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Chapter 2

Plant Growth-Promoting Bacteria: The Role of Chemotaxis in the Association Azospirillum Brasilense-Plant

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Abstract

The genus *Azospirillum* belongs to the plant growth-promoting bacteria group, capable of positively influence the growth and yield of numerous plant species, many of them with agronomic and ecological importance. Plant growth promotion is largely determined by efficient colonization of the rhizosphere (soil influenced by roots and microorganisms). Root exudates constitute the most significant source of

nutrients in the rhizosphere and seem to participate in the early colonization by inducing the chemotactic response of bacteria. Therefore, chemotaxis is considered an essential mechanism for the successful root colonization by *Azospirillum.*

In this chapter we present a background and new insights on *Azospirillum* chemotaxis, concerning the genetic aspects and its use addressing biotechnological application. First, we demonstrate that genetic complementation of a mutant strain, impaired in surface motility led to identification of the gene *chsA* (chemotactic signaling protein). The deduced translation product, ChsA protein, contained a PAS sensory domain and EAL active site domain. This latter has phosphodiesterase activity (PDE-A) for the hydrolysis of c-di-GMP [cyclic-bis (3' –5') dimeric GMP], a compound known to function as a second messenger in different cellular processes including motility, biofilm formation and cellular differentiation.

After cloning *chs*A, ChsA protein was expressed and purified by affinity chromatography. ChsA activity in presence of *bis*-*p*-nitro phenyl-phosphate was 0.59-ÂµM min^{-1} mg^{-1} protein, demonstrating that it displayed phosphodiesterase activity. This suggests that ChsA is a component of the signaling pathway controlling chemotaxis in *Azospirillum.* Then we propose that the redox state of the cell is sensed through the PAS domain and directly coupled to the transmitter EAL module, showing PDE-A activity.

Second, the chemotaxis of different strains of A. brasilense toward strawberry root exudates was investigated. The agar-plate assay was used, including two concentrations of exudates from three commercial varieties of strawberry, collected at different time intervals. To quantify the chemotactic response, the capillary method was used. In all cases, a positive chemotactic reaction was found, revealing higher responses in endophytic than in rhizospheric strains, being this strain-specific. Furthermore, the variation of the chemotactic response observed depended on the concentration and time to collect the exudates, as well as the total sugars content. Considering that A. brasilense, posses biotechnological application, addressing to a sustainable agriculture, determining the genes and mechanisms involved in chemotaxis response, as well as the level of activity of strains to root exudates may represent an initial step in selecting them for use as inoculants in different crops.

Introduction

Up to date, biofertilizers are considered a component of the integrated plant nutrition management. They are defined as substances that contain live

microorganisms which, when applied to seeds, roots, plant surfaces or soil, colonize the rhizosphere or inside the plant and promote growth by increasing the availability of nutrients and plant protection in the host (Vessey, 2003). In this context, the plant growth-promoting bacteria are considered as an alternative to the use of chemical fertilizers and pesticides (Kloepper and Beauchamp, 1992).

At laboratory and at field experimental conditions, the effect of biofertilizers has been recognized as a form of sustainable management in agroecosystems (Dobbelaere et al., 2003, Lucy et al., 2004); however, the success of using these biofertilizers lies in the study of compatibility strains and specificity for certain crops and environmental soil conditions.

The large scale use of beneficial microorganisms as biofertilizers in any agricultural production system would bring great benefits because they are cheaper than those of synthetic origin; have positive effects on plants (similar to a chemical fertilizer) and do not exert harmful ecological impacts on the environment or human health.

Most of the bacterial-plant associations occur at the rhizosphere, the soil area that is strongly influenced by plant roots (Vanbleu and Vanderleyden, 2003). These associations are initiated in response to exchange of signals triggered from the microbe-plant interaction (Vanbleu and Vanderleyden, 2003). The rhizosphere is rich in nutrients, due to the accumulation of a variety of organic compounds released by root through exudation, secretion, and deposition (Curl and Truelove, 1986). These compounds can be used as carbon and energy sources by microorganisms, stimulating the microbial activity, particularly intense in the rhizosphere. This was reflected by the number of bacteria found around the roots that are generally 10 to 100 times higher than outside the rhizosphere (Weller and Thomashow, 1994).

The root-associated bacteria capable to stimulate plant growth are generally known as plant growth-promoting rhizobacteria (PGPR) (Davison, 1988; Kloepper *et al*., 1989). Among them are found species belonging to several genera: *Azotobacter*, *Azospirillum*, *Azoarcus*, *Klebsiella*, *Bacillus*, *Pseudomonas*, *Arthrobacter*, *Enterobacter*, *Burkholderia*, *Serratia* and *Rhizobium*.

The mechanisms by which PGPR promote plant growth are not fully elucidated but it is known they include: phytohormones production (Egamberdiyeva, 2007; Shaharoona *et al.,* 2006), biological nitrogen fixation (Mrkovacki and Milic, 2001; Salantur *et al*., 2006), antagonism against pathogens by production of siderophores, the synthesis of antibiotics, enzymes

and/or antifungal compounds (Ahmad *et al*., 2006; Jeun *et al*., 2004), and also by the solubilization of phosphates and other nutrients (Cattelan *et al*., 1999).

In many crops of agronomic importance significant increases in growth and yield in response to inoculation with PGPR has been reported (Asghar *et al*., 2002; Bashan *et al*., 2004; Biswas, *et al*., 2000). The application of this type of rhizobacteria has resulted in an evident plant growth-promotion, observing an increase in the emergence, vigour, biomass production, root system development and enhancement of up to 30% in the production of commercially important crops, such as sorghum (Raju *et al*., 1999; Vikram, 2007), tomato (Gravel *et al*., 2007; Siddiqui and Shaukat, 2002), maize (Kozdroja *et al*., 2004), wheat (Ozturk *et al*., 2003; Khalid, 2004), and various grains (Dobbelaere *et al*., 2001).

The genus *Azospirillum* belongs to the α-subdivision of Protobacteria and has been isolated from the rhizosphere of many plant species in different regions of the world, including tropical and temperate climates. The species belonging to the genus are motile and exhibit chemotaxis to several compound found in roots exudates. The bacterial mobility in the rhizosphere responds to the chemotaxis, which allows them to move towards the roots to obtain benefits from root exudates (source of carbon and nitrogen).

Azospirillum was found in places where the oxygen concentration is optimal for biological nitrogen fixation (Barak *et al*., 1982); some associate on the root surface and others manage to colonize the root interior (Döbereneir and Pedrosa, 1987), where there is less competition for available substrates, which is very important due to the high demand for energy required for nitrogen fixation (Falk *et al*., 1985).

Motility provides a survival advantage under a wide variety of environments, allowing bacteria to respond to beneficial or negative conditions and to compete successfully with other microorganisms. *Azospirillum brasilense* possesses a polar flagellum in all culture conditions, and synthesizes lateral flagella when growing on semisolid media (Hall and Krieg, 1983). The flagella of *Azospirillum* are one of the most complex and extremely effective organelle of locomotion, capable of propelling the bacterium through liquids (swimming) and through viscous environments or over surfaces (swarming). In addition, these organelles play an important role in adhesion to substrates and biofilm formation contributing to the interaction with the plant (Croes *et al.* 1993). The colonization of at least part of the root system is required for the beneficial effects of *Azospirillum* inoculants preparations for applications such as biofertilization and phyto-stimulation (Okon and Vanderleyeden 1997).

Many bacteria use a complex behaviour called taxis to sense specific chemicals or environmental conditions and move towards attractants and away from repellents (Adler, 1966). Bacterial taxis is directly involved in interactions with both animal and plant host (Kato *et al.* 2008; de Weert, 2002). Taxis, especially chemotaxis, together with the mechanism of signal transduction and response regulation, have been well studied in *Escherchia coli* and *Salmonella enterica* serovar Typhimurium (Stock and Surette, 1996). Chemotactic ligands are detected by cell surface chemoreceptors called methyl–accepting chemotactic proteins (MCPs). Upon binding a chemotactic ligand, MCPs generate chemotactic signals that are communicated to the flagellar motor via a series of chemotaxis (Che) proteins, *E. coli* possesses 6 Che proteins, one of them, the CheA, is a histidine protein kinase that autophosphorylates at a specific histidine residue to form CheA~P. The phosphoryl group of CheA~P is transferred to a specific aspartate residue of CheY, to form activated CheY~P which is a response regulator of a two component regulatory system, that interacts directly with the flagellar motor switch protein to control the direction of flagellar rotation. CheZ acts as a negative regulator involved in inactivation of CheY~P by dephosphorylation of CheY~P to CheY. MCPs, with help from CheW, modulate the autophosphorylation activity of CheA in response to temporal changes in stimuli intensity. MCPs undergo reversible methylation at several glutamate residues mediated by CheR and CheB that are methyltransferase and methyl esterase enzymes, respectively. CheB is another response regulator, which is phosphorylated by CheA~P to form CheB~P. CheA~P exhibits higher methylesterase activity than CheB and is not affected by environmental stimuli. CheR continually adds methyl groups to MCPs, and constitutes the via of adaptation response. The methylation level of MCPs is controlled in response to environmental stimuli and affects their conformation. This reversible methylation of MCPs is required for temporal sensing of chemical gradients (Stock and Surette, 1996).

Pedraza *et al.* (2007) demonstrated the natural occurrence of *A. brasilense* colonizing strawberry plants, including inner tissues of roots and stolons. The latter would provide an additional agronomic advantage, considering the asexual propagation of those plants in commercial nurseries, by stolon fixation on the ground. Thus, if the strawberry plants are inoculated with *Azospirillum* strains selected by their PGPR characteristics, the presence of this bacterium could be insured in their descendants.

From the results of inoculating strawberry plants with different strains of *Azospirillum*, and considering that the best plant growth depends on the

interaction between specific genotypes of bacteria and plants (Pedraza *et al.* 2009), the use of this genus represents an interesting option for higher agricultural production and significant ecological advantages. Hence to increase the knowledge of the *Azospirillum*-plant interaction, in the present chapter we consider the chemotaxis as a starting point for a successful partnership.

Physiology of *Azospirillum* Chemotaxis

It has been demonstrated that *A. brasilense* polar flagellum rotates in both clockwise and counterclockwise directions. The last one rotation causes forward movement of free-swimming cells, while the change in the direction of rotation to clockwise cause a reversal in swimming direction. When *Azospirillum* cells are exposed to malate, a strong attractant, some effects in swimming behaviour are observed: suppression of direction change, the chemotaxis, and a long term-increase in swimming speed as chemokinesis (Zhulin and Armitage, 1993). In fact, *Azospirillum* strains responded chemotactically to temporal gradient of some effectors such as amino acids, sugars and organic acids, as well as to maize mucilage. This behaviour is strain dependent, suggesting that a certain degree of specificity exists in the establishment of plant-bacteria interaction (Reinhold *et al.* 1985; Mandimba, *et al.* 1986). The presence of oxidizable substrates increased the number of attracted bacteria only 1.2 to 3-folds, a rather low ratio, compared with other bacteria. Further work revealed that the attraction was to oxygen gradient dissolved in water, and the exposure of the cells to an oxygen gradient followed by aerotaxis, masked the chemotactic response of the bacteria (Barak *et al.* 1982). Therefore, aerotaxis is an important response in *A. brasilense,* which guide the bacterial to a preferred low oxygen concentration for energy generation (3 to 5 μM). Indeed, the proton motive force was lower at oxygen concentrations that were higher or lower than the preferred oxygen concentration. It was suggested for *A. brasilense* that to reach the optimal oxygen concentration was relevant for energy generation and nitrogen fixation in the rhizosphere (Zhulin *et al.* 1996).

It was described that there are several ways to sense chemicals: Chemotaxis usually is referred as to metabolism-independent behaviour, while the use for “energy taxis” denotes metabolism-dependent behaviour. Energy taxis is broadly defined as a behavioural response to stimuli that affect cellular energy levels. It includes responses directly linked to electron transport/energy

generation, such as aerotaxis, redotaxis and phototaxis (Taylor and Zhulin, 1998). The most important behaviour in *A. brasilense* is energy taxis and it was demonstrated that the compounds which are attractants for *Azospirillum* cells are metabolizable subtracts, while their nonmetabolizable analogues are not attractants. On the other hand, the inhibition of the metabolism of a chemical attractant completely abolishes chemotaxis to this compound. Moreover, it was observed the correlation between the efficiency of a chemical as a growth substrate and as a chemoeffector (Alexandre *et al,* 2000). Chemicals that interact directly as inhibitors of electron transport were found to be strong repellents, and most important, a mutant lacking the cytochrome ccb_3-type terminal oxidase had significantly diminished chemotaxis to all major attractants, but only under microaerobic conditions. When it was assayed under fully aerobic conditions, where this respiratory system is not functional, chemotactic responses in the mutant and wild-type strain were identical. The results showed that the signal for chemotaxis toward major attractants and repellents is originated within a functional electron transport system in *A. brasilense* (Alexandre *et al,* 2000).

The Research for the Chemoreceptors in *Azospirillum*

Characterization of chemoreceptors capable of measuring changes within the electron transport system is required in order to conclusively establish sensing mechanisms. The search for plant-inducible bacterial genes from *A. brasilense* led to identification of a 40kDa protein, which was induced in presence of wheat root exudates. The protein was proteolytic cleavage and the sequence of two peptides was used to obtain the corresponding gene, named *sbpA*. The cloning, sequencing and bioinformatics analysis of the coding DNA region revealed significant homology with ChvE protein from *Agrobacterium tumefaciens.* ChvE is a periplasmic sugar-binding protein, also functions in the uptake of sugars and chemotaxis of *A. tumefaciens* towards sugars (Van Bastelaere *et al.* 1999). Further, it was determined the role of SbpA in chemotaxis activity towards D-galactose, L-arabinose and D-fucose. It was interesting to note that the response of *A. brasilense* to sugars was inducible. This was confirmed by the expression analysis of a transductional fusion *sbpA::gusA*. D-galactose, L-arabinose and D-fucose strongly induced the gene expression. Furthermore, the mutant *sbpA::km*^{R} was severely affected in uptake of D-galactose. Then, SbpA is part of the binding protein-dependent, a high affinity uptake system, and is required for chemotaxis towards D-

galactose. Additionally, the chemosensory pathway seems to be dependent on the uptake and metabolism of the attractant (Van Bastelaere *et al.* 1999).

A protein acting as energy taxis chemoreceptor named Tlp1 (for transducer-like protein 1) was identified in *A. brasilense* Sp7 strain. The protein structure comprises the functional domains characteristics of chemoreceptors. It has a membrane topology typical of classical membrane chemoreceptors, the N-terminal periplasmic sensing domain, and a C-terminal region which consists of a HAMP (histidine kinase, adenylcyclase, methyl binding proteins, and phosphate) domain and a signalling module containing the HCD (highly conserved domain) and two methylation regions typical of chemosensors (Greer-Phillips, *et al.* 2004). The gene mutated *tlp1* exhibited a phenotype deficient in chemotaxis to several oxidizables subtracts, taxis to terminal electron acceptor such as oxygen and nitrate, and redox taxis to substituted quinones. Altogether, suggested that Tlp1 mediates energy taxis in *A. brasilense*. Furthermore, the *tlp1* mutant was severely affected in colonization to wheat root as defined by qualitative and quantitative ß-galactosidase assays. This indicated that Tlp1 protein was acting as chemoreceptor guiding the bacterium by allowing it to locate and navigate towards a habitat optimal for growth. Considering that root exudates could serve as growth substrates, the energy taxis might be involved in root colonization and the establishment of *Azospirillum*-grass associations (Greer-Phillips, *et al.* 2004).

Genetics and Biochemical of Determinants Involved in Chemotaxis in *Azospirillum*

The identification of genes encoding by the excitation (CheA, CheW and CheY) and adaptation (CheB and CheR) chemotaxis pathways from *A. brasilense* was obtained by genetic complementation of two generally non-chemotactic mutants. The genes identified showed high identities (>50%) with the corresponding genes to α-proteobacterias, and were located in tandem, suggesting an operon structure (Hauwaerts *et al.* 2000). Although a previous work has showed that the chemotactic response of *A. brasilense* to most strong attractant malate has been methylation independent (Zhulin & Armitage, 1993), the presence of the *cheR* (coding for a methyl esterarse), and *cheB* (coding for a methyltransferase) genes, indicates that responses to at least some stimuli requires methylation and demethylation of the chemotaxis transducers (Hauwaerts *et al.* 2000).

Further work revealed that CheR, CheB and CheBR contributed to chemotaxis and aerotaxis significantly in *A. brasilense* but they are not essential for these behaviors to occur (Stephens *et al.* 2006). The chemotacting rings formed by the corresponding mutants were significantly smaller than those formed by the wild-strain for the strong attractants tested (malate succinate, fructose). Using the spatial gradient assay *cheB* and *cheR* mutant were unable to form aerotaxis bands. However the *cheRB* mutant formed band, but farther away from the meniscus than the one formed by the wild-type. None of the three mutants responded to either addition or removal of oxygen for aerotaxis, whereas the wild-type had a positive and negative response, respectively. The liberation profile of methanol, that is a measure of methyl estearase (CheB) activity (involved in the adaptive response) was very different with the attractants tested to those obtained with *E. coli.* Interestingly, the wild-type strain on the addition or removal of oxygen and succinate did not induce the production of methanol, indicating that in this bacterium there is a methylation-independent pathway for those molecules, confirming the early results obtained (Zhulin & Armitage, 1993). Furthermore, the *cheBR* mutant release methanol upon both in addition or removal of succinate, suggesting that there are more than one methylation/demethylation system(s) in *A. brasilense*. In order to test this hypothesis the authors, constructed a Δ*cheA::gusA-km* mutant. The resulting *che* operon mutant was impaired in, but not null for chemotaxis. Taking together all this data strongly suggesting that: i).The operon under study is partially involved in aerotaxis and quimiotaxis, but it is not its principal function. ii). The aerotaxis in *A. brasilense* appears to be methylation independent. iii).The data strongly suggested that in *A. brasilense* occur several quimiotaxis pathways (Stephens *et al.* 2006). Analysis of the draft genome sequence of *A. brasilense* Sp245 strain suggests the presence of four *che*-like signal transduction pathways, multiple homologous of adaptation proteins and about 30 chemotaxis transducers (Alexandre. 2007).

Identification of Chsa, A Novel Protein Involved in Chemotaxis

It was recently obtained the characterization of a mutant strain of *A. brasilense* Sp7S impaired in surface motility and chemotactic response (Carreño-López *et al.* 2009). The genetic complementation of Sp7S strain (Fig 1) and the nucleotide sequencing of the complementing region conducted to the identification of *chsA* gene coding for a putative signal transduction

protein designated ChsA. The nucleotide sequence of the *chsA* region has been deposited in the GenBank database with accession number AM408892.

The inferred ChsA protein (63,714 Da, 586 residues) has characteristics similar to cytoplasmic signalling proteins. It contains two domains: a PAS sensory domain near the N terminus region (from residues 50 to 160) and an EAL transmitter domain in the C-terminus part (from residues 330 to 560). The EAL domain is present in proteins with phosphodiesterase activity (PDE-A) involved in the hydrolysis of c-di-GMP (cyclic-bis (3'-5') dimeric GMP), a compound known to function as a second messenger (Figure 1) in a broad spectrum of cellular processes including motility, biofilm formation and cellular differentiation (Jonas *et al.* 2009). Proteins containing an EAL domain often possess another domain GGDEF, carrying guanylate cyclase activity, but this domain is not present in ChsA. The PAS sensory domain is found in a variety of proteins with redox functions, including NifL, Dos and Aer, which typically bind heme, and flavines (Taylor and Zhulin 1999; Greer-Phillips *et al* 2003). Databases searches using the BLASTP program revealed that ChsA shared similarity with a limited number of uncharacterized ORFs from α Proteobacteria, phylogenetically close to *Azospirillum*, such as *Magnetospirillum magnetotacticum* (YP_421664.1) and *Rhodospirillum rubrum* (YP_426135.1), (Carreño-López *et al.* 2009).

It was constructed the *chsA::km*^{R} mutant and its phenotype determined. The presence of flagella in the mutant strain was confirmed by Western blot analysis, using antisera raised against the polar and lateral flagellins, and by electron microscopy. It displayed the same growth rate and motility as the wild type in liquid medium; however, showing a defect in motility in semi-solid minimal medium added with several metabolizable subtracts, defined as strong chemoattractants for *Azospirillum,* such as: to three organic acids, malate, succinate and pyruvate and the amino acids glutamate and proline. It was employed as a negative control a mutant lacking the sigma N factor (σ^{54}); the *rpoN* gene controls the expression of both flagella in *Azospirillum* (Milcamps *et al.* 1996). As expected, strain *rpoN* was completely non-chemotactic. It was observed a significant decrease (c.a. of 20 %) in the chemotactic response with all the substrates used, in the case of *chsA*-Tn*5* mutant strain (Sp74031) as compared to the wild type. A partially reduced chemotactic response was also reported for *cheB* and *cheR* mutants for the adaptation pathway, suggesting multiple chemotaxis systems in *Azospirillum* (Stephens *et al.* 2006; Bible *et al.* 2008)

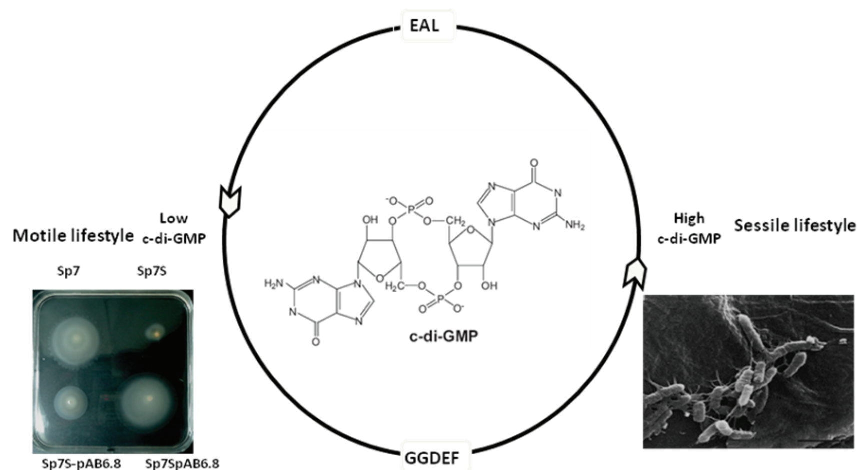

Figure 1. The role of c-diGMP in bacterial sessile and motile lifestyle. In *A. brasilense* Sp7, regulates swimming motility (left, genetic complementation of Sp7S mutant), and biofilm formation (right) in response to changing cellular pool of c-di-.GMP. On left an image of genetic complementation of *A. brasilense* Sp7S strain mutant by the plasmid pAB6.8, which carried the gene *chsA*. The strains were grown on K-malate minimal medium with 0.25% agar for 48h. On the right is a SEM of *A. brasilense* Sp7 biofilm

In addition, it was analyzed the enzymatic activity of ChsA as an avenue to gain information about the functional characteristics of this novel chemotaxis signal transduction protein, and to expand our knowledge on the behavior of an EAL protein. The *chsA* gene was cloned, the protein expressed and purified for the determination of phosphodiesterase activity. For that, the genomic DNA of *A. brasilense* Sp7 was isolated following standard procedures and the gene was amplified by PCR. The amplicon was cloned in the expression vector pBAD (Invitrogen). The plasmid harboring the gene and the C-terminal His_6 tag-encoding sequence were transformed into *E. coli* strain BL21(DE3). The expression results are presented in Figure 2.

The protein purification was done from 500ml bacterial culture (LB medium), which was grown to an optical density of 0.8 (OD_{600} nm) before being induced with 0.08% L-arabinose. The culture was shaken at 16°C for 12 h before being pelleted by centrifugation. The cells were lysed in 20 ml lysis buffer (20 mM Tris [pH 8.0], 500 mM NaCl, 5% glycerol, 0.1% β-mercaptoethanol, 0.1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride). After centrifugation at 25,000g for 30 min, the supernatant was filtered and then incubated with 2 ml of Ni_2-nitrilotriacetic acid resin (Qiagen)

for 1 h at 4°C. The resin was washed with 50 ml of W1 buffer (lysis buffer with 20 mM imidazole) and 20 ml of W2 buffer (lysis buffer with 50 mM imidazole). The proteins were eluted using a stepped gradient method with the elution buffer containing 20 mM Tris (pH 8.0), 500 mM NaCl, 5% glycerol, and 200 mM, 300 mM, or 500 mM imidazole. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, fractions with purity higher than 95% were pooled, and the enzymatic activity determined (Figure 3).

Using a purified his-tag fusion of ChsA, it was demonstrated that this protein has phosphodiesterase (PDE-A) activity in the presence of Mn^{2+} against the artificial substrate bis(*p*-nitrophenyl) phosphate (bis-*p*NPP). This *in vitro* activity was considerably higher at 37°C than at room temperature. In this study we have determined that *in vitro* the pH optimum for the PDE activity of EAL-ChsA was between pH 8 and 9 (Figure 3). Since the proposed function of EAL-domain proteins is to linearize c-di-GMP (Schmidt *et al*. 2005; Schimer and Jenal, 2009), this is a direct demonstration of the required phosphodiesterase activity of ChsA purified EAL-domain protein, which likely degrades the c-di-GMP.

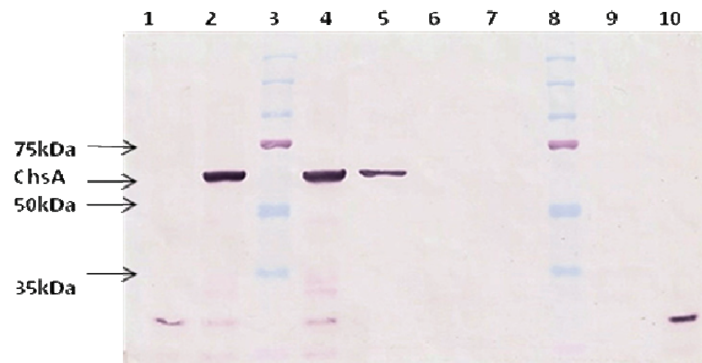

Figure 2. Expression of ChsA in *E. coli* BL21(DE3) strain. Transformed BL21(DE3) cells were grown at 30°C for 3h. The expression was performed in the presence of 0.08 % of L-arabinose at 16°C for 5 h post-induction. Expression from each sample was tested with 50µg of protein. Representative Western blot probed with an anti-His antibody showing the expression of the C-terminal His_6 ChsA after induction of expression, as well as the cellular localization of ChsA. Lane 1. *E. coli* pBAD (vector), pellet; lane 2. ChsA (pellet); lane 3. M.M.; lane 4. ChsA (pellet); lane 5. ChsA (soluble fraction); lane 6. *E. coli* pBAD (vector, soluble fraction; lane7. empty; lane 7 M.M; lane 10 pBAB (vector, pellet)

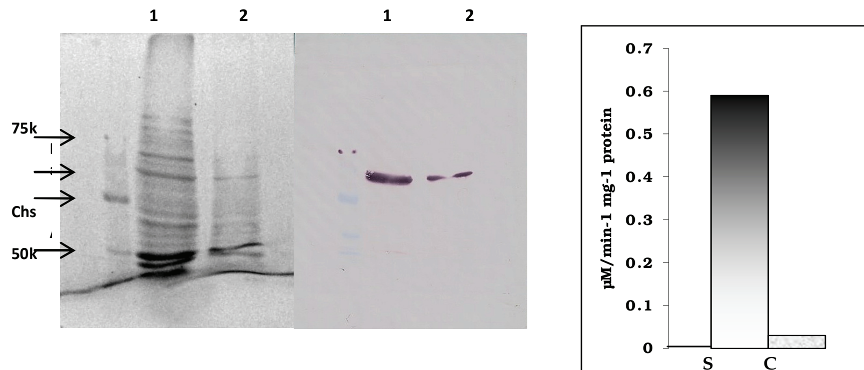

Figure 3. Purification and activity of recombinant ChsA by affinity chromatography. (a) Protein was detected by Western blotting of purified His-tag-ChsA, using antiserum against His6 (Sigma chemical Co. USA) (b). 10 µg of affinity purified, dialyzed ChsA was tested for PDE activity in a reaction buffer (0.05 M Tris, 0.05 M Bis-Tris, 0.1M Na acetate, 1mM MnCl2, 2.5 mM of bis-pNPP) at pH 8.5, as previously described by Bobrov et al. (2005); S = Sample, C = Control

It is worth noting to find that others genes such as *chsA* might contribute to chemotaxis response, as revealed for this gene. Many of the proteins involved in the metabolism of the second messenger compound c-di-GMP contain both EAL and GGDEF domains, responsible respectively for phosphodiesterase and diguanylate cyclase activities (Schmidt *et al*. 2005; Schimer and Jenal, 2009), however, ChsA only contains the EAL domain. It has been shown that the YhjH and EAL domain proteins with phosphodiesterase activity from *S. enterica* sv Typhymurium are implicated in the transition between sessility to motility (Simm *et al.* 2004). Surveys of bacterial genomes revealed that the number of genes encoding proteins with GGDEF or EAL domains could vary from none to up to 99, suggesting that the role of each individual protein may be extremely complex and that their inactivation is unlikely to result in clear-cut phenotype in case of high multiplicity (Galperin 2005). Deduced translation of *chsA* indicated that ChsA product contains a PAS domain. Proteins containing a PAS domain are present in numerous bacteria and are involved in sensing a variety of environmental signals (Taylor and Zhulin 1999). It is tempting to speculate that the PAS domain in ChsA protein senses the redox state of the cell through a cytoplasmic signaling molecule directly coupled to the transmitter EAL module. Here, we presented biochemical data to demonstrate that the EAL domain of ChsA is catalytically active, with phosphodiesterase activity.

However, the relationship between ChsA and the regulation of the chemeotactic machinery still remains unknown.

The Role of Bacterial Motility and Chemotaxis in *Azospirillum*-Plant Interaction

It has been reported that certain strains of *A. brasilense* show positive chemotaxis towards various attractants such as sugars, amino acids, organic acids (Okon *et al*., 1980; Barak *et al*., 1982; Reinhold *et al*., 1985; Zhulin and Armitage, 1992), as well as to root exudates (Heinrich and Hess, 1985; Mandiambra *et al*., 1986; Okon *et al*., 1980; Bacilio-Jiménez *et al*., 2003; Pedraza *et al*. 2009). This mobility is important as it allows the bacterial access to a more suitable habitat and become more competitive with other microorganisms in the root.

In natural environments, soil moisture is a limiting factor in the migration of *A. brasilense* to the roots of plants (Bashan, 1986). This suggests that the "swimming movement" plays an important role in such environments. The ability of *A. brasilense* to start the root colonization of wheat was investigated with different mutant strains (Vande Broek *et al*., 1998). Only non-flagellate and non-chemostatic mutants showed a strong reduction in the capability for colonization, which showed the requirement of bacterial mobility to initiate root colonization of wheat (Vande Broek *et al*., 1998).

Bashan and Holguin (1994) compared the interroot movement of *A. brasilense* wild-type and its isogenic non-motile strain, which were inoculated to the root systems of soybean and wheat seedling, in presence of chemoattractants and repellents. They showed highly differences between both strains, no movement of *A. brasilense* Mot^- cells from inoculated roots was detected. The inoculated *A. brasilense* Mot^- bacteria remained at the seed inoculation and did not migrate with the root tips; whereas wild-type strain showed a differential pattern, Mot^+ cells migrated for several centimetres from inoculated to non-inoculated roots, irrespectively of plants species. Indicating, that motility and chemotaxis are actives processes involved in efficient colonization (Bashan and Holguin, 1994)

By a different approach using the ß-galactosidase activity as a reporter system it was showed that only motility and chemotactic mutants from *A. brasilense* Sp7 wild-type strain were affected in their capacity to initiated wheat root colonization at the root hair zones. Indeed the ß-galactosidase activity determined by qualitatively and quantitatively assays from NM313

mutant strain (altered in chemotaxis to succinate and citrate), and Sp7p90Δ84 strain (a non-flagellated mutant; van Rhijn *et al.* 1990), differ significantly from Sp7wild-type strain in colonization to root wheat. This indicates that chemotactic movement is important in the initiation of wheat root colonization (Vande-Broek, 1998).

In the rhizosphere, it may be advantageous to respond positively to any compound that increases metabolic rates, such as organic acids and oxygen. Low oxygen concentrations typically of the rhizosphere are seen to be one of the main stimuli that attract bacteria to plant roots. Aerotaxis in the rhizosphere would be expected to provide a real advantage to bacteria when searching for nutrients and in competition with other microorganisms (Barak *et al.*1982; Zhulin and Armitage, 1993).

Chemotaxis of *A. Brasilense* towards Strawberry Root Exudates Assessed by the Agarose-Plate Method

Strawberry *in vitro* plants of three commercial varieties (‘Camarosa’, ‘Milsei’ and ‘Selva’) were aseptically grown in 25 ml of diluted (1:2) Hoagland solution (Hoagland 1975) and maintained in a growth-chamber at 25°C, 70% of relative humidity and a photoperiod of 16 h light. The root exudates were collected from the liquid nutrient medium (Hoagland solution) used by plants after 7, 14, and 28 days of growth. The nutrient medium (25 ml) containing the root exudates was removed and sterilized by filtration (0.2 µm Millipore), lyophilized and kept at -20°C for total protein and sugar determination and chemotaxis test. Sterility of each solution was verified by plating samples in LB medium and incubated 72 h at 30°C.

Chemotaxis was evaluated on SM medium (Reinhold et al. 1985), without malic acid, yeast extract, neither NH_4Cl, and supplemented with 0.3% agarose (w/v). Lyophilized extracts containing root exudates from each cultivar were resuspended in distilled sterile water to obtain two concentrations: 8x (d1) and 4x (d2). Root exudates (0.1 ml) was added to 7 ml of SM medium (kept at 45°C), vigorously mixed and poured into sterile Petri dishes (60 mm diameter). Once at room temperature 0.01 ml of previously grown and washed bacteria (see below) was placed in the centre of the plates. Plates were incubated at 30°C and the halo diameter measured (mm^2) after 48 h. Mobile bacteria were obtained from 48 h old cultures grown in SM medium (Reinhold et al. 1985). Cells were collected by centrifugation (15 min at 15,000xg) and washed three times with potassium phosphate (60 mM pH 7.0)/Na–EDTA (0.1

mM) buffer. The cells were finally resuspended in phosphate buffer without EDTA and the concentration adjusted to 10^8 cells ml^{-1} (OD_{600} = 1.0). Cell motility was controlled by contrast phase microscopy (Olympus BH-2). For chemotaxis test, cell suspensions were used within 1 h after washing to avoid motility loss.

Chemotaxis assays were carried out on a complete randomized factorial design, including four factors: bacterial strains, strawberry cultivars, root exudates dilution (d1, d2), and the time the root exudates were collected (t1, t2, t3). ANOVA was performed, and the main effect of the different treatments was evaluated by the Wald Test ($P \leq 0.05$), using the software Infostat 2.0.

The positive chemotactic response was visualized by halo formation on culture medium surface. Table 1 shows the Wald test results conducted from data obtained in measuring the surface of the halos formed. These show that the main effects of factor variety ($p < 0.01$) and the dilution factor ($p < 0.05$) were statistically significant, as well as the interaction between bacterial strain and strawberry variety ($p < 0.05$), as indicating that the chemotactic response of the strain depends on the variety of the plant. One factor affecting the size of the halo was the consistency of the agarose, therefore, the addition of Tween 80, recommended by Niu *et al*. (2005) decreased the tension surface, thus favouring the displacement motion by microorganisms and the formation of larger, regular-shape and easy to measure halos.

Table 1. Test of significance for the coefficient model of main effects and interaction of two factors on the response of halo formation

1	F. D.	Wald test	P
Coefficient	1	235.5059	0.000
Strain	3	6.8742	0.070
Variety	2	12.2044	0.002
Strain*Variety	6	13.3457	0.040
Time	2	3.2217	0.200
Variety*Time	4	2.8608	0.600
Strain*Time	6	4.5185	0.600
Dilution	1	5.3759	0.020
Time*Dilution	2	1.5212	0.500
Variety*Dilution	2	0.6355	0.700
Strain*Dilution	3	0.4640	0.900

F.D.: fredon degree; P:probability.

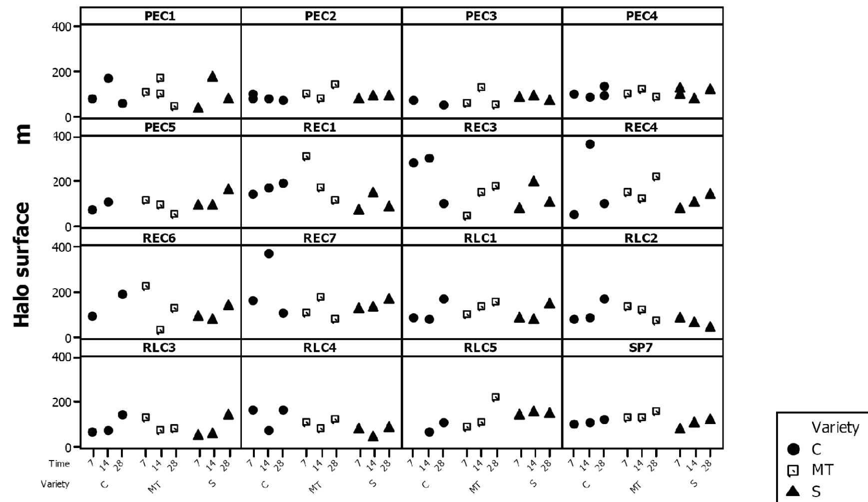

Figure 4 . Time effect of root exudates collection in different strawberry varieties on 16 strains of *A. brasilense* using a 2x exudates concentration

Once the optimal concentration of exudates for chemotaxis assay on agarose plates was determined, 15 strains, characterized in a previous work (Pedraza *et al*., 2007), were assessed: REC1, REC3, REC4, REC6, REC7, PEC1, PEC2, PEC3, PEC4, PEC5, RLC1, RLC2, RLC3, RLC4, RLC5, and the strain Sp7 as a control. With them, it was determined the chemotactic response towards root exudates in a 2x concentration of each variety of strawberry, taken during three periods, with the aim of selecting strains with high and low chemotactic response to be evaluated quantitatively.

Figure 4 shows the effect of time of collection of root exudates of different strawberry varieties in the response of different strains of *Azospirillum brasilense*. In all cases, we observed a higher number of positive responses to root exudates taken at 7 and 14 days. Endophytic strains (REC1, REC3, REC4, REC6 and REC7), isolated from sterile roots, showed a higher chemotactic response with formation of halos with areas greater than 200 mm^2. The lower chemotactic activity was observed in strains isolated from the rhizosphere and the first stolon (RLC2, RLC3, RLC4, PEC2, PEC3 and PEC4), which showed in all cases halos surfaces less than 200 mm^2. The best chemotactic response was obtained with strain REC3, which showed halos grater than 200 mm^2 for root exudates taken at 7 and 14 days of plant growth, from variety Camarosa. The strain RLC3 showed halos formation with surfaces smaller than 200 mm^2, being the one with less chemotactic response

towards the root exudates of the three strawberry varieties collected at different times.

Chemotaxis of *A. Brasilense* Towards Strawberry Root Exudates Assessed by the Capillary Method

From the results obtained in testing agarose-plates, three bacterial strains were chosen to quantify the chemotactic response by capillary method and determine the R_{chem} (chemotaxis index) of them: REC3 as the strain of higher chemotactic response, RLC3 as the lowest response, and Sp7 as control. The quantification of the chemotactic effect was performed by using the capillary method according to Guocheng and Cooney (1993). For that, a sterile plastic chamber with multiple wells was used. The capillaries (1µl of capacity) were placed in the centre of each well and filled using a sterile syringe. To observe the filling of capillaries, the attractant solution was mixed with Comassie blue. One end of the capillary carrying the attractant solution was introduced through the central hole silicone septa in the cell suspension (250 µl) contained in the chamber well. The free end of the capillary was sealed with plastic putty to avoid the aerotaxis effect. The chamber was incubated during one hour at 30°C. Then, the content of the capillary were expelled using a sterile syringe into microtubes (1.5 ml) with 1ml of chemotaxis buffer and serial dilutions were made (10^{-3}, 10^{-4}, 10^{-5}). Dilutions were plated on solid NFb medium (described in Pedraza *et al*. 2007). Each strain was processed with a capillary control that contained chemotaxis buffer without the attractant solution. The plates were incubated for 72 h at 30°C. After that time the CFU/ml was determined. The ratio between the number of bacteria in the capillary due to chemotaxis and the number due to random motion is known as R_{chem} (chemotaxis index), representing the chemotactic activity of the microorganism towards a particular substance (Barbour *et al*., 1991). Results are shown in Table 2 and they show concordance with those obtained on agarose-plate method.

The strain REC3 showed the highest chemotactic activity with exudates from the three varieties of plants taken after 7 days of growth. The highest values were obtained with the variety 'Camarosa' (11.98 ± 2.39), followed by the variety 'Milsei' (6.33 ± 0.59) and 'Selva' (3.11 ± 0, 45). These results agree with those obtained by the plate method. The minimum value obtained for this strain was 1.47 with exudates of the variety 'Selva' taken at 28 days.

Table 2. Chemotactic response of *A. brasilense* strains towards root exudates of three varieties of strawberry: Camarosa (C), Milsei (M) and Selva (S), collected in three periods: t7, t14 and t28 (7, 14, 28 days of plant growing), assessed by the capillary method (R_{chem}: chemotactic index; SD: standard deviation)

Strain-Strawberry var.-Time of collecting root exudates	Chemotactic response
	R_{chem} ± SD
REC3 M t7	6,33 ± 0,59
REC3 M t14	2,82 ± 1,53
REC3 M t28	3,97 ± 0,41
REC3 C t7	11,98 ± 2,39
REC3 C t14	6,69 ± 3,22
REC3 C t28	3,67 ± 2,62
REC3 S t7	3,11 ± 0,45
REC3 S t14	4,51 ± 1,52
REC3 S t28	1,47 ± 0,00
RLC3 M t7	0,90 ± 0,39
RLC3 M t14	1,18 ± 0,17
RLC3 M t28	2,06 ± 0,06
RLC3 C t7	0,00
RLC3 C t14	0,93 ± 0,11
RLC3 C t28	1,37 ± 0,89
RLC3 S t7	0,75 ± 1,06
RLC3 S t14	0,55 ± 0,05
RLC3 S t28	0,70 ± 0,28
Sp7 M t7	0,00
Sp7 M t14	0,52 ± 0,05
Sp7 M t28	0,58 ± 0,07
Sp7 C t7	0,00
Sp7 C t14	0,47 ± 0,20
Sp7 C t28	1,60 ± 1,29
Sp7 S t7	2,28 ± 1,01
Sp7 S t14	0,65 ± 0,44
Sp7 S t28	1,55 ± 1,48

The strain RLC3 showed a maximum R_{chem} (1.37 ± 0.89) for the variety 'Camarosa' at 28 days as in the previous test; this value was lower than the

value obtained with strain control Sp7 (1.60 ± 1.29). Chemotactic response was not detected by this method when using exudates collected from variety ‘Camarosa’ 7 days growth, as well as the control strain Sp7 with exudates from 'Milsei'. The results determined by the capillary method confirmed that the chemotactic activity depends on the bacterial strain, plant variety, and time of collection of the exudates. Although the chemotactic response towards strawberry root exudates was performed through two approaches: the method of plates and the quantitative method of capillaries, similar results were obtained in both cases.

Sugars and Proteins Determination of Strawberry Root Exudates

To explain the chemotactic behaviour previously observed, total sugars and proteins content of root exudates were determined. Total sugars was determined from lyophilized samples of root exudates suspended in sterile distilled water as indicated in Pedraza *et al.* (2009). Briefly, 0.64 ml of phenol 80% and 2.5 ml of concentrated H_2SO_4 (80%) was added to 1 ml of each sample, mixed for 1 min with vortex and kept at 30°C for 10 min. After colour development OD_{490} was measured and sugar content evaluated with a standard curve made with different glucose concentrations. Three determinations were performed for each sample (root exudates and glucose standards).

The major amount of sugars was detected in the root exudates obtained at 7 days of plant growing of the three cultivars (‘Milsei’, ‘Selva’, ‘Camarosa’), then, a diminution of them was observed at 14 and 28 days. The amount of total sugars varied among plant cultivars, being the cv ‘Milsei’ the highest producer at 7, 14 and 28 days as compared with the cvs ‘Camarosa’ and ‘Selva’. The maximum concentration of sugar (0.0806 mg/ml) was found in root exudates of the variety 'Milsei' taken at 7 days of plant growth, followed by exudates from the variety 'Selva' (0.0629 mg/ml), taken in the same period, and finally in the variety 'Camarosa' (0.0466 mg/ml). The minimum value of sugar concentration (0.0219 mg/ml) was determined in root exudates taken at 28 days from variety 'Camarosa'. The variety 'Selva' showed its lowest concentration (0.0245 mg/ml) in exudates obtained at 14 days and in the variety 'Milsei' the lowest value (0.0358 mg/ml) was detected at 28 days of plant growth.

In contrast, protein concentration of the root exudates, determined according to Bradford (1976) using bovine-serum albumin as standard, was

detected only after 14 and 28 days of plant growth. At 28 days, in 'Camarosa' it was determined the maximum value (0.030 mg/ml), followed by the variety 'Milsei' (0.024 mg/ml) and finally the variety 'Selva' with a concentration of 0.019 mg/ml. The minimum value detected was 0.018 mg/ml in the variety 'Selva', then 'Milsei' with a value of 0.024 mg/ml and finally 'Camarosa' (0.025 mg/ml), all detected at 14 days of plant growth.

Conclusion

Azospirillum as a motile genus bacteria are capable of navigating in gradients of oxygen, redox molecules, and nutrients by constantly monitoring their environment in order to inhabit where it is optimal for survival and growth. Although there is not strict host specificity in *Azospirillum*-plant associations, a strain-specific chemotaxis was reported. Strains isolated from the rizosphere of a particular plant demonstrated preferential chemotaxis towards chemicals found in root exudates of that plant. These results suggested that chemotaxis may contribute to host-plant specificity and could largely be determined by metabolism.

Until know it appears that the genetics and molecular traits determined in *Azospirillum* are enough complex. It has been reported that *Azospirillum* undergoes methylation-dependent and independent chemotaxis. Not all chemotactic responses require methylation-dependent. For example in *E. coli*, aerotaxis and chemotaxis to phosphoenolpyruvate transport system sugars are methylation independent (Alexandre & Zhulin, 2001). It has been shown that quimiotaxis for such as succinic acid and oxygen are methylation-independent (Stephen *et al.* 2006). On the other hand the genome sequence of *A. brasilense* Sp245 strain under progress has showed to contain several *che*-like genes, which could be probably involved in the chemotactic behaviour.

C-di-GMP is an intracellular signaling molecule that has been proposed to control the transition between biofilm and planktonic mode of growth (Römling *et al.* 2005). High intracellular c-di-GMP promotes the production of biofilms, adhesives organelles such as pili or stalks, and reduces motility in a certain number of species (Hickman *et al.* 2005; Jonas *et al.* 2009). The analysis of completely sequence bacterial genomes has revealed that some bacterial species have multiples homologous chemotaxis-like signal transduction pathways (Szurmant and Ordal, 2004). Interestingly, Che-like pathways have been recently implicated in controlling cellular functions other

than motility, including flagellum biosynthesis and biofilm formation (Hickman *et al.* 2005; Bible *et al.* 2008). Indeed a chemosensory system that regulates biofilm formation through modulation of cyclic c-di-GMP has been described in *Pseudomonas aeruginosa*. The Wsp system includes a predicted membrane-bound [methyl-accepting chemotaxis protein (MCP)]-like receptor (WspA), CheW-like scaffolding proteins (WspB and WspD), a CheA/Y hybrid histidine sensor kinase with a received domain (WspE), a methyltransferase CheB homologue (WspC), a methylesterase CheB homologue (WspF), and a two domain response regulator GGDEF protein WspR, which catalyzes the synthesis of c-di-GMP. The system stimulates production of biofilm and suppresses motility, by formation of c-di-GMP intracellular levels (Hickman *et al.* 2005). Although it is not already verified, it is speculated that ChsA contributes to motility interacting with the chemotactic machinery as was described in *P. aeruginosa*.

The bacterial chemotaxis provides a resource to respond to gradients of potential nutrients in the environment, by moving towards or away from these substances (Adler, 1966). The root exudates are an important source of nutrients for the microorganisms in the rhizosphere and participate in the colonization process through chemotaxis of microorganisms in the soil (Campbell and Greaves, 1990; Hiroyuki *et al*., 1998; Lynch and Whipps, 1990).

It is shown in this chapter that root exudates from different varieties of strawberry caused positive chemotaxis of different strains of *A. brasilense*. This effect was greater with endophytic strains than with those that colonize externally the root surface. Similar results were observed in the study of the chemostatic response of endophytic and rhizospheric bacteria toward root exudates in rice (Bacilio-Jiménez *et al*., 2003). It was observed that the chemotactic effect of local strains of *A. brasilense* towards strawberry root exudates depended on the plant variety, where the best chemostactic response was obtained with exudates of the variety ‘Camarosa’. However, differences in responses may be due to the origin of the bacterial strains (rhizospheric or endophytic). Furthermore, the specificity of strains probably reflects the adaptation of the bacterium to the nutritional conditions provided by the plant and can, thus, play an important role in the establishment of *Azospirillum* in the rhizosphere of the host, as previously indicated by Reinhold et al. (1985).

It was also verified that the concentration of root exudates affected chemotaxis response, as the best response was obtained when using a low concentration. These results agree with those previously reported in other studies regarding the ability of *Azospirillum* to detect potential sources of

nutrients at lower concentrations than other soil microorganisms, favouring its survival in natural environments (Chet and Mitchell, 1976; Roszak and Colwell, 1987). Also, the production time of the exudates had effects on chemotaxis as a major chemotactic result was toward the exudates produced within the first two weeks of plant growth. This also coincides with the observations of Bacilio-Jimenez et al. (2003), working with root exudates of hydroponic rice.

In this case, the crop root exudates from strawberry in hydroponic conditions showed a decreased concentration of carbohydrates with the production time thereof, and an increase in protein concentration. This would indicate that the sugar residues have a greater attractive effect than protein, al least in the early stages of the partnership. These results partially coincide with those obtained by Bacilio-Jimenez et al. (2003) in rice hydroponics, who detected a higher concentration of sugars and amino acids in the exudates obtained during the first two weeks of cultivation. In other works it has been observed that during germination of wheat seeds, the concentration of carbohydrates was higher in the first seven days than in the second and fourth weeks of plant growth (Jones and Darrah, 1993; Prikryl and Vankura, 1980).

The decrease in total sugar concentration determined in hydroponic culture after the second week, probably was caused by two potential mechanisms: the accumulation of high levels of organic substances in the vicinity of the roots that suppressed the release of additional organic compounds (Jones and Darrah, 1993; Prikryl and Vankura, 1980), and the re-absorption of organic compounds by the plant (Guckert *et al*., 1991; Jones and Darrah, 1993). Moreover, the growing conditions conducted in this study could have influenced the amount and composition of the exudates released by the roots, as also indicated by Jones and Darrah (1993). Besides, the lack of enough oxygen in the tubes containing the plants may have contributed to the decrease in root exudation over the time.

However, the results showed in this chapter are consistent with those previously reported by Reinhold et al. (1985) for different organic compounds of root exudates of other crops; they indicate that the association of *Azospirillum* with the host plant is of type strain-specific.

By assessing the chemotactic activity of different strains of *A. brasilense* it was possible to observe the affinity of certain genotype in the association *Azospirillum*-strawberry. Considering that *A. brasilense*, posses biotechnological application, addressing to a sustainable agriculture, determining the genes and mechanisms involved in chemotaxis response, as

well as the activity of strains to root exudates may represent an initial step in selecting them for use as inoculants in different crops.

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