Bacterial traits and genes involved in rhizosphere colonization

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ABSTRACT

Plant roots are colonized by microbes which are supposed to grow on exudate compounds. Among these microbes are soil-borne pathogens which cause diseases. The process of root colonization can be applied for beneficial purposes such as biocontrol, biofertilization, bioremediation and phytostimulation. In attempts to identify bacterial genes and traits involved in root colonization we have used two approaches. By predicting possible colonization traits and subsequently testing mutants impaired in that trait on their ability to colonize in competition with the parental strain, we have identified the following colonization traits: the syntheses of flagella, the O-antigen of LPS [lipopolysaccharide], amino acids and vitamin B1, and utilization of major exudate carbon sources. Using another approach, we tested random Tn5[*lacZ*] mutants on their competitive colonization ability and found that mutations in the following genes resulted in impaired colonization: [*i*] A two-component system [*colR/S*] of which neither the stimulus nor the trait has been identified yet; [*ii*] *sss*, encoding site-specific recombinase belonging to the lambda integrase gene family; [*iii*] *nuo*, which in *E. coli* consists of a 14-gene operon encoding NADH:ubiquinone oxidoreductase. One of the two enzymes involved in the generation of the proton motive force.

Introduction

The enormous impact that soil bacteria can have on plants is illustrated in an experiment of Campbell and Greaves [3], who isolated 150 fluorescent *Pseudomonas* sp. from field-grown wheat and reintroduced these strains separately on wheat seedlings; 40% of the strains stimulated root growth, 40% inhibited it, whereas 20% showed no effect.

Effective rhizosphere colonization by bacteria can contribute to the following processes: (*i)* Causing disease by plant pathogens; (*ii*) disease control by microbes that produce anti-fungal factors (AFFs); (*iii*) phytostimulation, e.g. plant growth promotion by the production of phytohormones; (*iv*) biofertilisation, i.e. the process in which micro-organisms increase the availability of nutrients [17]; (*v*) bioremediation, i.e. the microbial degradation or inactivation of hazardous chemicals in the environment.

Strategy for studying root colonization at the molecular level

Despite its important role in all processes mentioned previously, bacterial traits involved in the molecular basis of rhizosphere colonization are still hardly known. Therefore we set out to study traits involved in competitive root-tip colonization. *Pseudomonas fluorescens* WCS365, a biocontrol strain in a tomato-*Fusarium* system [6], was chosen as the parental strain because it appeared to be an efficient root colonizer [2, 15]. Because applications of

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beneficial bacteria often make use of coated seeds, we chose to start with colonized seeds or seedlings. A gnotobiotic system was developed to study root colonization [20] and has been used successfully for tomato, potato, radish and wheat [4, 7, 8, 9, 12, 19, 20, 21]. Two different approaches were used to identify traits and genes involved in competitive root colonization. In the predictive approach mutants were isolated in traits suspected to be involved in colonization and mutants were subsequently tested for their colonization ability. Secondly, individual random transposon mutants were screened for their colonization ability in competition with the parental strain WCS365. Subsequently these mutants were genetically and physiologically characterized [7, 8, 9].

To study the efficiency of colonization we analyzed the root-tip rather than the whole root since cells that reach the root-tip are actively participating in the colonization process.

Traits and genes involved in root colonization

In this paper colonization traits are defined as traits that appear to be defective in mutants with reduced ability to colonize the root tip after application to the seed or seedling. The most severely impaired colonization mutants from our screening studies appeared to be non-motile mutants. Although the role of **motility** in colonization could not be established in some studies [16, 18] we consistently observed that *Pseudomonas* motility mutants are impaired in colonization, independent of: (*i*) the chosen parental strains [13, 20]; (*ii*) whether the mutants were isolated as non-motile or as colonization deficient; (*iii*) whether tested in sand [9, 20] or soil [13]; or (*v*) whether tested on potato [13] or tomato [9, 20].

Mutants of *P. fluorescens* impaired in the synthesis of the **O-antigen of lipopolysaccharide** (LPS) are impaired in root colonization, both when tested alone in soil on potato [10] and tomato [9] as well as when tested in competition in the gnotobiotic tomato system [9, 20]. Using a sensitive test system, we could show that mutants lacking the O-antigen of LPS have a decreased growth rate compared to their parental strains [9]. Also, one mutant with a shifted O-antigen ladder pattern had a decreased growth rate [9]. The mutation appeared to be located in a gene homologous to *htrB* that encodes a lauroyl transferase that functions in lipid A biosynthesis [5]. All O-antigen mutants as well as the *htrB* mutant showed a strongly defective growth in exudate, especially when tested in competition with the parental strain.

In contrast, a newly isolated colonization mutant, containing a shortened O-antigen of LPS [9] did not have a decreased growth rate. These results suggest that in addition to a possible role through growth rate, the O-antigenic side chain itself must also, in some other way, be involved in root colonization.

The ability to **synthesize amino acids and vitamin B1** was shown to be essential for root colonization. This indicates that the amounts of these amino acids and vitamin B1 exuded by the root is too low to physiologically complement the mutations. Genes involved in the synthesis of amino acids and vitaminB1 are therefore essential for colonization [19].

Mutants of WCS365 impaired in their ability to grow on some sugars present in exudate were shown to behave similarly to the wild type in competitive colonization. In contrast, mutants impaired in the utilization of organic acids had reduced colonization, indicating that utilization of organic acids is the major nutritional basis for root colonization by strain WCS365.

Novel traits obtained by screening random transposon mutants of P. fluorescens *WCS365*

After screening of 2200 mutants [7, 9, 11], sixteen novel competitive colonization mutants were isolated. The observation that **growth rate** contributes to colonization [20] is underpinned by the fact that of these mutants five had a slightly decreased growth rate in competition in rich medium [9]. The remaining novel colonization mutants had no detectable defect in motility, the synthesis of O-antigen of LPS, amino acids, vitamin B1, and growth rate [7, 8, 9]. All were severely impaired in competitive root colonization on tomato, wheat, and radish, indicating broad host range mutations. All these mutants colonize the root well when inoculated alone, indicating that they are not super sensitive to compounds exuded by the root [7, 8, 9].

In one of the novel mutants an insertion was found in the *nuo4* gene encoding one of the subunits of NADH:ubiquinone oxidoreductase, **NADH dehydrogenase**. This protein is involved in the generation of proton motive force that is used for the synthesis of ATP, active transport of various nutrients and ATP-dependent rotation of the flagella [1]. In *Escherichia coli* a second NADH dehydrogenase is present encoded by the *ndh* gene, this might explain the lack of a defect in competitive growth. Expression of this *ndh* is reduced during anaerobic growth. The colonization defective phenotype of the *nuo* mutant could be explained by the presence of a similarly regulated *ndh* gene in *Pseudomonas*. Experiments showed that under micro-aerobic conditions the *nuo* mutant indeed grows slower then WCS365. Therefore it is tempting to speculate that an oxygen limitation is present in the rhizosphere that is too low to maintain wild type growth rate and motility and that, as a result, the *nuo* mutant loses its rhizosphere competence [9].

A **two-component system** is shown to be involved in the colonization ability of *P. fluorescens* WCS365. A transposon insertion was found to be present in the sensor kinase member [ColS] of a two-component system [7]. No evidence was found that the ColR/ColS two-component system was involved in suspected colonization traits (chemotaxis and transport of exudate compounds [7]). Therefore we conclude that competitive root colonization by *P. fluorescens* WCS365 is an active process in which an environmental stimulus plays a major role. Recent preliminary results indicate that some exudate compounds are able to induce the promoter of ColR/S and we suspect that the system may play a role in maintaining the cells internal pH.

The action of a member of the lambda integrase family of **site-specific recombinases [xerC/sss]** also seems to be essential for colonization [8]. Members of the lambda integrase family of site-specific recombinases promote conservative reciprocal recombination between two small homologous DNA repeats. Depending on the orientation of these two sequences, this will either lead to inversion or to excision of the DNA fragment situated between these sites. Site-specific recombinases have been implicated in the production and regulation of fimbriae in *E.coli*, the production of two different forms of LPS in *Francisella tularensis*, antigenic variation of surface lipoprotein antigens in *Mycoplasma bovis* and the production of two flagellin genes in *Salmonella typhymurium*. This suggests that lacking the ability for DNA rearrangements can affect one or more traits already described to be important for root colonization.

DNA rearrangements in bacteria are presumed to play a role in the formation of sub-populations. Dybvig [14] suggested that the ability to generate sub-populations enables bacteria to adjust to sudden environmental changes. We interpret the colonization defect of the *xerC/sss* mutant as being locked in a genetic arrangement not suitable for colonization.

Colonization is often considered the limiting step in biocontrol. Introduction of the operon containing *xerC/sss* into two other *Pseudomonas* strains leads in both cases to a 10-20 fold enhanced colonization ability. Moreover, in one case it improved biocontrol abilities of the strain [6].

What is colonization?

In an attempt to speculate on bacterial traits important for colonization one can imagine that root exudate compounds are very important for rhizosphere bacteria to grow and maintain themselves in a competitive manner on the root system. In order to react adequately to these compounds, bacteria need an intact chemotactic apparatus. For chemotaxis towards root exudates, bacteria need to be motile. **Motility** requires the use of flagella. The function of several colonization genes can be explained in relation to motility. In some bacteria such as *Salmonella typhimurium* production of flagella is regulated by a **site-specific recombinase**. Rotation of flagella requires ATP, which is generated by **NADH dehydrogenase**. In some bacteria mutations which result in the absence of the **O-antigen of LPS** are associated with a decrease in **motility**. Export of A-band lipopolysaccharide in *P. aeruginosa* requires an ATP binding cassette transport system.

Growth rate in the rhizosphere is the second trait to which genes found to be involved in colonization can be linked. The growth rate will depend on the ability to take up components essential for cell growth and/or maintenance. The ColR/S **two-component** system could indirectly be involved in the uptake of certain root exudate compounds. The proton motive force generated by the **NADH dehydrogenases** encoded by the *nuo* operon is likely to be involved in transport of some exudate compounds. The simplest explanation for the colonization traits identified so far is that they play a role in chemotaxis towards exudate compounds and in initiating a high growth rate in the rhizosphere.

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