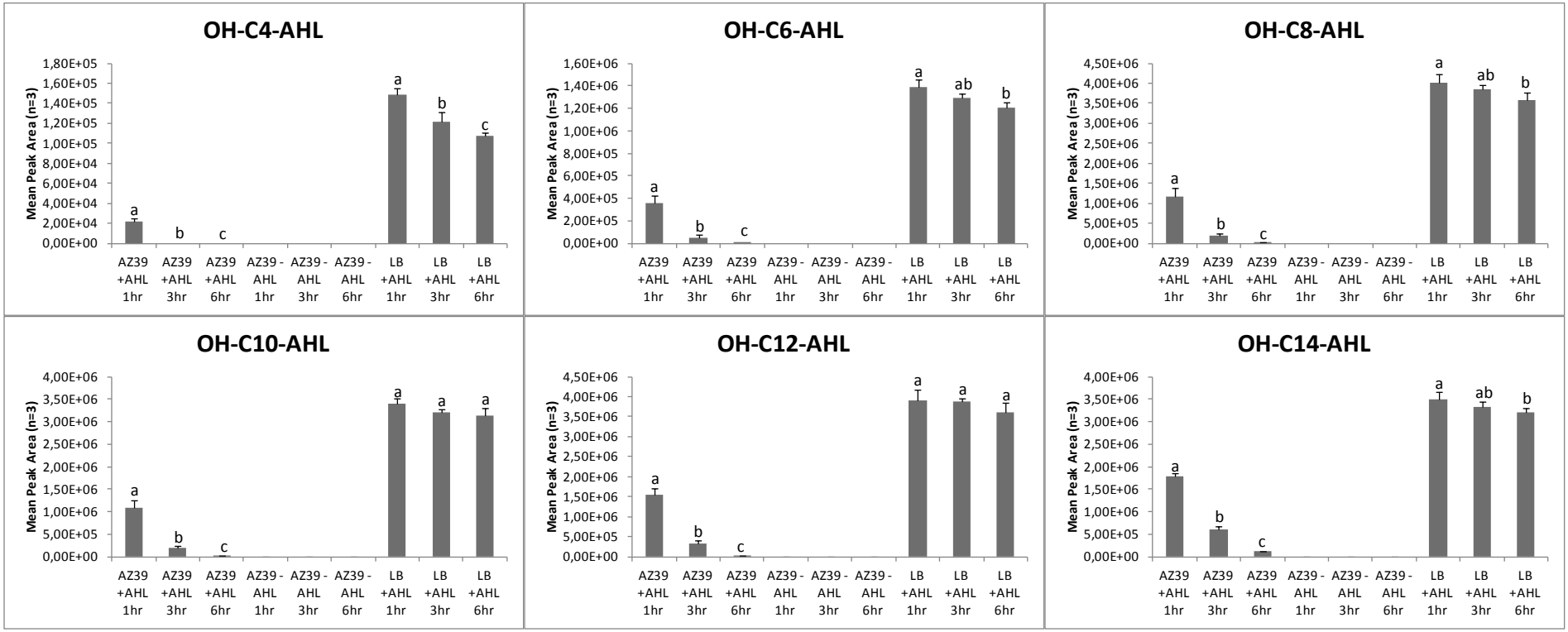


Figure S2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2,00E+04
0,00E+00
a
b
c
a
b
c
AZ39 +AHL 1hr
AZ39 +AHL 3hr
AZ39 +AHL 6hr
AZ39 - AHL 1hr
AZ39 - AHL 3hr
AZ39 - AHL 6hr
LB +AHL 1hr
LB +AHL 3hr
LB +AHL 6hr
OH-C6-AHL
Mean Peak Area (n=3)
1,60E+06
1,40E+06
1,20E+06
1,00E+06
8,00E+05
6,00E+05
4,00E+05
2,00E+05
0,00E+00
a
b
c
a
ab
b
OH-C8-AHL
Mean Peak Area (n=3)
4,50E+06
4,00E+06
3,50E+06
3,00E+06
2,50E+06
2,00E+06
1,50E+06
1,00E+06
5,00E+05
0,00E+00
a
b
c
a
ab
b
OH-C10-AHL
Mean Peak Area (n=3)
4,00E+06
3,50E+06
3,00E+06
2,50E+06
2,00E+06
1,50E+06
1,00E+06
5,00E+05
0,00E+00
a
b
c
a
a
a
OH-C12-AHL
Mean Peak Area (n=3)
4,50E+06
4,00E+06
3,50E+06
3,00E+06
2,50E+06
2,00E+06
1,50E+06
1,00E+06
5,00E+05
0,00E+00
a
b
c
a
a
a
OH-C14-AHL
Mean Peak Area (n=3)
4,00E+06
3,50E+06
3,00E+06
2,50E+06
2,00E+06
1,50E+06
1,00E+06
5,00E+05
0,00E+00
a
b
c
a
ab
b

Figure S3

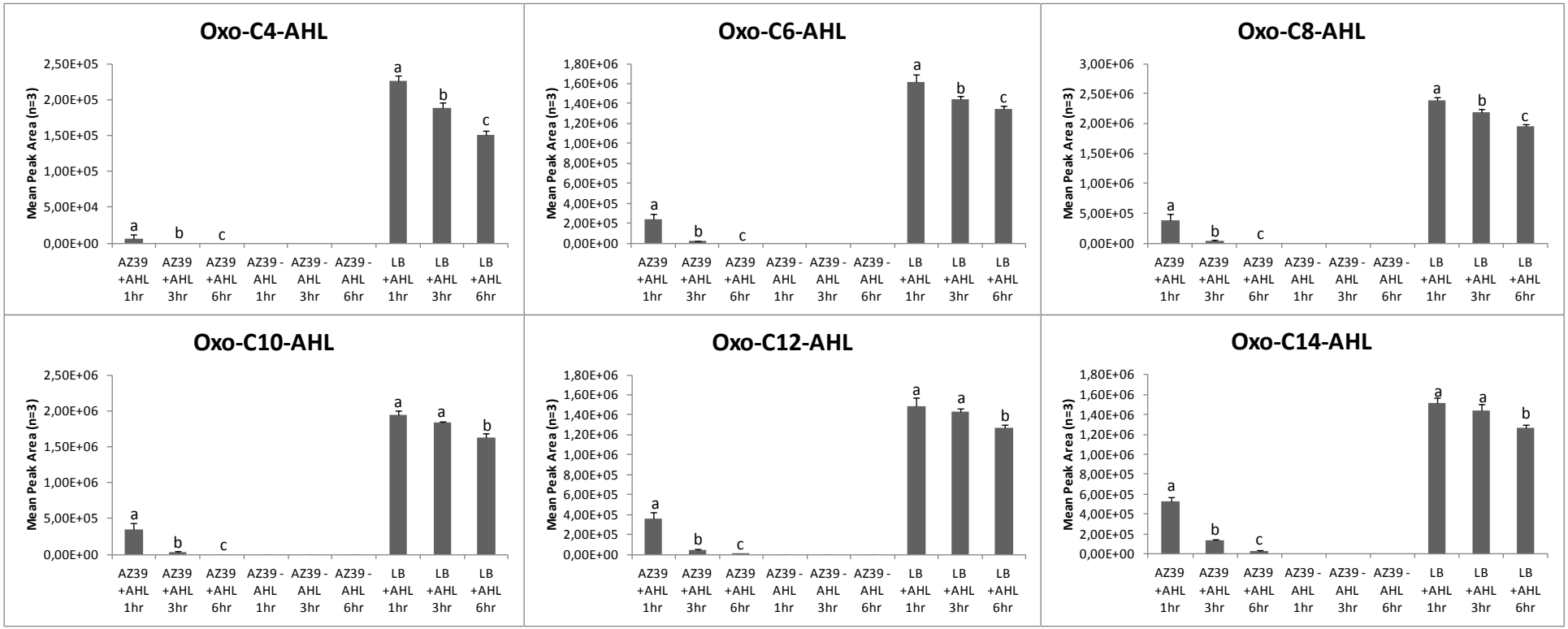
Oxo-C4-AHL
Mean Peak Area (n=3)
2,50E+05
2,00E+05
1,50E+05
1,00E+05
5,00E+04
0,00E+00
a
b
c
a
b
c
AZ39 +AHL 1hr
AZ39 +AHL 3hr
AZ39 +AHL 6hr
AZ39 - AHL 1hr
AZ39 - AHL 3hr
AZ39 - AHL 6hr
LB +AHL 1hr
LB +AHL 3hr
LB +AHL 6hr
Oxo-C6-AHL
Mean Peak Area (n=3)
1,80E+06
1,60E+06
1,40E+06
1,20E+06
1,00E+06
8,00E+05
6,00E+05
4,00E+05
2,00E+05
0,00E+00
a
b
c
a
b
c
AZ39 +AHL 1hr
AZ39 +AHL 3hr
AZ39 +AHL 6hr
AZ39 - AHL 1hr
AZ39 - AHL 3hr
AZ39 - AHL 6hr
LB +AHL 1hr
LB +AHL 3hr
LB +AHL 6hr
Oxo-C8-AHL
Mean Peak Area (n=3)
3,00E+06
2,50E+06
2,00E+06
1,50E+06
1,00E+06
5,00E+05
0,00E+00
a
b
c
a
b
c
AZ39 +AHL 1hr
AZ39 +AHL 3hr
AZ39 +AHL 6hr
AZ39 - AHL 1hr
AZ39 - AHL 3hr
AZ39 - AHL 6hr
LB +AHL 1hr
LB +AHL 3hr
LB +AHL 6hr
Oxo-C10-AHL
Mean Peak Area (n=3)
2,50E+06
2,00E+06
1,50E+06
1,00E+06
5,00E+05
0,00E+00
a
b
c
a
a
b
AZ39 +AHL 1hr
AZ39 +AHL 3hr
AZ39 +AHL 6hr
AZ39 - AHL 1hr
AZ39 - AHL 3hr
AZ39 - AHL 6hr
LB +AHL 1hr
LB +AHL 3hr
LB +AHL 6hr
Oxo-C12-AHL
Mean Peak Area (n=3)
1,80E+06
1,60E+06
1,40E+06
1,20E+06
1,00E+06
8,00E+05
6,00E+05
4,00E+05
2,00E+05
0,00E+00
a
b
c
a
a
b
AZ39 +AHL 1hr
AZ39 +AHL 3hr
AZ39 +AHL 6hr
AZ39 - AHL 1hr
AZ39 - AHL 3hr
AZ39 - AHL 6hr
LB +AHL 1hr
LB +AHL 3hr
LB +AHL 6hr
Oxo-C14-AHL
Mean Peak Area (n=3)
1,80E+06
1,60E+06
1,40E+06
1,20E+06
1,00E+06
8,00E+05
6,00E+05
4,00E+05
2,00E+05
0,00E+00
a
b
c
a
a
b
AZ39 +AHL 1hr
AZ39 +AHL 3hr
AZ39 +AHL 6hr
AZ39 - AHL 1hr
AZ39 - AHL 3hr
AZ39 - AHL 6hr
LB +AHL 1hr
LB +AHL 3hr
LB +AHL 6hr

Figure S4