Root colonization by *Pseudomonas putida*: love at first sight

Fluorescent pseudomonads are an important part of the soil microbiota, frequently found in association with plant roots. This interaction between bacteria and plants is often beneficial for both partners. The surface of roots and the surrounding soil areas (the rhizosphere) constitute an environment where nutrients released by the plant in the form of root exudates are available to the microorganisms. In turn, some *Pseudomonas* strains can improve plant growth and play a protective role against pathogens. These activities and their potential use in agriculture have prompted an increasing interest in the study of mutualistic plant-*Pseudomonas* interactions. One of the most relevant aspects is the process of bacterial establishment in the rhizosphere, since an effective biocontrol depends on the efficiency of root colonization (2). Most studies of factors potentially involved in root colonization efficiency draw their conclusions from looking at well-established populations, i.e. after one to four weeks of seedling inoculation and sowing. Comparatively less attention has been paid to the early stages of colonization. How do these root-*Pseudomonas* interactions begin?

Like in any love story, attraction is probably key for the initiation of mutualistic plant–bacterial interactions. Bacterial chemotaxis on plates towards different nutrients known to be present in root exudates has been demonstrated (9). In *Azospirillum brasilense*, non-flagellated and non-chemotactic mutants showed reduced ability to colonize wheat roots (10). Although the question of bacterial motility in soil is still under debate, a series of experiments performed by Bashan (1) with *Pseudomonas fluorescens* and *A. brasilense*, pointed out the existence of a directed motion of bacterial cells towards wheat roots in soil. However, this motion was heavily influenced by soil composition and humidity (1). The role of motility in attachment and colonization has been recently examined in detail by Turnbull et al. (7, 8). Motility seems to be important for competitive root colonization by *P. fluorescens* (8), as well as for attachment of *Pseudomonas putida* to wheat roots under conditions of nutrient limitation (7).

As part of our on-going interest in mutualistic plant–*Pseudomonas* interactions, we have now examined the initiation of root colonization by *P. putida* in an *in vitro* system that allows direct observation by time-lapse microscopy.

The bacterium *Pseudomonas putida* KT2440, a derivative of the soil isolate mt-2 (5), is able to colonize the root system of a number of different plants, establishing and persisting in the rhizosphere at a relatively high population density (6). The initial establishment of *P. putida* KT2440 around corn roots was followed by phase-contrast microscopy using a Nikon Diaphot 200 inverted microscope. Corn seedlings (germinated for 4 days) were placed on Petri plates with 0.4% agar (w/v in sterile deionized water). A small amount of bacterial culture was then inoculated ~1 mm away from the root with a sterile toothpick. Images were captured at 20 s intervals with a CCD72 camera integrated with a Power Macintosh 8600/300 computer, processed using SCION IMAGE software (Scion), a modification of NIH IMAGE (NIH), and mounted as three different movies, 25 s long, each corresponding to 1.5 h periods. These movies are available at http://mic.sgmjournals.org

The bacteria immediately responded to the presence of the plant, migrating from the point of inoculation towards the root. During the first hour after inoculation, motion of the bacterial cells towards the root could be clearly observed (Fig. 1a, b), suggesting a rapid chemotactic response of KT2440 to the presence of the plant (which of course would depend on the distance from the root). After this first stage of bacterial motion, microcolonies start to form on the surface and in the vicinity of the root, their size steadily increasing during the following hours (Fig. 1c, d). This increase in size may result both from the recruitment of new members into the colony as well as from cell growth and division. Dividing cells were in fact observed during this period, suggesting that there is a relatively abundant release of nutrients in the form of root exudates. An intriguing question raised by these microscopy data is if bacterial aggregation and microcolony formation responds to the relative accumulation of nutrients around the root, or if there is a specific signal triggering it.

After this fast establishment of the bacteria

GUIDELINES

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in the vicinity of the root, the population reached an equilibrium. Two days after inoculation, microscopy revealed a stable and somewhat static population of bacteria around the root, with only a small proportion of free-swimming cells visible (not shown).

We compared these microscopy observations with a short-term study of bacterial growth on the root from inoculated seedlings, determined by viable counts. In this system, the initial association takes place during incubation in liquid medium, and is reflected in the number of bacteria attached to the seedlings.

Corn seedlings (germinated for 3 d) were inoculated with a suspension of KT2440 [5 × 10⁶ c.f.u. (g seedling)⁻¹, Fig. 1c], and sown in pots containing vermiculite. At different times, plants were removed and the number of bacteria associated with the root was determined by plating on selective medium as described (3, 4). Around 1% of the inoculated bacteria attached to the seedlings after 1 h incubation in minimal medium. Interestingly, after sowing the seedlings, there was a drop in the number of viable cells recovered from them with respect to the initial attached population. This phenomenon was observed in all the experiments and could be explained either by the bacteria spreading in the solid substrate or by death of part of the population. To determine which of these possibilities was true, samples of vermiculite accounted almost completely for the reduction in the number of cells recovered from the seedlings (data not shown). The reasons why this ‘detachment’ from the seedlings takes place in the solid matrix remain to be clarified.

Following this initial drop, the population associated with the seedlings rapidly grew (τ ~ 85 min during the first 3 h and ~ 60 min the following 3 h), reaching ~ 2 × 10⁹ c.f.u. (g seedling)⁻¹ after 24 h and a maximum of ~ 5 × 10⁹ c.f.u. (g root)⁻¹ after 48 h. From then on the number of c.f.u. (g root)⁻¹ stayed essentially steady with slight fluctuations for over a week (not shown), suggesting that although seed and root exudates are released constantly by the plant, the amount of nutrients present in them determines a maximal population size per weight unit of plant tissue.

The experiments presented here may not be a direct reflection of the ‘real world’, but they offer an interesting perspective of what happens during the processes ordinarily used to study root colonization in the lab. The establishment of P. putida on plant roots appears as a very fast and dynamic process in which three stages can be defined: i) an ‘attraction phase’ of bacterial movement probably resulting from chemotactic responses to the presence of the root; ii) a rapid ‘settlement phase’, in which bacteria grow, divide and aggregate to form microcolonies around the root; and iii) a ‘residence phase’, where the established population reaches the maximal size relative to root weight and its further growth couples with plant growth, so that the bacterial numbers relative to root weight remain constant afterwards. In the experiments presented here, starting from inoculated seedlings (a protocol routinely used in colonization studies), this maximum is attained as soon as 48 h after seed inoculation. The dynamic nature of the early colonization stages suggests they may be substantial for the later events, and so their study could answer key questions with respect to the mechanisms of mutualistic plant–Pseudomonas interactions.

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Fig. 1. Establishment of P. putida KT2440 around corn roots. Microscopy images were captured every 20 s for 4.5 h from the time of inoculation. Selected images are shown. (a, b) Motility of KT2440 towards the root (dark area) (a) 5 min and (b) 1 h after inoculation. (c, d) Establishment and growth of microcolonies of KT2440 around the corn root tip (c) 2.5 h and (d) 4.5 h after inoculation. Examples are indicated by arrows. (e) Corn root colonization by P. putida KT2440 from inoculated seedlings. No. cells in the bacterial suspension used to inoculate seedlings [log c.f.u. (g seed)⁻¹]; bacteria associated with the seedling or root [log c.f.u. (g root)⁻¹]. Inoculation and determination of the number of cells associated with the seeds/roots was done as described (3, 4). Results are the average of two assays where each data point corresponds to duplicate samples from three seedlings. Error bars are indicated.
New chlamydial lineages from freshwater samples

Chlamydiae are important obligate intracellular bacteria, causing a variety of diseases in vertebrates, including humans. Small subunit ribosomal RNA gene (16S rDNA) sequence analyses have allowed the identification of new chlamydial lineages; at present four families, for a total of six genera and 13 species, have been described (6, 11, 15). Among the new lineages the family Parachlamydiaceae that includes endosymbionts of amoebae of the group Acanthamoeba – Hartmannella is of interest. Parachlamydiaceae have been identified in various strains of amoebae isolated from soil, water conduit systems, sewage sludge, corneal/contact lens samples and nasal mucosa of healthy individuals (1, 9, 11), and there is evidence for their implication in human respiratory infections (2, 4). 16S rDNA analyses showed that this group of chlamydiae is very rich, probably including several species and genera (10). Simkania negevensis is another emerging chlamydia, isolated initially as a cell culture contaminant and successively associated with human respiratory infection in Israel, Great Britain and USA (11, 15, 19, 21). The diversity within chlamydiae is likely to be more important: using PCR, new 16S rDNA phytoplasts have been detected in clinical samples (4, 14), and sequence databases contain over 100 partial 16S rDNA sequences apparently belonging to distinct chlamydial species. Increasing diversity within Chlamydiales is also shown by results reported by Bowman et al. (3), who found 16S rDNA sequences from sediments of Antarctic marine salinity lakes (Burton-46; 999 bp) and a basin (Taynaya-24; 995 bp). The sequence Burton-46 seems to belong to an organism related to parachlamydiae and Waddlia chondrophila, while the sequence Taynaya-24 probably represents a new lineage distantly related to the other chlamydiae.

Herein we report for the first time the presence of a Simkaniaceae-related sequence in an environmental sample, as well as the presence of another sequence representing a novel chlamydial lineage distantly from the other chlamydiae described until now. These sequences were named cvE6 (GenBank accession no. AF448722) and cvE9 (GenBank accession no. AF448723), and were detected in freshwater samples from two independent ponds, one located in France, the other in Italy.

Total DNA was extracted from 1 ml aliquots of subsurface shore samples by the classic phenol-chloroform method after proteinase K digestion. Chlamydial 16S rDNA was searched for by using a pan-chlamydia primer set amplifying almost all the gene (nucleotide sequences for forward and reverse primers were 5′-CGT GGA TGA GGC ATG C/A/G) A GTC G-3′ and 5′-GTC ATC (A/G)GC C/T/C/T/C ACC TT A/C/G C/G/ C/A/G C/A/G (T/C/T/C/T/C TCT-3′, positions 35–1481, Parachlamydia acanthamoebae 16S rDNA numbering). Manipulations were performed according to recommended guidelines and included negative controls starting from the DNA extraction step. Both strands of an inner portion (~1100 bp) of the PCR products were sequenced (three repetitions) using a series of inner primers, and the partial 16S rDNA sequences were aligned with all chlamydial 16S rDNA sequences available in the database. The sequences cvE6 and cvE9 showed 90.1% similarity with each other, and values of 87.9 and 92.7%, respectively, with that of S. negevensis. Sequence similarities of cvE6 and cvE9 with other chlamydiae were 85.7% and 86.4% for W. chondrophila, 86.9–89.5% and 89.1–91% for Parachlamydiaceae, and 84–85% and 86.5–87% for Chlamydiaceae. In phylogenetic reconstructions our sequences cluster with S. negevensis showing high bootstrap values (Fig. 1a).

Phylogenetic analysis was also performed on a 770 bp portion common to all chlamydial 16S rDNA sequences, including Antarctic sequences (Fig. 1b). The sequence Burton-46 showed similarity values of 89.4–92.2% to those of parachlamydiae, and 88.7% to that of W. chondrophila. However, the branching pattern was not clearly defined. Even if based on partial sequence analysis only, it seems clear that our sequence cvE9 was from an organism specifically related to S. negevensis (92.7% similarity, 100% bootstrap), representing either a new member of the Simkaniaeae or an organism of a new, closely related lineage. This is the first report, to our knowledge, on a Simkania-like organism from environmental samples. On the contrary, the sequence cvE6 seems to be distantly related to the Antarctic sequence Taynaya-24 (87.9% similarity), and both seem to form new independent lineages, perhaps distantly related to Simkaniaeae. Amplification with S. negevensis-specific primers (12) was negative in both our samples.

The presence of a Simkania-related organism in an environmental habitat might be surprising. S. negevensis is at present the only known member of the family Simkaniaeae. Its natural host is unknown, and there is evidence that human infections are widespread (8). Recently Kahane et al. (13) demonstrated S. negevensis being able in vitro to infect and to multiply within Acanthamoeba. It seems then conceivable that our sequence cvE9 was from a protistan endosymbiont. In addition, at least one member from the Chlamydiaceae, Chlamydothelia pneumoniae, may also grow in vitro within acanthamoebae (5). Intra-amoebal growth does not seem to be restricted to parachlamydiae, and amoebae and perhaps other protists (e.g. ciliates) may be natural or occasional hosts of chlamydiae, as it occurs for a large variety of bacteria. Similarly, the most plausible explanation for the origin of our second sequence, cvE6, as well as of Antarctic sequences (3), is that they are from chlamydial endosymbionts of protists. In addition, a rich mosaic of chloroplast and eukaryotic gene homologues has been

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Fig. 1. Phylogenetic reconstructions for partial 16S rDNA sequences using the neighbour-joining method (Jukes & Cantor reoption), generated by Molecular Evolutionary Genetics Analysis (MEGA). Topology stability was evaluated by bootstrapping (200 replicates), only values of 50% or more are indicated. Sequences cvE6 and cvE9 are indicated in bold. Uncultured bacterium vadinBE97 (vadin lineage), verrucomicrobial strain VeCb1 (Verrucomicrobia), and Pirellula staiare (Planctomycetes) sequences were used as outgroups. Bars indicate estimated genetic distance. Nucleotide positions are 16S rDNA numbering. (a) Phylogenetic tree based on a 1053 bp fragment (nt positions 230–1284). (b) Phylogenetic tree based on a 770 bp fragment (nt positions 512–1284) to include also 16S rDNA sequences Burton-46 and Taynaya-24 from Antarctic samples.

The ability to use intra-amoebal growth may be considered as a preadaptation for successful infection of mammalian cells, including phagocytic ones. Previous reports on new chlamydial organisms were from clinical samples (2, 4, 8, 12, 14, 15), and environmental isolates concerned exclusively new phylotypes of para-chlamydia (9–11). Our data and those of Bowman et al. (3) indicated that new distinct chlamydial lineages exist in natural environments as different as Antarctic marine salinity and freshwater ponds. All the chlamydiae described until now are able to infect vertebrates and to induce diseases: it may be of interest to estimate their prevalence and diversity in various natural habitats and in this way to identify possible sources of infections for both known and new chlamydiae.

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the sequenced genomes of Chlamydiaceae (16), and the 23S rDNA of S. negevensis possesses an unspliced group I intron that is closely related to introns found in chloroplasts of some green algae (Chl-

mydomonas spp.) and in mitochondria of Acanthamoeba (7). Such a genetic mosaicism has led to the supposition that lateral gene transfer events may have occurred in the ancestors of present-day chlamydiae living probably as endosymbionts of protists. Searches in environmental habitats might lead to the discovery of new chlamydial lineages and/or new natural hosts other than acanthamoebae. For example, the recently discovered Neochlamydia hartmannellae, belonging to Parachlamydiaceae, is able to infect both Hartmannella and Dictyostelium, but not Acanthamoeba (11). This implies that other eukaryotes may harbour chlamydial organisms. Chlamydia-like organisms have been morphologically described in some invertebrates as hydrids and mussels, but confirmation by phylogenetic analyses is lacking. It may be interesting also to evaluate the protist host range of chlamydiae by experimental infections of various types of protists other than amoebae, including ciliates and algae. In this way it has already been shown that both the respiratory pathogens C. pneu-

miae and S. negevensis may survive and multiply within Acanthamoeba (5, 13). They might then utilize amoebae as a reservoir vector, like parachlamydiae or legionellae.