RESEARCH LETTER

Different, overlapping mechanisms for colonization of abiotic and plant surfaces by *Pseudomonas putida*

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Received 26 May 2008; accepted 15 August 2008.
First published online 9 September 2008.

DOI:10.1111/j.1574-6968.2008.01339.x

Editor: David Clarke

Keywords
biofilm; rhizosphere; spermosphere; adhesion.

Abstract

Mechanisms governing biofilm formation have generated considerable interest in recent years, yet comparative analyses of processes for bacterial establishment on abiotic and biotic surfaces are still limited. In this report we have expanded previous information on the genetic determinants required for colonization of plant surfaces by *Pseudomonas putida* populations and analyzed their correlation with biofilm formation processes on abiotic surfaces. Insertional mutations affecting flagellar genes or the synthesis and transport of the large adhesin LapA lead to decreased adhesion to seeds and biofilm formation on abiotic surfaces. The latter also causes reduced fitness in the rhizosphere. Decreased seed adhesion and altered biofilm formation kinetics are observed in mutants affected in heme biosynthesis and a gene that might participate in oxidative stress responses, whereas a mutant in a gene involved in cytochrome oxidase assembly is affected in the bacterium–plant interaction but not in bacterial establishment on abiotic surfaces. Finally, a mutant altered in lipopolysaccharide biosynthesis is impaired in seed and root colonization but seems to initiate attachment to plastic faster than the wild type. This variety of phenotypes reflects the complexity of bacterial adaptation to sessile life, and the partial overlap between mechanisms leading to biofilm formation on abiotic and biotic surfaces.

Introduction

It is now generally accepted that the formation of multicellular, matrix-encased communities associated with solid surfaces (biofilms) is an intrinsic feature of the bacterial life cycle and a common strategy for microbial persistence in a variety of environments. A number of genetic determinants participating in this process have been described and characterized in different microorganisms (for reviews, see Kierek-Pearson & Karatan, 2005; Lasa, 2006), and global changes in gene expression in biofilms are being studied (Prüss *et al.*, 2006). However, one of the areas that still remains to be fully explored is the elucidation of the similarities and differences between pathways leading to bacterial colonization of abiotic and biotic surfaces. Apart from its implications in medical microbiology, this information is relevant in the study of plant–bacterial interactions, given the impact that colonization of plant surfaces by pathogenic or mutualistic bacteria can have on plant health, nutritional status or stress responses. The correlation between biofilm formation and bacterial adhesion to plant surfaces may be reflected by the appearance of microcolonies and biofilm-resembling structures of both epiphytic (i.e. colonizers of the aerial parts of the plant) and root-associated bacteria (Ramos *et al.*, 2000; Monier & Lindow, 2004). In the latter case, traits that are of relevance for bacterial fitness in the rhizosphere (surface of roots and surrounding soil area) have been studied to a significant extent in plant-beneficial *Pseudomonas* (Lugtenberg *et al.*, 2001) but there is less information on elements directly involved in the ability of bacterial cells to attach to and establish on the plant surface.

We have previously identified functions required for adhesion of *Pseudomonas putida* to corn seeds (Espinosa-Urgel *et al.*, 2000). Only two of the mutants then analyzed were defective both in seed colonization and in biofilm formation on different abiotic surfaces, suggesting the existence of specific determinants for colonization of the plant surface as well as certain common elements for both processes. These mutants were affected in an 8682-amino

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acid protein, termed LapA, which was subsequently identified and characterized as a key element for adhesion of *P. putida* to seeds and for the early stages of *Pseudomonas fluorescens* biofilm formation on abiotic surfaces (Hinsa *et al.*, 2003). This information has now been expanded by isolating and characterizing a new battery of *P. putida* mutants that confirm the importance of flagella and the large adhesin LapA as general adhesion factors and reveal new functions involved either in both processes or specifically in the establishment and survival of *P. putida* on plant surfaces.

Materials and methods

Strains and culture conditions

Pseudomonas putida KT2440 is a plasmid-free derivative of *P. putida* mt-2, a strain that was isolated from a vegetable-planted field (Nakazawa, 2002). *Escherichia coli* HB101 (RK600) and CC118λ*pir*(pUT-Km1) were from our laboratory collection. *Pseudomonas putida* strains were grown at 30 °C, in Luria–Bertani (LB) medium or M9 minimal medium (Sambrook *et al.*, 1989) with glucose (10 mM) or sodium citrate (15 mM), supplemented with 1 mM $MgSO_4$, 0.06% (w/v) Fe-citrate and 0.25% (v/v) trace elements solution (final concentrations, in $\mu g\,L^{-1}$: HBO_3, 75; $ZnCl_2$, 12.5; $MnCl_2$, 7.5; $CoCl_2$, 50; $CuCl_2$, 2.5; $NiCl_2$, 5; and $NaMoO_4$, 7.5). *Escherichia coli* strains were grown at 37 °C in LB medium. When appropriate, antibiotics were added at the following concentrations ($\mu g\,mL^{-1}$): kanamycin (Km), 25; ampicillin (Ap), 100; streptomycin (Sm), 100; and chloramphenicol (Cm), 30.

Transposon mutagenesis and selection of seed adhesion-deficient mutants

Transposon mutagenesis with mini-Tn*5*[Km1] (de Lorenzo *et al.*, 1990) was performed by triparental mating, with KT2440 as the recipient and *E. coli* CC118λ*pir*(pUT-Km1) and HB101(RK600) as the donor and helper strains, respectively. Selection of mutants with reduced seed colonization capacity was essentially performed as described previously (Espinosa-Urgel *et al.*, 2000). Six independent matings were performed, and the mutants obtained in each case were pooled. The pools were grown in LB medium to mid-exponential phase and diluted in 5 mL of M9 basal medium, and then introduced in syringes filled with hydrated, surface-sterilized corn seeds. After incubation for 1 h at room temperature, syringes were opened and washed with 5 mL of sterile M9 basal medium. The flow through was collected and dilutions were plated on selective minimal medium. The resulting colonies were collected and passed once more through seed columns and the clones thus obtained were then assayed individually. Qualitative evaluation of adhesion to seeds was performed as described (Espinosa-Urgel *et al.*, 2000), discarding those clones with obvious growth defects in LB medium. Clones showing a consistent phenotype through three rounds of assays were kept for further analysis.

Identification of mini-Tn*5* insertion sites

Transposon insertion sites were determined by arbitrary PCR, followed by sequencing, in a manner similar to that described previously (Espinosa-Urgel *et al.*, 2000). Primer sequences and PCR conditions are available upon request. Comparison of the obtained sequences with genome databases was performed using BLAST programs available at the NCBI website (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Seed attachment and competitive rhizosphere colonization assays

Quantitative seed attachment assays were performed following previously described protocols (Espinosa-Urgel & Ramos, 2004). For rhizosphere colonization experiments, surface-sterilized seeds were allowed to germinate at 30 °C in the dark for 48 h. An otherwise isogenic KT2440 derivative carrying a streptomycin resistance gene in single copy in the chromosome (KT2440-Sm), generated by a site-specific insertion of miniTn*7*-ΩSm1 (Koch *et al.*, 2001) at an extragenic location near *glmS*, was used for these competitive colonization studies. Where indicated, strains labeled in a similar manner with miniTn*7* derivatives harboring constitutively expressed *dsred* or *gfp* were used.

Strains were grown in LB medium and diluted in M9 to an $OD_{660\,nm} = 1$. KT2440-Sm and each mutant were then mixed in a 1 : 1 proportion (*c.* 5×10^6 CFU of each strain) in 10 mL plant nutrient solution (Ramos-González *et al.*, 2005). This mix was poured on 50 mL Sterilin tubes containing 40 g of sterile washed silica sand, where germinated seeds were then sown. Plants were maintained in a controlled chamber at 24 °C and 55–65% humidity with a daily light period of 16 h. After 1 week, plants were collected, shoots were discarded and the roots were placed in tubes containing 20 mL of M9 basal medium and 4 g of glass beads (diameter, 3 mm). Tubes were vortexed for 2 min, and dilutions were plated on selective media (LB medium with Km or Sm, respectively).

Motility, Congo red binding and biofilm formation assays

Swimming motility was tested by spotting 1 μL of overnight cultures in the center of LB plates with 0.3% agar and measuring movement halos after 6 h at 30 °C. Swarming was assayed at 25 °C as described previously (Matilla *et al.*,

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Table 1. Characteristics of mus mutants

Model mutant	Number≠hits/ total mutants	Locus (gene)	Function	Swim*	Swarm	Congo red†
mus-42	4/11	PP_0168 (*lapA*)	Large surface adhesin	+	+	Red and spread
mus-59	1/2	PP_0167 (*lapB*)	LapA secretion	+	+	Red and spread
mus-46	2/4	PP_0166 (*lapC*)	LapA secretion	+	+	Red and spread
mus-49	2/3	PP_0165 (*lapD*)	Possible LapA regulation	+	+	Red and spread
mus-64	1/1	PP_4380 (*flgL*)	Hook–filament junction	+ –	+	As wt
mus-65	1/2	PP_4341 (*fliA*)	Flagellar sigma factor	–	+	Spread
mus-69	1/1	PP_4373 (*fleQ*)	Flagellar regulator	+ –	–	As wt
mus-70	1/1	PP_4366 (*flil*)	Flagellar secretion system	–	+ –	As wt
mus-73	1/1	PP_4357 (*fliN*)	Flagellar motor switch	–	–	As wt
mus-74	1/1	PP_4389 (*flgD*)	Flagellar hook scaffolding protein	–	+ –	As wt
mus-72	1/1	PP_5101 (*hemN2*)	Coproporphyrinogen III oxidase	+	+	As wt
mus-53	1/1	PP_0110 (*coxE*)	Protoheme farnesyl transferase	+	–	As wt
mus-40	1/1	PP_3821 (*galU*)	UDP-glucose pyrophosphorylase	+ –	–	Red and rough
mus-63	1/1	PP_5311	Unknown (oxidative stress)	+	–	As wt

*Swimming and swarming patterns relative to the wild type; +, similar to KT2440; –, no movement observed; + –, reduced swimming halos or swarming motility; ND, not determined.
†Colonies of the wild type (wt) are pink, with a smooth appearance.

2007b). Congo red binding was assayed on tryptone medium ($10\,g\,L^{-1}$ tryptone, 1% agar) with $40\,\mu g\,mL^{-1}$ Congo red. Attachment to abiotic surfaces and biofilm formation were tested in LB medium in 96-well microtiter plates, using the crystal violet staining and quantification method described by O'Toole & Kolter (1998), or during growth in borosilicate tubes under orbital shaking at 40 r.p.m.

Sensitivity to UV light and hydrogen peroxide (H_2O_2)

To test bacterial sensitivity to UV, cultures were grown to the mid-exponential phase, centrifuged, resuspended in M9 basal medium and plated on LB medium after 0, 5, 10, 20 and 30 s of exposure to UV light (354 nm). Plates were incubated at 30 °C in the dark and the number of viable counts were recorded. Sensitivity to H_2O_2 was analyzed by measuring the turbidity of overnight cultures grown in LB medium with different concentrations of H_2O_2.

Results and discussion

Isolation and characterization of mutants defective in adhesion to corn seeds

In a previous work we had identified eight different mutants that showed reduced capacity to colonize corn seeds (Espinosa-Urgel *et al*., 2000). Thirty-five new mutants deficient in adhesion to seeds were isolated after transposon mutagenesis and several independent rounds of selection using a similar approach, with the difference that in this case enrichment and selection experiments were carried out with exponentially growing cells instead of stationary-phase cells. In this way, we intended to identify functions that might not have been represented in that previous screen due to differential expression under various growth situations. All the selected mutants showed growth rates similar to the wild type in different media (data not shown). The site of insertion of the minitransposon in the chromosome could be determined for all the mutants except two. Data are presented in Table 1, which also includes phenotypic characteristics such as swimming and swarming motility and Congo red binding.

Most mutants fall in two categories, in terms of functions affected by the transposon insertion. A significant proportion of mutants present insertions at several positions in the genes coding for LapA, as well as the LapB and LapC proteins, which are part of the ABC transporter responsible for its export (Hinsa *et al*., 2003). No mutant was isolated affecting *lapE*, which, in *P. fluorescens*, forms an operon with *lapB* and *lapC* and encodes the outer membrane component of this ABC transporter. In *P. putida* KT2440, the homolog of *lapE* is located in a different chromosomal region. Three other mutants in this group correspond to the adjacent *lapD* gene (PP_0165) that, in *P. fluorescens*, has been described to modulate LapA secretion (Hinsa & O'Toole, 2006). As in *P. fluorescens*, *P. putida* LapD shows noncanonical GGDEF and EAL domains. These domains are involved in the turnover of cyclic di-GMP, modulating the transition between planktonic and sessile lifestyles as well as other multicellular behaviors (Römling *et al*., 2005).

A second group corresponds to genes involved in the synthesis, regulation or functionality of the flagellar apparatus. As expected, all these mutants were impaired in swimming motility to different degrees, and some of them also

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showed defects in swarming motility (detailed below). The remaining mutants were affected in functions such as lipopolysaccharide and heme biosynthesis, cytochrome oxidase assembly and a hypothetical protein of unknown function. Consistent with previous observations on the role of lipopolysaccharide (Matilla *et al.*, 2007b), mus-40 was deficient in swarming motility. This mutant is affected in *galU*, encoding UDP-glucose pyrophosphorylase, which, in *Pseudomonas aeruginosa*, has been shown to be required for the synthesis of intact lipopolysaccharide (Dean & Goldberg, 2002), a role that we could confirm in *P. putida* by electrophoretic analysis of lipopolysaccharide of the wild type and mus-40 (data not shown). The mutant also showed differences with respect to the wild type on Congo red plates, colonies being darker and less smooth. Defects in swarming were also observed in mutants affected in the flagellar motor switch protein FliN and in the FleQ regulator, as well as in mus-53 (mutated in PP_0110, a gene that we have renamed *coxE*, because it encodes a protoheme IX farnesyl transferase, presumably involved in assembly of the aa_3-type cytochrome oxidase Cox) and mus-63. This mutant is affected in a gene encoding a hypothetical 467-amino acid protein (PP_5311), highly conserved in *Pseudomonas*. Analysis of the genomic region surrounding PP_5311 revealed that this ORF is 78 bp downstream of the *oxyR–recG* operon and transcribed in the same direction. Given the regulatory role of OxyR in oxidative stress response, and the involvement of RecG in DNA replication and repair (Ochsner *et al.*, 2000), we considered the possibility that PP_5311 could also contribute to these processes. Hence the tolerance of KT2440 and mus-63 to H_2O_2 or to UV irradiation was tested. No significant differences in sensitivity to UV could be observed. However, growth of mus-63 (PP_5311) was impaired above 0.35% (v/v) H_2O_2, whereas the minimum inhibitory concentration was 0.7% in the case of the wild type. These data suggest that the protein encoded by PP_5311 may participate in oxidative stress responses.

Mutants representing each class were selected for a quantitative analysis of their seed adhesion capacity. As shown in Fig. 1, the ability of all these strains to attach to corn seeds is significantly reduced in comparison with the wild type.

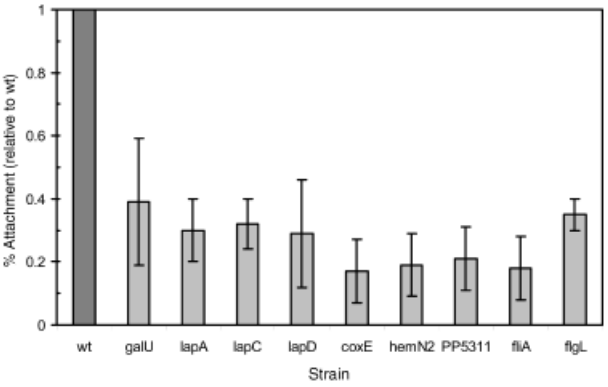

Fig. 1. Adhesion of representative mus mutants to corn seeds, relative to the percentage of *Pseudomonas putida* KT2440 cells attached after 1 h of incubation. Results are averages and SDs of at least three independent experiments (three seeds per experiment).

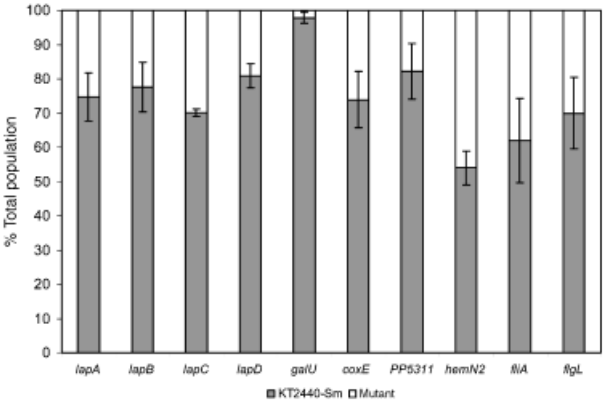

Fig. 2. Competitive rhizosphere colonization by KT2440-Sm and different mus mutants. Data are given as the proportion of wild type and mutant strain in the total population and correspond to averages and SD of at least six plants.

Competitive rhizosphere colonization

The potential role played by the identified functions in rhizosphere colonization was also tested. It is worth noting that expression of *coxE* (PP_0110), *lapD* (PP_0165) and several flagellar genes is upregulated in *P. putida* in the corn rhizosphere (Matilla *et al.*, 2007a). Competitive colonization assays were performed with several representative mutants, mixed individually in a 1 : 1 proportion with the wild type. The mix was directly inoculated in sand tubes before sowing corn seedlings to ensure that the observed results reflected actual differences in rhizosphere fitness and were not only a consequence of the initial seed adhesion defects of the mutants. Bacteria were recovered after 1 week and the number of wild type and mutant cells was determined. Results are shown in Fig. 2. Mutant mus-40 (*galU*) was the most severely affected in competition with the wild type. The number of cells of this mutant recovered from the rhizosphere was two orders of magnitude lower than that of KT2440-Sm cells (4.36×10^5 vs. 2.95×10^7 CFU g^{-1}). This is consistent with the previously reported influence of mutations affecting the O-antigen of lipopolysaccharide in tomato root tip colonization by *P. fluorescens* (Dekkers *et al.*, 1998). Mutants in *lap* genes, as well as mus-53 (*coxE*) and mus-63 (PP_5311), also showed reduced colonization capacity, but to a lesser extent. No significant reduction in

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competitive colonization capacity was observed in the case of mus-72, affected in a gene, *hemN-2* (PP_5101), encoding a putative coproporphyrinogen III oxidase, presumed to participate in heme biosynthesis. A second locus in the chromosome of *P. putida* has the same predicted role (*hemN*, PP_4264); hence, it is possible that PP_5101 is relevant under the experimental conditions set for seed adhesion but its function is replaced by PP_4264 in the rhizosphere. In fact, a 7.6-fold induction of the expression of this gene has been observed in the rhizosphere in comparison with sessile populations in sand (Matilla *et al.*, 2007a), which seems to support this hypothesis. Similarly, the differences in rhizosphere fitness between KT2440 and mus-65, affected in *fliA*, were not significant, whereas a mutant in a structural flagellar gene (mus-64, affected in *flgL*) showed a larger decrease in competitive colonization. Several reports have revealed the importance of flagellar motility for root colonization by other *Pseudomonas* species (Capdevila *et al.*, 2004; Martínez-Granero *et al.*, 2006). It should be noted, however, that the data presented here correspond to the whole root system, while most previously published results refer solely to root tip colonization. One possibility was that although the total numbers are similar, the distribution of KT2440 and mus-65 (*fliA*) along the root was different. To test this hypothesis, KT2440 and mus-65 were differentially labeled with *dsRed* and *gfp*, respectively, and root distribution was visualized by fluorescence microscopy. As shown in Fig. 3, KT2440(*dsRed*) cells were significantly more abundant in the root tip than mus-65(*gfp*), while this did not occur in older parts of the root.

Biofilm formation on abiotic surfaces

In order to define which of the identified functions has a general role in adhesion of *P. putida* to solid surfaces and which are specific to the bacterium–plant interaction, the ability of different mutants to attach to abiotic surfaces was tested under static conditions in microtiter plates (Fig. 4a), where KT2440 reaches the maximum observed attachment after *c.* 6 h of growth, followed by detachment from the surface at later times. All flagellar mutants showed a significant reduction in their adhesion capacity with respect to the parental strain, as did the *lapD* mutant, especially at 3 h, but its phenotype is less marked than that described previously for *lapA* mutants (Espinosa-Urgel *et al.*, 2000; Hinsa *et al.*, 2003). Biofilm formation did not progress further in any of these mutants. Mutants in *coxE* and PP_5311 (mus-53 and mus-63), on the other hand, showed attachment kinetics similar to the wild type, although with a slight delay in the second case. The capacity to initiate biofilm formation of the *hemN2* mutant mus-72 was comparable to that of KT2440, but it does not progress further, so that the quantity of biomass attached to the surface is the same after 3 or 6 h. A very different phenotype was observed for the *galU* mutant mus-40. Although data were highly variable, this strain always showed a significant increase in early attachment, followed by a decline in biomass associated with the surface after 6 h. It is possible that lipopolysaccharide hinders initial adhesion, but is required at later stages of biofilm development. This contrasts with the recently reported defect of certain lipopolysaccharide-deficient mutants in the early stages of biofilm formation by *Klebsiella pneumoniae* (Balestrino *et al.*, 2008).

Biofilm formation was also followed during growth in rotating glass tubes (Fig. 4b). Under these conditions, the delayed attachment of mus-63 (PP_5311) was more evident, whereas the differences between the wild type and mus-40 (*galU*) were not as significant as in microtiter plates. The most severe defects were observed in the *fleQ* and *lapD* mutants. Intriguingly, in both cases, a thick biofilm could be observed after 24 h of growth (data not shown), as if the regular kinetics of KT2440 (attachment during exponential growth of the liquid culture and detachment in the stationary phase) were reversed in these mutants, a phenotype that was only observed in glass tubes and not in plastic. It seems possible that not only the surface but aeration conditions can explain the differences between the two experiments.

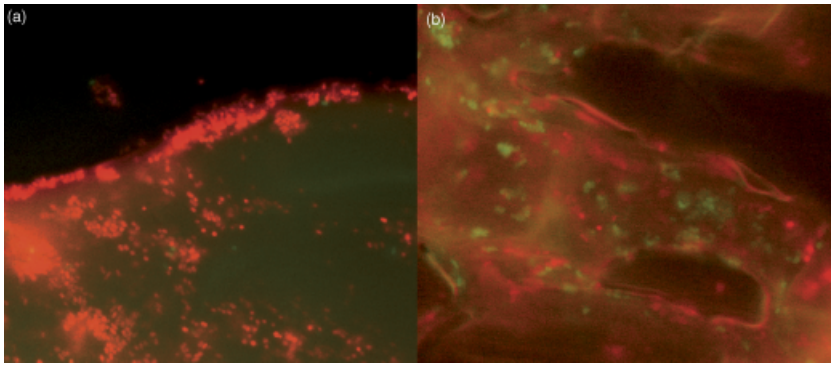

Fig. 3. Distribution of KT2440(*dsRed*) and mus-65(*gfp*) on corn roots (a) close to the root tip or (b) at older parts. Each image is a composite of two pictures of the same field taken with specific filter sets for each fluorescent protein. Pictures were taken with a Nikon CCD camera mounted on a Zeiss Axioscope fluorescent microscope. Magnification: × 1000.

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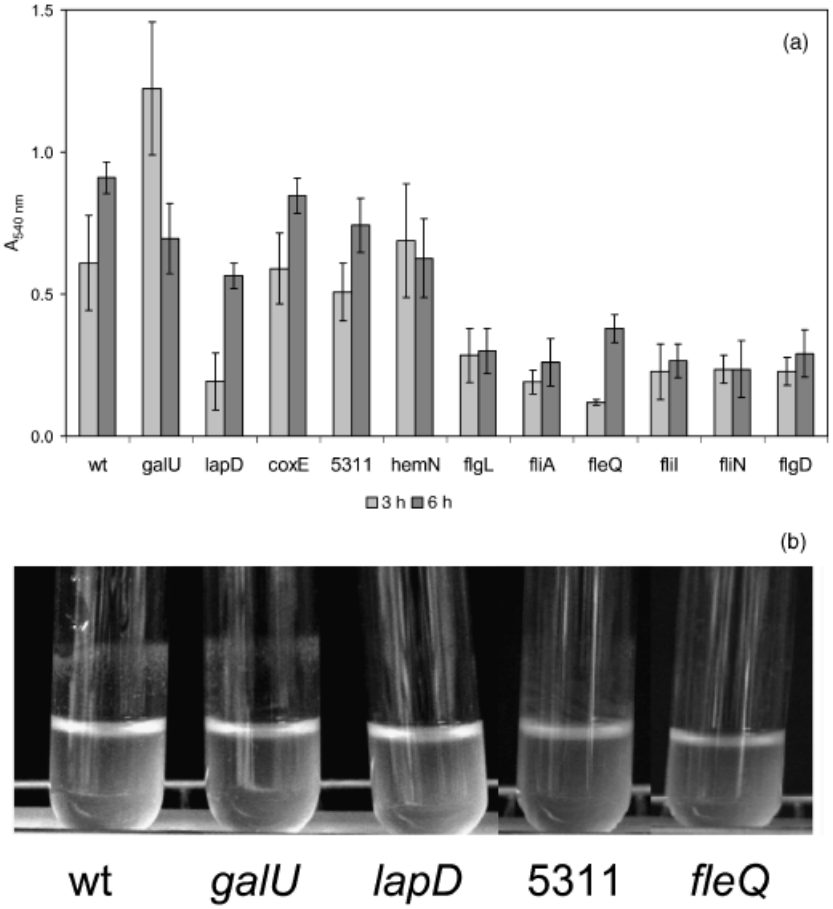

Fig. 4. (a) Biofilm formation in 96-well microtiter plates, quantified as $A_{540\,nm}$ after staining the biofilm with crystal violet and redissolving the stain with ethanol. Measurements were done on a Sunrise microplate reader (Tecan) and are the averages and SDs of three independent assays, each with six replicas per strain. (b) Biofilm formation in borosilicate glass tubes after 3 h of growth in LB medium with orbital shaking.

Concluding remarks

The results presented here give an idea of the hierarchy of functions participating in the establishment of sessile communities of *P. putida* in different environments. They support previous data on the role of flagella in attachment to abiotic and seed surfaces (DeFlaun *et al.*, 1990; Toutain *et al.*, 2007) and reveal the importance of the LapA/LapBC/LapD system both in biofilm formation and in bacterial fitness in the plant environment. These elements are common to the pathways for biofilm initiation regardless of the surface and can be considered global adhesion traits. Large surface proteins with a structure resembling that of LapA are widespread among prokaryotes (Yousef & Espinosa-Urgel, 2007), suggesting the existence of similar molecular mechanisms for biofilm initiation in different bacteria. We have also identified genes with a differential or a surface-dependent role. A specific function in colonization of plant surfaces can be ascribed to *coxE*, while PP_5311 also influences adhesion to hydrophylic abiotic surfaces (glass). HemN2, on the other hand, plays a role in seed colonization and biofilm development on hydrophobic abiotic surfaces (plastic), but does not affect rhizosphere fitness or adhesion to glass. The role of lipopolysaccharide appears to be more complex. Intact lipopolysaccharide is key for colonization of plant surfaces, but has a limited influence on attachment to glass and seems to hamper initial attachment to plastic, while being required at later stages. This suggests a time- and surface-dependent participation of lipopolysaccharide in the settlement of sessile *P. putida* populations. It is worth noting that, apart from LapA, no common functions were identified here with respect to our previously published analysis (Espinosa-Urgel *et al.*, 2000). This is consistent with our observation that actively growing *P. putida* show higher seed attachment rates than stationary-phase bacteria (Espinosa-Urgel & Ramos, 2004). Thus, the physiological state of the bacteria (stationary phase vs. exponentially growing phase) also seems to delimit the requirements for seed colonization by *P. putida*.

Acknowledgements

This work was funded by grants BFU2004-03038 and BFU2007-64270 from the Plan Nacional de I+D+I. F.Y.C is the recipient of an FPI fellowship from the Ministerio de Educación y Ciencia. We thank Dr Søren Molin for the generous gift of Tn*7* derivatives and Dr M.I. Ramos-González for critically reading the manuscript. The support

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of Prof. Juan L. Ramos to this line of research is gratefully acknowledged.

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