Available online at www.sciencedirect.com
ScienceDirect
Research in Microbiology 157 (2006) 867–875
Research in Microbiology
www.elsevier.com/locate/resmic

Effects of nutritional and environmental conditions on *Sinorhizobium meliloti* biofilm formation

Luciana Rinaudi [a], Nancy A. Fujishige [b], Ann M. Hirsch [b,c], Erika Banchio [a], Angeles Zorreguieta [d], Walter Giordano [a,*]

[a] *Departamento de Biología Molecular, Universidad Nacional de Río Cuarto, 5800-Río Cuarto, Córdoba, Argentina*
[b] *Department of Molecular, Cell and Developmental Biology, University of California-Los Angeles, 405 Hilgard Avenue, Los Angeles, CA 90095-1606, USA*
[c] *Molecular Biology Institute, University of California-Los Angeles, 405 Hilgard Avenue, Los Angeles, CA 90095-1606, USA*
[d] *Fundación Instituto Leloir, CONICET and Instituto de Investigaciones Bioquímicas, FCEyN, Universidad de Buenos Aires, Patricias Argentinas 435, C1405BWE-Buenos Aires, Argentina*

Received 15 February 2006; accepted 8 June 2006

Available online 12 July 2006

Abstract

Rhizobia are non-spore-forming soil bacteria that fix atmospheric nitrogen into ammonia in a symbiosis with legume roots. However, in the absence of a legume host, rhizobia manage to survive and hence must have evolved strategies to adapt to diverse environmental conditions. The capacity to respond to variations in nutrient availability enables the persistence of rhizobial species in soil, and consequently improves their ability to colonize and to survive in the host plant. Rhizobia, like many other soil bacteria, persist in nature most likely in sessile communities known as biofilms, which are most often composed of multiple microbial species. We have been employing in vitro assays to study environmental parameters that might influence biofilm formation in the *Medicago* symbiont *Sinorhizobium meliloti*. These parameters include carbon source, amount of nitrate, phosphate, calcium and magnesium as well as the effects of osmolarity and pH. The microtiter plate assay facilitates the detection of subtle differences in rhizobial biofilms in response to these parameters, thereby providing insight into how environmental stress or nutritional status influences rhizobial survival. Nutrients such as sucrose, phosphate and calcium enhance biofilm formation as their concentrations increase, whereas extreme temperatures and pH negatively affect biofilm formation.
© 2006 Elsevier Masson SAS. All rights reserved.

Keywords: Biofilm; *Sinorhizobium meliloti*; Environmental signals

1. Introduction

The symbiosis between Gram-negative bacteria of the family Rhizobiaceae and the roots of legume plants (family Fabaceae) leads to the formation of nitrogen-fixing nodules, in which the differentiated bacteria (bacteroids) reduce atmospheric nitrogen to ammonia.

The study of root colonization, and subsequent nodule development and occupancy, has contributed a great deal to our understanding of the nitrogen-fixing association between rhizobia and legumes. Nevertheless, much is still unknown about rhizobial attachment to roots and the importance of its role in subsequent stages of nodulation and nitrogen fixation. Many methods have been employed to study rhizobial attachment, but most are indirect, involving initial detachment of the microorganisms from the root surface and then counting of them. Other indirect procedures, such as radiolabelling bacteria, enzyme-linked immunosorbent assays or other biological assays, estimate the number of attached organisms in situ by measuring some attribute of the attached organism [2]. Methods involving direct observation, such as light-, laser-scanning confocal-, transmission electron- and scanning electron microscopy, detect microbial attachment directly and have greatly improved our understanding of root colonization [13].

In a previous publication, we utilized a modified microtiter plate assay to study biofilm formation by *Sinorhizobium meliloti* and *Rhizobium leguminosarum* bv. *viciae* [14].

* Corresponding author.
E-mail address: wgiordano@exa.unrc.edu.ar (W. Giordano).

0923-2508/$ – see front matter © 2006 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.resmic.2006.06.002

Biofilms are surface-attached communities of bacteria contained within a self-produced extracellular polymeric matrix; they are composed either of a single species or, more often in nature, of multiple bacterial species [15,49]. Indeed, the majority of bacteria appear to form biofilms, and this multicellular mode of growth predominates in nature, most likely as a protective mechanism against hostile environmental conditions [7,20]. Biofilm behavior affords bacteria, and especially non-spore formers such as rhizobia, a number of survival benefits, since biofilms can be established on both abiotic and biotic surfaces, often under stressful conditions. Research on a number of different bacteria indicates that biofilms exist as a mass of microcolonies in a single layer or as three-dimensional structures with vertical and horizontal channels allowing liquid flow and dispersion of nutrients and waste components [2]. The involvement of extracellular exopolysaccharides, pili, flagellae and quorum sensing signals in biofilm formation has been revealed by molecular genetic analyses [12,40]. Also, recent research suggests that the environmental signals regulating whether bacterial cells will initiate a biofilm differ from one bacterial species to another, thereby allowing each bacterial species to efficiently colonize its preferred environment [46].

The plant pathogen *Agrobacterium tumefaciens*, which persists as a surface-associated population of cells, develops biofilms on both inert surfaces such as soil particles, and living plant tissues [43]. Similarly, we previously reported that *R. leguminosarum* bv. *viciae* and *S. meliloti* establish biofilms on both roots and abiotic surfaces [13,14]. Other studies have demonstrated that bradyrhizobia and azorhizobia form biofilms on fungal mycelia [44]. Although only a limited amount of information is known thus far about biofilm formation in rhizobial species, it is tempting to speculate that biofilm formation is important for the overall fitness of rhizobia in the soil and in rhizosphere microenvironments, thereby contributing to efficient symbiosis [54].

In the present report, we have expanded our analysis to study *S. meliloti* biofilm formation under various conditions of environmental stress and nutrient status. These experiments lend support to our hypothesis that rhizobial biofilm formation is important for the survival of these non-spore forming bacteria in soil in the absence of a legume host.

2. Materials and methods

2.1. Bacterial strain, culture media and growth conditions

S. meliloti Rm1021 [29] was used in this study and was grown in minimal medium RDM (*Rhizobium* Defined Medium) [51] supplemented with streptomycin (100 $\mu g\,ml^{-1}$) at 28 °C. For strain maintenance, the medium was solidified with 1.5% Bacto-agar (Difco Laboratories). Bacterial liquid cultures comprising 10–15% of the flask volume were grown in a New Brunswick shaker at 200 rpm to mid- or late-log phase, diluted to an optical density at 600 nm (OD_{600}) of approximately 0.2, and used in the biofilm assay [14]. Bacterial growth and biofilm formation were measured to determine their relationship under the different conditions. RDM was supplemented with various sugars, osmotic agents or salts as indicated in the Results section. When RDM was made with low phosphate levels (RDM normally has a phosphate concentration of 12.5 mM), the pH was adjusted with 2 M Tris–HCl, pH 6.8.

2.2. Biofilm formation assay

2.2.1. Microtiter plate method

The biofilm formation assay used is based on the method of O'Toole and Kolter [38] with modifications [14]. This assay relies on the ability of the cells to adhere to the wells of 96-well microtiter dishes made of polyvinylchloride. We found that polystyrene plates gave similar results (data not shown). To each well, 150 µl from an overnight culture was added. After inoculation, the plates were covered with plastic to prevent evaporation and incubated without agitation at 30 °C for a minimum of 24 h or for the time indicated in each experiment. Then, the contents of each well were gently aspirated with an automatic hand pipette or a Pasteur pipette. The wells were washed three times with 180 µl of sterile physiological saline solution and the plates were vigorously shaken in each wash in order to remove all non-adherent bacteria. The plates were emptied, left to dry, and stained for 15 min with 150 µl per well of 0.1% CV. They were then rinsed thoroughly and repeatedly with water and scored for biofilm formation.

2.2.2. Quantification of biofilm formation

Biofilm formation was quantified by addition of 150 µl of 95% ethanol to each CV-stained microtiter dish well, and the absorbance of solubilized CV was determined with a MicroELISA Auto Reader at 560 nm (series 700 microplate reader, Cambridge Technology) or at 570 nm, where indicated, in a BioRad microtiter plate reader (Model no. 680). Alternatively, and in order to compare the results from the microtiter plate reader, CV was solubilized in 200 µl of 95% ethanol. The entire contents were then transferred to a 1.5 ml Eppendorf tube, the volume was brought to 1 ml with distilled water and absorbance was determined at 560 nm in a spectrophotometer (DU-640 Spectrophotometer, Beckman Instruments). Before the addition of CV, cells were homogenized manually by repeated pipetting of the contents in each well and bacterial growth was quantified by measuring absorbance at 600 nm. Bacterial growth and adherence measurements were performed in triplicate and repeated at least three times; values were then averaged.

2.3. Potassium assay

Cells from 1 ml portions of bacterial suspensions ($OD_{600} =$ 0.8–1.0) of free-living and sessile bacteria were harvested by centrifugation for 1 min, supernatants were removed and pellets were suspended in 1 ml of deionized water. The suspensions were boiled for 1 min to release potassium from the cells, and the potassium concentrations (mM K^{+}/g protein) were determined by flame photometry as previously described [50].

2.4. Protein quantification

The protein concentration was determined by the method of Bradford [5] using bovine serum albumin (Sigma) as the standard.

2.5. Microscopy

Biofilms were established on 1 cm round glass cover slips, following our previously published procedure for using plastic tabs [14]. For microscopy, substrates and their associated biofilms were washed with copious amounts of RDM to remove planktonic cells. The samples were placed on a depression slide and viewed by fluorescence microscopy using a Zeiss Axiophot microscope. Images were taken with Kodak Ektachrome Tungsten 160T slide film and processed using Adobe Photoshop.

Biofilm cells were stained with the LIVE/DEAD® BacLight™ bacterial viability kit according to the manufacturer's instructions (Molecular Probes, Eugene, OR). Briefly, biofilms grown on glass cover slips were incubated in the staining solution (RDM containing 5 μM SYTO9 dye and 30 μM propidium iodide) for 15 min in the dark at room temperature. These were examined under fluorescence microscopy as described above.

2.6. Statistical analysis

Experiments were conducted in a completely randomized design and were repeated. Values presented are the means of repeated experiments. Data were subjected to one-way ANOVA followed by comparison of multiple treatment levels with the control using *post hoc* Fisher's LSD test. All statistical analyses were performed with Infostat software version 1.0.

3. Results

We had previously found that *S. meliloti* biofilm formation was greater when the bacteria were grown in RDM rather than in richer media such as LB or TY [14], indicating that a nutritionally limiting environment increases the transition from planktonic to a sessile mode of life, i.e., a biofilm. This observation suggested that biofilm formation may represent a survival strategy in a nutritionally limited environment because surface colonization would provide a number of advantages such as increased capture of nutrients that may be absorbed to surfaces [55]. Because the nutrient content of the growth medium has been found to regulate the development of biofilms by other organisms [38,55], we tested various nutrients as well as environmental conditions for their effects on the ability of *S. meliloti* to form biofilms in the wells of the microtiter plate dishes.

Fig. 1 shows that maximal biofilm formation in RDM was observed 72 h after incubation in the microtiter plate wells, followed by a marked decrease in the amount of biofilm formation. One explanation for the decrease in biofilm formation is that it is related to the decrease in growth at later time points. Alternatively, cells may detach from the wells at these time points. Shirtliff et al. [47] have shown that *Pseudomonas aeruginosa* cycles in between increased biofilm biomass and a significant detachment phase.

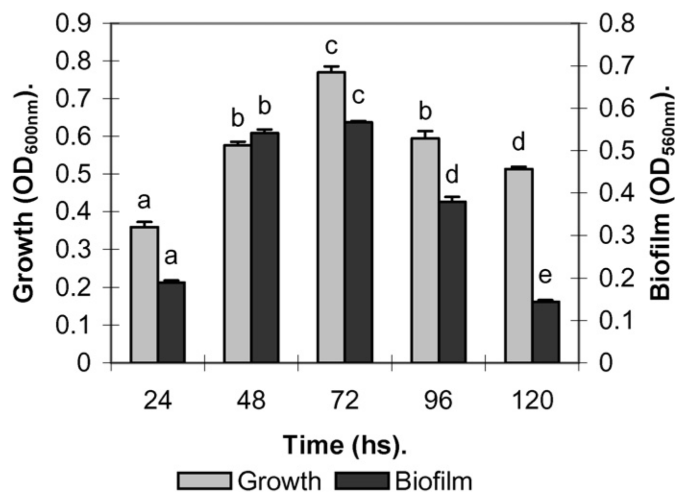


Fig. 1. Quantification of bacterial growth and biofilm formation of static cultures of *S. meliloti* following different times of incubation. All assays were performed in triplicate, and mean values and standard deviations are shown. Values having different letters are significantly different from each other according to Fisher's LSD test ($P < 0.05$).

RDM normally contains 0.5% (ca. 0.015 M) of sucrose. As the concentration of sucrose was increased to 0.3 M (approximately 10%), biofilm formation was augmented, by almost twofold, as assayed by CV staining. In contrast, the highest increase in rhizobial growth occurred at 0.06 M sucrose (Fig. 2A). At 0.6 M sucrose, both bacterial growth and biofilm formation were significantly diminished.

To determine whether the increase in biofilm formation at 0.3 M sucrose was correlated with a concomitant increase in the osmolarity of the medium, we assessed the intracellular potassium content in free-living bacteria. Accumulation of K^+ followed by glutamate is a primary response to osmotic upshift, by counteracting the loss of water, in both *Rhizobium* and *Agrobacterium* when these bacteria are grown in minimal salt medium [32]. A high value (2.16 mmol K^+/g of protein) was observed for cells grown in RDM containing 0.3 M sucrose compared to those grown in medium containing 0.015 M sucrose (1.29 mmol K^+/g of protein), indicating that bacteria undergo osmotic adaptation in RDM containing 0.3 M sucrose. Intracellular K^+ concentrations were also measured for attached and planktonic *S. meliloti* incubated in RDM containing 0.015 M sucrose. The K^+ content in free-living bacteria was 1.29 mmol K^+/g of protein, whereas a value of 1.88 mmol K^+/g of protein was obtained for attached bacteria. Similar results had been found previously for *E. coli*: the intracellular content of potassium ions was higher in attached bacteria than in free-living bacteria [42].

We next assessed whether osmolarity had an effect on the ability of *S. meliloti* to form biofilms, by using NaCl and D-sorbitol as osmotic agents. Across a range of NaCl concentrations (0 to 0.3 M), Rm1021 growth was practically unaffected except at 0.3 M NaCl, where growth was reduced (Fig. 2B). This is not surprising, as *S. meliloti* is reported to be tolerant to 0.3 to 0.7 M NaCl (see references in [56]). In contrast,

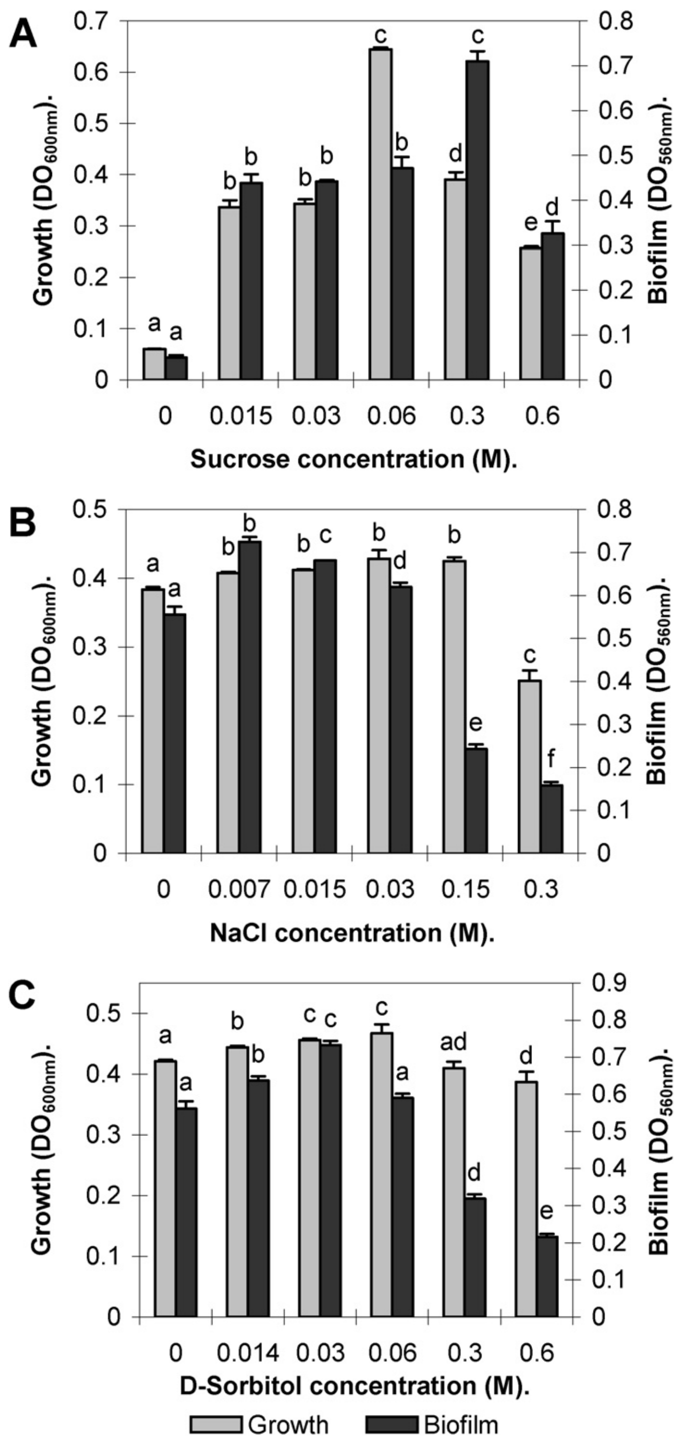


Fig. 2. Bacterial growth and biofilm formation of *S. meliloti* in RDM supplemented with (A) sucrose at the indicated concentrations and (B) different levels of NaCl and (C) D-sorbitol. All assays were performed in triplicate, and mean values and standard deviations are shown. Values having different letters are significantly different from each other according to Fisher's LSD test ($P < 0.05$).

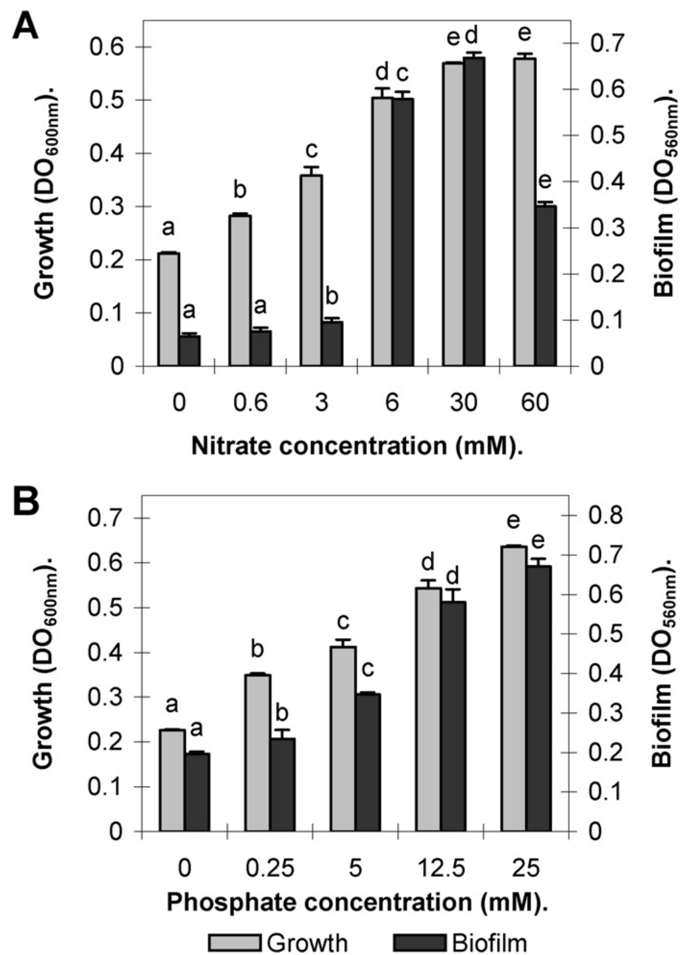


Fig. 3. Bacterial growth and biofilm formation of *S. meliloti* in RDM supplemented with (A) KNO_3 at the indicated concentrations and (B) different levels of phosphate. The biofilm was allowed to develop for 48 h before quantification. All assays were performed in triplicate, and mean values and standard deviations are shown. Values having different letters are significantly different from each other according to Fisher's LSD test ($P < 0.05$).

biofilm formation significantly decreased at higher concentrations of NaCl (Fig. 2B). The inhibitory effect of NaCl on rhizobial biofilm formation could be due to an osmotic effect, since biofilm formation was highest in 0.3 M sucrose (equivalent to the osmotic potential of 0.15 M NaCl) (Fig. 1A). Alternatively, the inhibitory effect of NaCl could be attributed to a specific ion effect, as suggested by Elsheikh and Wood [10]. Hence, we tested the responses of *S. meliloti* to increasing concentrations (0–0.6 M) of another osmolyte, sorbitol. Compared to growth, biofilm formation was reduced in RDM containing 0.3 and 0.6 M sorbitol (Fig. 2C). Taken together, these observations suggest that both NaCl and sorbitol negatively affect biofilm formation through an osmotic effect, whereas the effect of sucrose on biofilm formation is most likely nutritional and not related to osmolarity.

Growth and biofilm formation were assessed using the microtiter plate assay in RDM (normally 6 mM KNO_3) with different nitrate concentrations (0–60 mM) (Fig. 3A). With increasing nitrate concentrations, *S. meliloti* growth increased, reaching a plateau at about 30 mM. Biofilm formation was low between 0 and 3 mM and increased significantly starting at 6 mM of nitrate, with a maximum at 30 mM. At 60 mM nitrate, biofilm formation significantly decreased in contrast to rhizobial growth, which remained at a high level (Fig. 3A).

Apart from carbon, nitrogen and oxygen, phosphorus is the most important nutrient for living cells. To test the importance

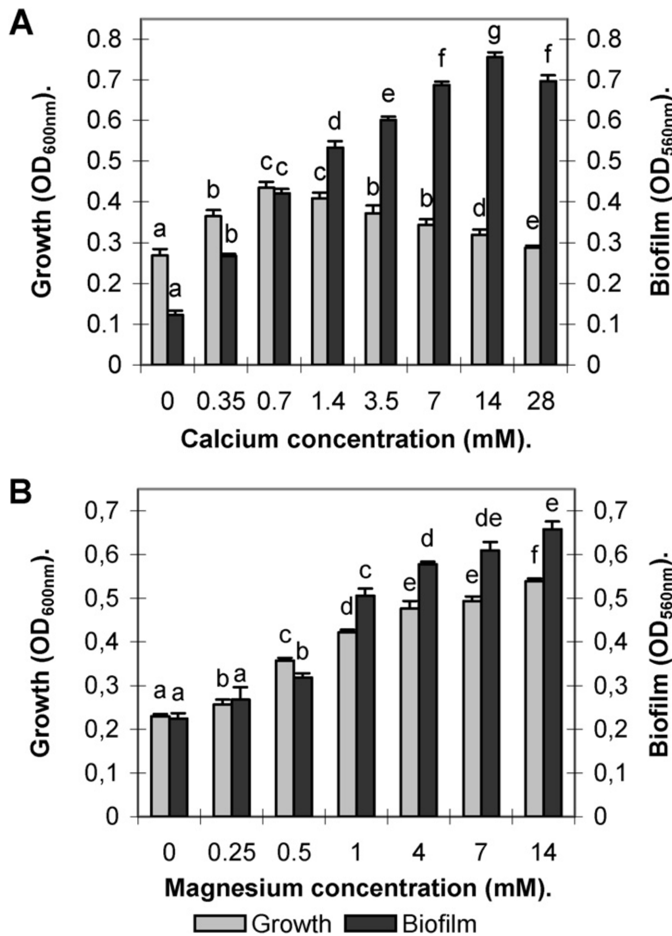
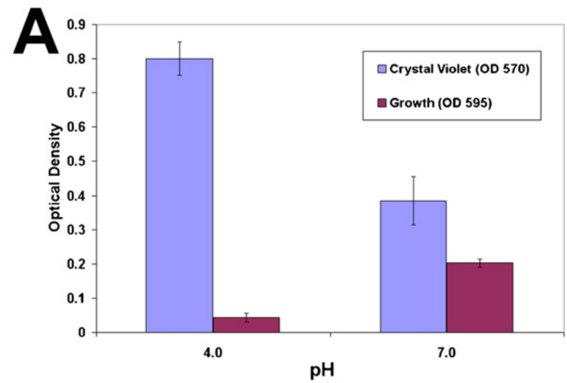


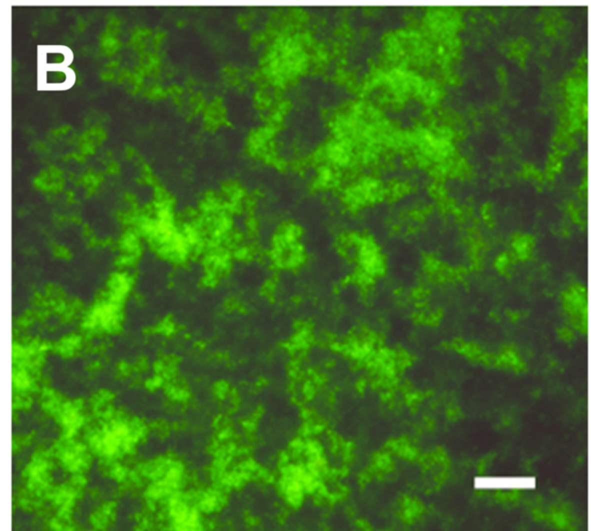
Fig. 4. Quantification of bacterial growth and biofilm formation of static cultures of *S. meliloti* (A) at different $CaCl_2$ and (B) $MgSO_4$ concentrations. All assays were performed in triplicate, and mean values and standard deviations are shown. Values having different letters are significantly different from each other according to Fisher's LSD test ($P < 0.05$).

of phosphate in biofilm formation, *S. meliloti* cells were grown in microtiter plate wells in RDM with phosphate levels ranging from 0 to 25 mM. At low phosphate concentrations (lower than the normal concentration in RDM, 12.5 mM), weak biofilm formation was observed compared to the higher phosphate concentrations (Fig. 3B). This increase in biofilm establishment correlated with an increase in rhizobial growth.

We next tested the involvement of Ca^{2+} and Mg^{2+} in biofilm formation. The optimal Ca^{2+} concentration for growth was 0.7 mM, which is the concentration of Ca^{2+} in RDM. Although increasing the Ca^{2+} concentration above this optimum had a slight deleterious effect on growth, it had a positive effect on biofilm formation (Fig. 4A). The same increase in biofilm formation was observed with increasing Mg^{2+} concentrations. Concentrations above 1 mM of Mg^{2+} (the concentration of Mg^{2+} in RDM) had little effect on growth (Fig. 4B).

S. meliloti strains are extremely sensitive to acidic pH and will show good growth only above pH 5.5 [17,18]. RDM, like most rhizobial media has a pH close to 7.0, and both biofilm formation and growth were high at this pH. In contrast, at pH 4.0, which is typically found in acidic soils, bacteria attached





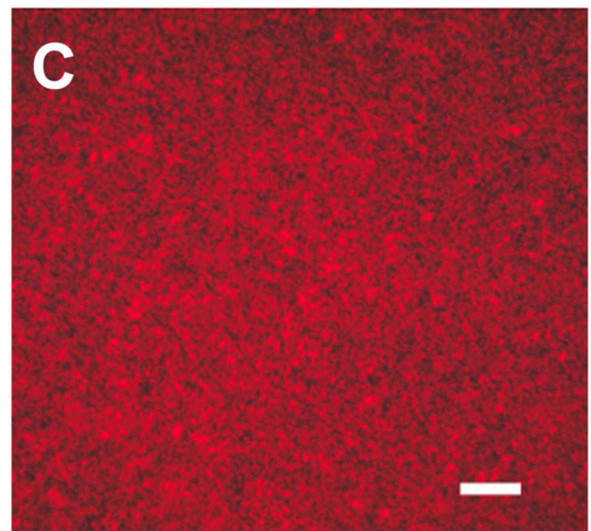


Fig. 5. (A) Microtiter plate assay of biofilm formation by Rm1021 grown for 24 h before staining with crystal violet. Each value point is the average of at least 16 wells. Error bars indicate the standard deviation from the mean. The biofilm levels were standardized to the different growth rates at each pH. (B, C) Fluorescent microscopy of biofilms grown on glass coverslips under different pH conditions for 6 d. Biofilms were stained with LIVE/DEAD fluorescent markers. Bar = 10 µm. (B) Cells grown at pH 7.0. (C) Cells grown at pH 4.0.

to the microtiter plate wells in numbers that were even greater than those observed at neutral pH (Fig. 5A). This was a surprising result, so we stained the biofilm cells with the LIVE/DEAD stain to determine cell viability. At neutral pH, the biofilm tow-

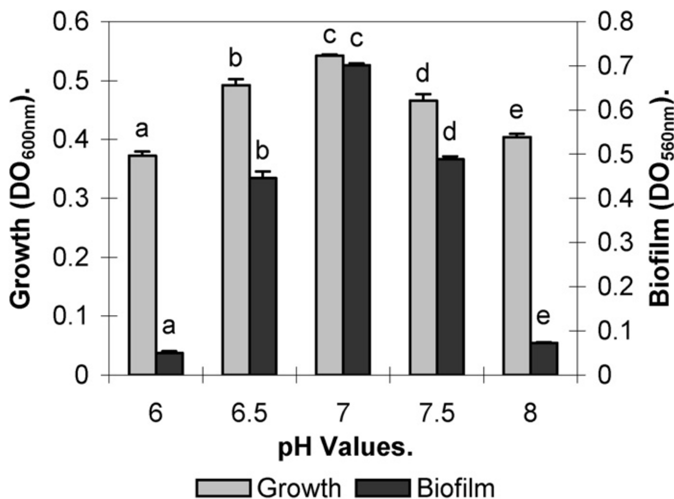


Fig. 6. Influence of pH on growth and biofilm formation by strain Rm1021 grown for 48 h in RDM at different pH values (6.0–8.0). All assays were performed in triplicate, and mean values and standard deviations are shown. Values having different letters are significantly different from each other according to Fisher's LSD test ($P < 0.05$).

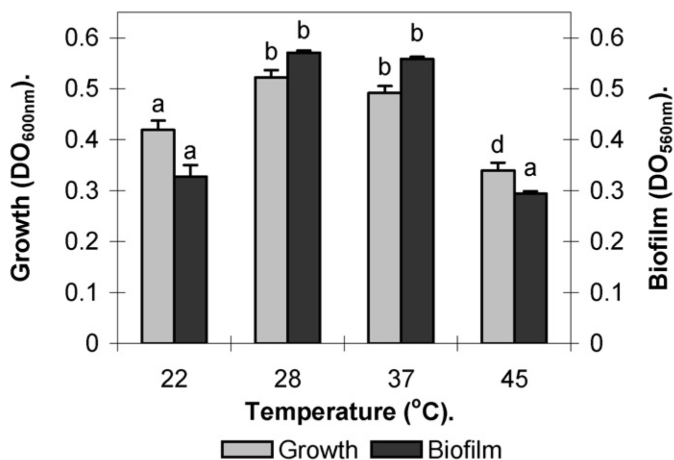


Fig. 7. Bacterial growth and biofilm formation of *S. meliloti* under different temperature regimes. Bacteria were grown at 22–45 °C. All assays were performed in triplicate, and mean values and standard deviations are shown. Values having different letters are significantly different from each other according to Fisher's LSD test ($P < 0.05$).

ers fluoresced green after staining, indicating that the cells were viable (Fig. 5B); a few cells in the older parts of the biofilm fluoresced red (data not shown). At pH 4.0, nearly all cells were in densely packed mats that stained red rather than in towers (Fig. 5C). However, these cells were not necessarily dead. Virtually all planktonic cells also fluoresced red at this pH, but they were observed to swim and twitch, indicating that the bacteria were viable. We also analyzed biofilm formation at initial pH values (from 6.0 to 8.0) that were less likely to inhibit rhizobial growth (Fig. 6). As expected, biofilm formation was significantly diminished at the extremes, pH 6.0 and 8.0, whereas the growth of *S. meliloti* cells, as measured by absorbance at OD_{600}, was not. Taken together, these data show that the optimal pH for growth is pH 7.0 (Fig. 6). However, at pH 4.0, although growth was low, the number of cells in the biofilm was very high.

We also studied the influence of temperatures ranging between 22 and 45 °C on growth and the ability of *S. meliloti* to form biofilms (Fig. 7). At 28 or 37 °C, no clear difference in growth or biofilm accumulation on microtiter plate wells was observed, but the temperatures at the extremes negatively affected both bacterial growth and biofilm formation. Interestingly, at 45 °C, biofilm formation was observed at levels comparable to 22 °C.

4. Discussion

Bacterial biofilms have a significant impact in medical, industrial and environmental settings. Moreover, a number of environmental parameters influence whether biofilms are successfully established in these settings. For example, osmolarity clearly influences rhizobial biofilm formation, as was found for *P. fluorescens* [38] and *E. coli* [41]. We found that growth at high osmolarity (and not simply ionic strength) inhibited biofilm formation in *S. meliloti*. *S. meliloti* cells within biofilms are likely to encounter higher osmolarity conditions. Indeed, the intracellular concentration of potassium ions, which is essentially proportional to the osmolarity of the external medium in the absence of exogenous solutes such as proline or betaine, is almost 1.5-fold higher in attached bacteria than in free-living bacteria (see results). Taken together, these results show that *S. meliloti* cells within the biofilm are under more osmotic stress than planktonic cells, and also that high osmotic potential inhibits biofilm establishment. Furthermore, the effects of sucrose on *S. meliloti* biofilm formation are not strictly related to osmolarity, but rather to the effects of this carbon source on growth, at least until very high osmotic potentials were reached, and then growth was inhibited.

Although we had previously found that nitrogen was required for proper biofilm formation [14], we did not determine the optimal concentration of nitrate. In this report, we found that for optimal biofilm formation a threshold of nitrate concentration between 3 to 6 mM is required, below which there is minimal biofilm formation and above which biofilm formation is at a maximum. The increase in biofilm establishment was unrelated to growth, since the biofilm/growth ratio in minimal medium containing 6 mM nitrate was close to 1.0, whereas in 3 mM nitrate, the ratio was ca. 0.22. This means that in RDM with 6 mM nitrate, that ratio was almost fivefold higher than in RDM containing 3 mM nitrate. This result is interesting in light of the fact that the addition of as little as 5 mM nitrate to seedling growth medium is reported to significantly decrease the number of rhizobial cells adhering to seedling roots [56]. Indeed, a number of studies have illustrated the negative effect of nitrate on root infection by rhizobia [48]. However, differences in tolerance to nitrate and ammonium have also been found among rhizobial isolates when investigated in nodulation systems [34]. For example, Gibson and Harper [16] reported that different strains of *B. japonicum* have varying tolerance to external nitrogen application in their nodulation and nitrogen fixation characteristics. The inhibitory effect of nitrogen on rhizobial cell adherence to roots and on nodulation may be in part plant-mediated because excess nitrate is known to influence

lectin activity [9], and legume lectins mediate a specific type of adherence of rhizobia in *Rhizobium*-legume symbiosis [22]. However, non-lectin-mediated bacterial attachment also occurs, and an excess of nitrate that inhibits the symbiotic process may not necessarily affect non-specific binding to abiotic surfaces. This would explain why adherence to microtiter plate wells is not inhibited by concentrations of nitrate that inhibit nodulation. Therefore, our observations are probably more relevant to the ability of rhizobia to adhere to different soil surfaces.

Availability of phosphate is critical for bacterial growth and metabolism. To some extent, the increase in *S. meliloti* growth correlated with augmented surface attachment as the phosphate concentration was increased. It is well recognized that rhizobia have developed physiological mechanisms for coping with phosphate starvation [26] because the concentration of free phosphate is usually low in natural environments, such as soil and water. For example, in *S. meliloti* Rm1021, exopolysaccharide biosynthesis is influenced by phosphate starvation, resulting in decreased production of succinoglycan (EPSI) and induction of galactoglucan (EPSII) biosynthesis [30,57]. The large difference in phosphate concentrations between the soil (typically 1 to 10 μM) [3] and the nodule (up to 100 mM) [25] is an excellent example of the strikingly dissimilar conditions that *Sinorhizobium* encounters during its lifetime, requiring certain physiological adjustments to occur in order to survive in these environments. For example, the phosphate concentration may serve as a signal to regulate which EPS is produced by *S. meliloti* according to cues taken from the surrounding environment [30]. The type of EPS produced may have an important effect on biofilm formation because EPS accounts for 50–90% of the total organic carbon of biofilms [11]. We previously showed that EPS-minus *S. meliloti* mutants are compromised in biofilm formation, whereas Exo-plus mutants make larger biofilms than wild-type *S. meliloti* [14]. Although EPS varies in chemical and physical properties, it is primarily composed of polysaccharides. Some of these polysaccharides are neutral or polyanionic, as is the case for the EPS of Gram-negative bacteria. The presence of uronic acids (such as D-glucuronic, D-galacturonic, and mannuronic acids) or ketal-linked pyruvates confers anionic properties to the EPS [49]. This is important because it allows the association of divalent cations, such as Ca^{2+} and Mg^{2+}, which have been shown to cross-link with EPS, thereby providing greater stability in a biofilm [11].

In bacteria, Ca^{2+} is implicated not only in EPS binding, but also in processes as important and diverse as the cell cycle and cell division, competence, pathogenesis, motility and chemotaxis, and quorum sensing [23,31,35,36,45]. In the extracellular space, Ca^{2+} also has an important structural role, maintaining the integrity of the outer lipopolysaccharide layer and the cell wall [45]. It is well known that the attachment ability of the bacteria to roots varies considerably depending on the bacterial strain, nutrient requirements and growth conditions. Caetano-Anollés et al. [6] suggested that Ca^{2+} could act as a bridge between negatively charged groups on plant and bacterial surfaces, and/or indirectly activate the bacteria for adhesion. When the medium was supplemented with Ca^{2+} ranging from 7 to 28 mM, a clear increase in the rate of accumulation of attached bacteria occurred in the microtiter plate wells. This may result from the interaction between EPS and Ca^{2+}. However, higher levels of Ca^{2+} negatively affected rhizobial growth, so we examined the effect of another divalent cation (Mg^{2+}) on biofilm formation, and found that high Mg^{2+}concentrations led to an increase in biofilm formation, but without an inhibitory effect on growth. The exact mechanism whereby the transition to the mature biofilm state occurs is unknown, but a decrease in growth may incite the bacteria to form a biofilm or, alternatively, the presence of high levels of divalent cations may stabilize EPS in the biofilm.

The optimal pH for *S. meliloti* growth formation was pH 7.0, but more bacteria attached to the microtiter plate well at pH 4.0. As observed with *Streptococcus gordonii* [27], *S. meliloti* biofilm formation is more sensitive to pH changes than bacterial growth. However, pH had no effect on biofilm formation in *P. fluorescens* [38]. Taken together, these results suggest that pH effects, in terms of establishing a biofilm, may differ from one bacterial species to another, thereby enabling each bacterial species to efficiently colonize its preferred environment.

Differences in tolerance of high temperatures among species and strains of *Rhizobium* have long been recognized [4,19, 24,28,37,39,52]. For most rhizobia, the optimum temperature range for growth in culture is 28–31 °C, and many are unable to grow at 37 °C [18], although Allen and Allen [1] noted that *S. meliloti* has an optimum growth temperature of 35 °C, and eight of eleven strains tested by Graham and Parker [19] grew at 39 °C. We found that *S. meliloti* grew in a wide range of temperatures and no differences were observed between 28 and 37 °C. Moreover, biofilm formation was also affected by extreme temperatures, in that more attachment was evident at 28 and 37 °C. The high tolerance of Rm1021 towards elevated temperatures may allow this strain to survive periods of thermal stress in the soil. For example, high (not extreme) soil temperatures delay nodulation or restrict it to the subsurface region [18]. Munns et al. [33] found that alfalfa plants grown in desert environments in California maintained few nodules in the top 5 cm of soil, but were extensively nodulated below this depth. Under these conditions, nitrogen fixation activity is shut off, but may resume when temperatures are lower.

The results of the present study show that external conditions exert an important influence on the partitioning of cells between planktonic and biofilm phases. At this point, it is not clear whether this partitioning is the simple result of a shift in physiology, possibly related to quorum-sensing cues, which are important for biofilm development in *P. aeruginosa* [8] and *Mesorhizobium huakuii* [54], or whether there is an underlying genetic switch, such as that seen with phase variation by some species facilitating adaptation to new ecological conditions [21], with the subsequent selection and outgrowth of adherent-phase variants in a time- and population-size-dependent manner. The former seems more likely, since several microarray studies comparing biofilm to planktonic bacteria have shown that there are significant differences in gene expression between these two states of existence [53], implying that there is a concomitant change in physiology.

In conclusion, a simple system for the analysis of biofilm formation by *S. meliloti* was utilized that permits the study of persistent colonization on artificial substrates by this organism. We demonstrate that the microtiter plate assay provides a convenient and quantitative means for studying biofilm formation by rhizobia growing under different environmental conditions. Coupling these types of analyses with microarrays or proteomics approaches will give us a better understanding of how rhizobial cells survive in the absence of their host legume.

Acknowledgements

We dedicate this paper to Gabriel Favelukes, a pioneer in the study of rhizobial attachment. This work was supported by grants from the Secretaría de Ciencia y Técnica de la Universidad Nacional de Río Cuarto (SECyT-UNRC) and the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) to WG, by a Shanbrom Family Foundation grant to AMH, and by a UCLA Doctoral Dissertation grant to NAF WG and AZ are Career Members of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) of the República Argentina. LR and EB were supported by a fellowship from the CONICET.

References

[1] E.K. Allen, O.N. Allen, Biochemical and symbiotic properties of rhizobia, Bacteriol. Rev. 14 (1950) 273–330.
[2] Y.H. An, R.J. Friedman, Handbook of Bacterial Adhesion: Principles, Methods, and Applications, Humana Press, Totowa, NJ, 2000.
[3] R. Bieleski, Phosphate pools, phosphate transport, and phosphate availability, Annu. Rev. Plant Physiol. 24 (1973) 225–252.
[4] G.D. Bowen, M.M. Kennedy, Effect of high soil temperature on *Rhizobium* spp., Qld. J. Agric. Sci. 16 (1959) 177–197.
[5] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[6] G. Caetano-Anollés, A. Lagares, A.G. Favelukes, Adsorption of *Rhizobium meliloti* to alfalfa roots: Dependence on divalent cations and pH, Plant Soil 117 (1989) 67–74.
[7] J.W. Costerton, Z. Lewandowski, D.E. Caldwell, D.R. Korber, H.M. Lappin-Scott, Microbial biofilms, Annu. Rev. Microbiol. 49 (1995) 711–745.
[8] D.G. Davies, M.R. Parsek, J.P. Pearson, B.H. Iglewski, J.W. Costerton, E.P. Greenberg, The involvement of cell-to-cell signals in the development of a bacterial biofilm, Science 280 (1998) 295–298.
[9] F.B. Dazzo, W.J. Brill, Regulation by fixed nitrogen of host symbiont recognition in the *Rhizobium*-clover symbiosis, Plant Physiol. 62 (1978) 18–21.
[10] E.A.E. Elsheikh, M. Wood, Response of chickpea and soybean rhizobia to salt: Osmotic and specific ion effects of salts, Soil Biol. Biochem. 21 (1989) 889–895.
[11] H.C. Flemming, J. Wingender, C. Griegbe, C. Mayer, Physico-chemical properties of biofilms, in: L.V. Evans (Ed.), Biofilms: Recent Advances in their Study and Control, Harwood Academic Publishers, Amsterdam, 2000, pp. 19–34.
[12] L. Friedman, R. Kolter, Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms, Mol. Microbiol. 51 (2004) 675–690.
[13] N.A. Fujishige, N.N. Kapadia, A.M. Hirsch, A feeling for the microorganism: Structure on a small scale. Biofilms on plant roots, Bot. J. Linn. Soc. 150 (2006) 79–88.
[14] N.A. Fujishige, N.N. Kapadia, P.L. DeHoff, A.M. Hirsch, Investigations of *Rhizobium* biofilm formation, FEMS Microbiol. Ecol. 56 (2006) 195–206.
[15] J.M. Ghigo, Biofilm-specific physiological pathways beyond a reasonable doubt? Res. Microbiol. 154 (2003) 1–8.
[16] A.H. Gibson, J.E. Harper, Nitrate effect on nodulation of soybean by *Bradyrhizobium japonicum*, Crop Sci. 25 (1985) 497–501.
[17] A.R. Glenn, M.J. Dilworth, The life of root nodule bacteria in the acidic underground, FEMS Microbiol. Lett. 123 (1994) 1–10.
[18] P.H. Graham, Stress tolerance in *Rhizobium* and *Bradyrhizobium*, and nodulation under adverse soil conditions, Can. J. Microbiol. 38 (1992) 475–484.
[19] P.H. Graham, C.A. Parker, Diagnostic features in the characterization of the root-nodule bacteria of legumes, Plant Soil 20 (1964) 383–396.
[20] J.J. Harrison, R.J. Turner, L.L.R. Marques, H. Ceri, Biofilms, Am. Sci. 93 (2005) 508–515.
[21] I.R. Henderson, P. Owen, J.P. Nataro, Molecular switches—the ON and OFF of bacterial phase variation, Mol. Microbiol. 33 (1999) 919–932.
[22] A.M. Hirsch, Role of lectins (and rhizobial exopolysaccharides) in legume nodulation, Curr. Opin. Plant Biol. 2 (1999) 320–326.
[23] I.B. Holland, H.E. Jones, A.K. Campbell, A. Jacq, An assessment of the role of intracellular free Ca^{2+} in *E. coli*, Biochimie 81 (1999) 901–907.
[24] S. Ishizawa, Studies on the root nodule bacteria of legume plants. I. Characters in artificial media. Part 5. Effect of temperature on growth of rhizobia, J. Sci. Soil Manure (Tokyo) 24 (1953) 227–230.
[25] D. Israel, Investigation of the role of phosphorus in symbiotic dinitrogen fixation, Plant Physiol. 84 (1987) 835–840.
[26] E. Krol, A. Becker, Global transcriptional analysis of the phosphate starvation response in *Sinorhizobium meliloti* strains 1021 and 2011, Mol. Gen. Genomics 272 (2004) 1–17.
[27] C.Y. Loo, D.A. Corliss, N. Ganeshkumar, *Streptococcus gordonii* biofilm formation: Identification of genes that code for biofilm phenotypes, J. Bacteriol. 182 (2000) 1374–1382.
[28] K.C. Marshall, Survival of root-nodule bacteria in dry soils exposed to high temperature, Aust. J. Agric. Res. 15 (1964) 273–281.
[29] H.M. Meade, S.R. Long, G.B. Ruvkun, S.E. Brown, F.M. Ausubel, Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis, J. Bacteriol. 149 (1982) 114–122.
[30] K.E. Mendrygal, J.E. González, Environmental regulation of exopolysaccharide production in *Sinorhizobium meliloti*, J. Bacteriol. 182 (2000) 599–606.
[31] J. Michiels, C. Xi, J. Verhaert, J. Vanderleyden, The functions of Ca^{2+} in bacteria: A role for EF-hand proteins? Trends Microbiol. 10 (2002) 87–93.
[32] K.J. Miller, J.M. Wood, Osmoadaptation by rhizosphere bacteria, Annu. Rev. Microbiol. 50 (1996) 101–136.
[33] D.N. Munns, H.H. Keyser, V.W. Fogle, J.S. Hohenberg, T.L. Righetti, D.L. Lauter, M.G. Zaruog, K.L. Clarkin, K.W. Whitacre, Tolerance of soil acidity in symbiosis of mung bean with rhizobia, Agron. J. 71 (1979) 256–260.
[34] L.M. Nelson, Response of *Rhizobium leguminosarum* isolates to different forms of inorganic nitrogen during nodule development in pea (*Pisum sativum* L.), Soil Biol. Biochem. 19 (1987) 759–763.
[35] V. Norris, M. Chen, M. Goldberg, J. Voskuil, G. McGurk, I.B. Holland, Calcium in bacteria: A solution to which problem? Mol. Microbiol. 5 (1991) 775–778.
[36] V. Norris, S. Grant, P. Freestone, J. Canvin, F.N. Sheikh, I. Toth, M. Trinei, K. Modha, R.I. Norman, Calcium signalling in bacteria, J. Bacteriol. 178 (1996) 3677–3682.
[37] P.S. Nutman, *Rhizobium* in the soil, in: N. Walker (Ed.), Soil Microbiology, Wiley, New York, 1975, pp. 111–131.
[38] G.A. O'Toole, R. Kolter, Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: A genetic analysis, Mol. Microbiol. 28 (1998) 449–461.
[39] C.A. Parker, M.J. Trinick, D.L. Chatel, Rhizobia as soil and rhizosphere inhabitants, in: R.W.F. Hardy, A.H. Gibson (Eds.), A Treatise on Nitrogen Fixation, Section IV, Agronomy and Ecology, Wiley, New York, 1977, pp. 311–352.
[40] L.A. Pratt, R. Kolter, Genetic analysis of *Escherichia coli* biofilm formation: Roles of flagella, motility, chemotaxis and type I pili, Mol. Microbiol. 30 (1998) 285–293.
[41] C. Prigent-Combaret, E. Brombacher, O. Vidal, A. Ambert, P. Lejeune, P. Landini, C. Dorel, Complex regulatory network controls initial adhesion

and biofilm formation in *Escherichia coli* via regulation of the *csgD* gene, J. Bacteriol. 183 (2001) 7213–7223.

[42] C. Prigent-Combaret, O. Vidal, C. Dorel, P. Lejeune, Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli*, J. Bacteriol. 181 (1999) 5993–6002.

[43] B.E. Ramey, A.G. Matthysse, C. Fuqua, The FNR-type transcriptional regulator SinR controls maturation of *Agrobacterium tumefaciens* biofilms, Mol. Microbiol. 52 (2004) 1495–1511.

[44] G. Seneviratne, H.S. Jayasinghearachchi, Mycelial colonization by bradyrhizobia and azorhizobia, J. Biosci. 28 (2003) 243–247.

[45] R.J. Smith, Calcium and bacteria, Adv. Microb. Physiol. 37 (1995) 83–133.

[46] N.R. Stanley, B.A. Lazazzera, Environmental signals and regulatory pathways that influence biofilm formation, Mol. Microbiol. 52 (2004) 917–924.

[47] M.E. Shirtliff, J.T. Mader, A.K. Camper, Molecular interactions in biofilms, Chem. Biol. 9 (2002) 1–20.

[48] J. Streeter, Inhibition of legume nodule formation and N_2 fixation by nitrate, Crit. Rev. Plant Sci. 7 (1988) 1–23.

[49] I.W. Sutherland, Biofilm exopolysaccharides: A strong and sticky framework, Microbiology 147 (2001) 3–9.

[50] L. Sutherland, J. Cairney, M.J.I. Elmore, R. Booth, C.F. Higgins, Osmotic regulation of transcription: Induction of the *proU* betaine transport gene is dependent on accumulation of intracellular potassium, J. Bacteriol. 168 (1986) 805–814.

[51] J.M. Vincent, A Manual for the Practical Study of Root-Nodule Bacteria, Blackwell Scientific Publications, London, 1970.

[52] J.M. Vincent, *Rhizobium*: General microbiology, in: R.W.F. Hardy, W.S. Silver (Eds.), A Treatise on Nitrogen Fixation, Section III, Biology, Wiley, New York, 1977, pp. 277–366.

[53] R.D. Waite, A. Papakonstantinopoulou, E. Littler, M.A. Curtis, Transcriptome analysis of *Pseudomonas aeruginosa* growth: Comparison of gene expression in planktonic cultures and developing and mature biofilms, J. Bacteriol. 187 (2005) 6571–6576.

[54] H. Wang, Z. Zhong, T. Cai, S. Li, J. Zhu, Heterologous overexpression of quorum-sensing regulators to study cell-density-dependent phenotypes in a symbiotic plant bacterium *Mesorhizobium huakuii*, Arch. Microbiol. 182 (2004) 520–525.

[55] J.W.T. Wimpenny, R.A. Colasanti, A unifying hypothesis for the structure of microbial films based on cellular automation models, FEMS Microbiol. Ecol. 22 (1997) 1–16.

[56] H.H. Zahran, *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate, Microbiol. Mol. Biol. Rev. 63 (1999) 968–989.

[57] H.J. Zhan, C.C. Lee, J.A. Leigh, Induction of the second exopolysaccharide (EPSb) in *Rhizobium meliloti* SU47 by low phosphate concentrations, J. Bacteriol. 173 (1991) 7391–7394.