Phenotypic variation of *Pseudomonas putida* and *P. tolaasii* affects attachment to *Agaricus bisporus* mycelium

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The effect of phenotypic variation on attachment of *Pseudomonas tolaasii* and *P. putida* to *Agaricus bisporus* mycelium was investigated. Quantitative studies demonstrated the ability of each isolate to attach rapidly and firmly to *A. bisporus* mycelium and significant differences in attachment of wild-type and phenotypic variant strains were observed. This was most pronounced in *P. tolaasii*, where the percentage attachment of the wild-type form was always greater than that of the phenotypic variant. The medium upon which the bacteria were cultured, prior to conducting an attachment assay, had a significant effect on their ability to attach. Attachment of the wild-type form of *P. putida* was enhanced when the assay was performed in the presence of CaCl_2 , suggesting the involvement of electrostatic forces. No correlation was observed between bacterial hydrophobicity and ability to attach to *A. bisporus* mycelium. Scanning electron microscopy confirmed the results obtained from the quantitative studies and provided further evidence for marked differences in the ability of the pseudomonads to attach to mycelium. Fibrillar structures and amorphous material were frequently associated with attached cells and appeared to anchor bacteria to each other and to the hyphal surface. A time-course study of attachment using transmission electron microscopy revealed the presence of uneven fibrillar material on the surface of cells. This material stained positive for polysaccharide and may be involved in ensuring rapid, firm attachment of the cells.

Introduction

Adherence of bacteria to surfaces of living organisms can be an important factor in determining the extent to which bacteria influence the growth and development of a 'host'. Colonization of legume roots by *Rhizobium* spp. is necessary before the bacterium can infect the host cells and incite formation of nitrogen-fixing nodules (Dazzo, 1984). Similarly, colonization of dicot root surfaces is necessary before *Agrobacterium tumefaciens* can infect its host (Lippincott & Lippincott, 1969; Matthysse *et al.*, 1978) and colonization of plant root surfaces by rhizobacteria must occur before subsequent effects of the bacteria on promotion, or limitation, of plant growth can occur (Schroth & Hancock, 1982; De Weger *et al.*, 1987; Bashan *et al.*, 1991).

Growth and development of the cultivated mushroom, *Agaricus bisporus*, is affected by several pseudomonads (see Grewal & Rainey, 1991), in particular *Pseudomonas putida*, which provides *A. bisporus* with the stimulus necessary to 'trigger' basidiome initiation (Eger, 1961; Hayes *et al.*, 1969; Rainey *et al.*, 1990; Rainey, 1991) and

P. tolaasii, the causal organism of brown blotch disease (Tolaas, 1915). Bacteria inhabiting the casing layer have been shown to exist in close proximity to *A. bisporus* hyphae (Stanek, 1976) and colonization of mycelium by *P. tolaasii* and *P. putida* has been observed (Preece & Wong, 1982; Masaphy *et al.*, 1987). Direct contact of bacteria with hyphae is necessary for basidiome initiation to occur (Rainey *et al.*, 1990) and colonization of mycelial surfaces by *P. tolaasii* is thought to be important in relation to the development of brown blotch disease (Preece & Wong, 1982).

An accompanying paper (Grewal & Rainey, 1991) describes the chemotactic response of mycelium-derived isolates of *P. putida* and *P. tolaasii* and a phenotypic variant of each species to mycelial exudates of *A. bisporus*. The work described here concerns attachment of the same bacteria (and phenotypic variants) to *A. bisporus* mycelium.

Methods

Strains and growth conditions. The wild-type strains *P. putida* (PMS118S) and *P. tolaasii* (PMS117S), and their respective phenotypic variants PMS118R and PMS117R, were as described by Grewal & Rainey (1991). *A. bisporus* was grown on compost malt medium (CMM; Rainey, 1989a) in 90 mm diameter Petri dishes for 10 d (25 °C) before being used in experiments. Casing soil extract medium (CX) was prepared by mixing 150 g of *A. bisporus*-colonized casing material

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Abbreviations: BATH, bacterial adherence to hydrocarbons; SAT, salt aggregation test; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

(3 parts peat : 1 part lime, v/v) with 200 ml distilled water. The slurry was filtered through Whatman no. 1 filter paper and the pH adjusted to pH 7.3 with 1 M-HCl. After addition of 1.5% (w/v) agar the medium was sterilized by autoclaving (120 °C, 15 min, 1.2 atm).

Determination of bacterial hydrophobicity. Three different methods were used: the salt aggregation test (SAT) (Lindahl *et al.*, 1981), bacterial adherence to hydrocarbons (BATH) (Dillon *et al.*, 1986), and bacterial adherence to polystyrene (Rosenberg, 1981). The SAT test relies on the ability of ammonium sulphate to cause hydrophobic surfaces to aggregate. The concentration of ammonium sulphate required to cause bacterial aggregation is dependent on surface hydrophobicity and decreases as hydrophobicity increases. The lowest concentration causing cell aggregation is the SAT value. The BATH test relies upon partitioning of cells into hydrocarbons. Cells are suspended in buffer and the hydrocarbon added and vortex-mixed. After separation of the phases the optical density of the aqueous phase is measured and the result recorded as the percentage optical density of the aqueous phase relative to the initial optical density of the bacterial suspension. The hydrophobicity of cells grown under nutrient-replete and nutrient-depleted conditions was examined by culturing cells on King's medium B (King *et al.*, 1954) (KB) and CX medium, respectively.

Quantitative determination of adherence to mycelium. (i) *Preparation of bacterial inoculum.* Bacteria were cultured on a range of media: KB, minimal medium (M63; Miller, 1972) and M63 medium in which the concentration of carbon and nitrogen were altered: 1/2-M63, glucose 1 g l⁻¹, (NH₄)₂SO₄ 1 g l⁻¹; 1/10-M63, glucose 0.2 g l⁻¹, (NH₄)₂SO₄ 0.2 g l⁻¹; M63-C (carbon limited), glucose 0.5 g l⁻¹, (NH₄)₂SO₄ 2.0 g l⁻¹; M63-N (nitrogen limited), glucose 2.0 g l⁻¹, (NH₄)₂SO₄ 0.5 g l⁻¹. Bacteria were also grown on CX medium. All media were solidified by the addition of 1.5% agar.

Bacteria were grown overnight (28 °C), harvested with an inoculating loop and suspended in sterile distilled water to give an OD₆₁₀ of 0.4 (4 × 10⁸ cells ml⁻¹), or if grown on media of reduced nutrient status (which resulted in smaller cells), to give an OD₆₁₀ of 0.3 (4 × 10⁸ cells ml⁻¹). The suspension was mixed well (but gently) to disrupt bacterial aggregates and motility was checked microscopically before performing each experiment. The effect of Ca²⁺ on bacterial adhesion was assessed by the addition of 5 mM-CaCl₂ to the distilled water.

(ii) *Preparation of A. bisporus cultures and attachment assay.* Petri dish cultures of *A. bisporus* were flooded with sterile distilled water and left at room temperature for 15 min to suppress aerial mycelium. Agar-mycelial plugs (10 mm diam.) were aseptically removed from the margin of the cultures and placed in sterile Petri dishes. After the surface of the agar-mycelial plugs had dried (15 min), a 20 µl drop of bacterial suspension was carefully applied and spread using a pipette tip (without touching the mycelium) to cover the entire mycelial surface. Inoculated plugs were incubated for 30 min at 25 °C and then any bacteria not firmly attached to the hyphal surfaces were dislodged by vigorously washing each plug in 10 ml phosphate-buffered saline (PBS; Na₂HPO₄ 7 g l⁻¹, KH₂PO₄ 3 g l⁻¹, NaCl 4 g l⁻¹, MgSO₄ · 7H₂O 0.2 g l⁻¹) for 1 min using a vortex mixer. The number of bacteria not firmly attached to the mycelium and the total number of bacteria applied in the 20 µl drop were determined using the drop method of Miles & Misra (1938). Dilutions were made in PBS and all equipment which made contact with the bacteria was manufactured from polypropylene (initial experiments revealed the inability of these bacteria to attach to polypropylene or to agar plugs).

The number of bacteria attached to hyphal surfaces after 30 min was calculated by subtracting the number of bacteria initially applied to the mycelial surface of the plug from the number recovered after vortex-mixing. The result was expressed as a percentage of the number of bacteria initially placed on the surface of the agar-mycelial plug; each treatment was replicated five times.

Electron microscopy. *A. bisporus* cultures were flooded with 10 ml bacterial suspension (overnight KB plate-cultured cells suspended in sterile distilled water to a concentration of 1 × 10⁸ cells ml⁻¹) and re-incubated at 25 °C. Plugs of agar and mycelium (5 mm diam.) were removed from the colony margin with a sterile cork borer 30 min, 3 h, 24 h and 72 h after inoculation. Plugs were washed vigorously in sterile distilled water (2 × 5 min) before fixing in glutaraldehyde. To investigate the attachment behaviour of bacteria under nutrient-depleted conditions some plugs were removed from the fungal culture 24 h after inoculation and placed in 5 ml sterile distilled water, where they were left for a further 48 h before fixation. Plugs incubated in the presence of bacteria for 24 h or longer were plated onto fresh CMM to check for fungal growth. All treatments were examined by scanning electron microscopy (SEM), but only the 30 min, 3 h and 24 h treatments were examined by transmission electron microscopy (TEM).

SEM. Mycelial-agar plugs were fixed by immersion in 3% (v/v) glutaraldehyde (in 0.075 M-potassium phosphate buffer, pH 7.2) for 3 h at 20 °C and washed in 0.075 M-potassium phosphate buffer (pH 7.2). Specimens were dehydrated in a series of ethanol/distilled water mixtures and put through a series of amyl acetate/ethanol mixtures (4 h in each solution). They were critical-point dried in a CO₂ atmosphere, mounted on stubs and gold-coated for 3 min in a 'Polaron' diode sputter device (E50000) and examined with a Cambridge stereoscan 250 Mk 2 scanning electron microscope.

TEM. Mycelial-agar plugs were fixed as for SEM and washed in 0.075 M-potassium phosphate buffer (pH 7.2), post-fixed in 1% (w/v) OsO₄ in the same buffer for 3 h, washed again in buffer and dehydrated in an acetone series. Dehydrated material was infiltrated and embedded in Spurr's low-viscosity resin and ultra-thin specimens were cut using an LKB 'Pyramitome' (type 11800). Specimens were routinely stained with uranyl acetate (Watson, 1958). Selected specimens were stained using the Thiéry reaction (TCH staining; Thiéry, 1967), with appropriate controls, to detect periodate-sensitive polysaccharide material. Specimens were examined in a JEM-1200EX electron microscope at an accelerating voltage of 80 kV.

Statistical analysis. Statistical analysis was performed using standard ANOVA techniques. Comparisons between means were made using Fisher's protected least significant difference (LSD) test.

Results

Hydrophobicity of *P. putida* and *P. tolaasii*

SAT assay. The results from the SAT assay are shown in Table 1. Both variants of *P. tolaasii* appeared moderately hydrophobic and the hydrophobicity of the wild-type

Table 1. *Hydrophobicity of wild-type and phenotypic variants of P. putida and P. tolaasii after growth on KB and CX as determined by the SAT assay*

Values are the lowest (NH₄)₂SO₄ molarity giving bacterial aggregation.

Bacterial isolate	Medium	
	KB	CX
<i>P. putida</i> PMS118S	> 4.0	4.0
<i>P. putida</i> PMS118R	> 4.0	4.0
<i>P. tolaasii</i> PMS117S	2.0	1.6
<i>P. tolaasii</i> PMS117R	2.0	2.0

Fig. 1. Adherence of wild-type and phenotypic variant forms of *P. putida* (PMS118S, ●; PMS118R, ○) and *P. tolaasii* (PMS117S, ■; PMS117R, □) to varying volumes of *p*-xylene and *n*-hexadecane after growth on (a) KB medium and (b) CX medium in the BATH assay. Data are means of duplicates.

form increased after growth on CX. An end-point was not produced for KB-cultured *P. putida*, but aggregation did occur in 4.0 M-ammonium sulphate after the cells were grown on CX, indicating that both *P. putida* forms were relatively hydrophilic.

BATH assay. Fig. 1 shows adherence of wild-type and phenotypic variants of *P. putida* and *P. tolaasii* to xylene and hexadecane. Adherence of the isolates to xylene after growth on KB suggested that the phenotypic variants of both *P. putida* and *P. tolaasii* were weakly hydrophobic. Hexadecane gave similar results. The hydrophobicity of all isolates increased after growth on CX.

Bacterial adherence to polystyrene assay. All isolates showed a limited ability to adhere to polystyrene and scored a weak positive result for this test, indicating that the cells were weakly hydrophobic.

Quantitative studies

Attachment of the wild-type and the phenotypic variant of *P. putida* to *A. bisporus* mycelium is shown in Fig. 2(a). A two-way ANOVA revealed no significant difference between the ability of the wild-type and phenotypic variant to adhere to mycelium, but the medium on which the cells were grown, prior to performing the attachment assay, had a significant effect on attachment ($P < 0.001$). The interaction between the different variants and media on which they were grown was also significant ($P < 0.001$).

Growth of both *P. putida* strains on CX caused the percentage attachment to increase and the percentage

attachment of the phenotypic variant (PMS118R) was significantly greater than on any other medium ($P < 0.001$). When the attachment assay was conducted in the presence of 5 mM- CaCl_2 there was a significant increase in adherence of the wild-type form (PMS118S) to mycelium ($P < 0.001$) when compared to adherence of this form after growth on KB or M63. Addition of CaCl_2 did not increase the percentage attachment of the phenotypic variant when compared to the percentage attachment of this form after growth on either KB or M63.

Adherence of the wild-type and the phenotypic variant of *P. tolaasii* to *A. bisporus* mycelium is illustrated in Fig. 2(b). A significant difference was found between the ability of the two forms to adhere to mycelium ($P < 0.001$) and the effect of different media on adherence was also significant. The interaction between these two factors was significant ($P < 0.001$).

Wild-type *P. tolaasii* (PMS117S) consistently attached to the mycelium in greater numbers than the phenotypic variant and attachment of the wild-type organism was greatest when cultured on nitrogen-limited M63 and CX. Compared with these two media, the percentage attachment of the wild-type form decreased significantly when the attachment assay was performed in the presence of CaCl_2 , but the percentage of bacteria which attached to the mycelium under these conditions was comparable to that observed after culture on KB or M63. On 1/10-M63, attachment of the wild-type form was significantly reduced ($P < 0.05$) compared to the attachment of this form after growth on KB and M63. The percentage attachment of the phenotypic variant (PMS117R) was

Fig. 2. Effect of culture conditions on attachment of wild-type and phenotypic variant forms of (a) *P. putida* (wild-type PMS118S, □; phenotypic variant PMS118R, ■) and (b) *P. tolaasii* (wild-type PMS117S, □; phenotypic variant PMS117R, ■) to *A. bisporus* mycelium. KB, KB medium; KB-Ca, KB-cultured cells, but assay conducted in the presence of 5 mM-CaCl₂; M, minimal M63 medium; 1/2M, 0.5 × M63; 1/10M, 0.1 × M63; M-C, carbon limited M63; M-N, nitrogen limited M63; CX, casing extract medium (see text for full description). Data are means and SE of five replicates.

greatest after culture on 1/10-M63. Conducting the attachment assay in the presence of 5 mM-CaCl₂, or growing the phenotypic variant on CX, had little effect on its ability to attach to mycelium.

Microscopic studies of adherence

SEM. Figs 3(a)–(f) show bacteria attached to *A. bisporus* mycelium after 30 min. Both the wild-type and phenotypic variant of *P. putida* attached to the mycelium in large numbers and in most instances the bacteria lay flat

against the hyphae (Fig. 3a, b), although some were seen attached by one end only. Wild-type *P. tolaasii* cells also attached to the mycelium in large numbers and were often orientated perpendicular to hyphal surfaces (Fig. 3c). Few cells of the phenotypic variant of *P. tolaasii* were seen attached to the mycelial surfaces (Fig. 3d).

Fibrillar rods were frequently associated with the attached bacteria (Fig. 3e, f) and appeared to join the bacteria both to the mycelial surfaces and to each other. These rod-like structures did not resemble flagella, or fimbriae.

Differences in the patterns of attachment, especially of the wild-type and phenotypic variants of *P. putida*, became evident after the bacteria were left for longer than 30 min in the presence of the mycelium. Examination of the mycelial plugs after 3 h and 24 h revealed little change, although the number of phenotypic variant *P. putida* cells attached appeared to decline. After 72 h in the presence of the mycelium (the last 48 h in distilled water), very few wild-type *P. putida* cells were seen attached to the mycelium and those present were confined to small micro-colonies (Fig. 4a). In contrast, a large number of *P. putida* variant cells were attached and were evenly distributed over the mycelial surface. These cells were orientated perpendicular to the mycelial surface (Fig. 4b). The number of cells of the wild-type form of *P. tolaasii* which remained in contact with the mycelium after this treatment appeared to decline, but not to the extent observed in *P. putida*, and distinct micro-colonies were not observed (Fig. 4e). Few cells of the phenotypic variant of this species were seen in contact with the mycelium (Fig. 4f). Similar results were observed when this material was examined by light microscopy, without prior fixation, or staining (results not shown).

Examination of the mycelial plugs 72 h after inoculation of CMM cultures with bacteria revealed a large number of wild-type *P. putida* and *P. tolaasii* cells attached to the mycelium and large amounts of amorphous material associated with the cells, possibly polysaccharide slime (Fig. 4c). The number of phenotypic variant *P. putida* cells which remained attached after 72 h under these nutrient-replete conditions declined (Fig. 4d) and few cells of the variant form of *P. tolaasii* were observed in contact with the mycelium.

Mycelial plugs removed from the 'broth' after 3 d grew when plated on CMM.

TEM. Figs 5(a)–(d) show attachment of the wild-type form of *P. putida* to *A. bisporus* mycelium. Initial attachment appeared to occur after contact was made between the uneven surface of the bacterium and the hyphae. Accumulation of amorphous material between the hyphal surface and attached bacteria was observed

Fig. 3. Scanning electron micrographs of *A. bisporus* mycelium colonized by *P. putida* and *P. tolaasii* 30 min after application of the bacteria. (a–e) Attachment of (a) wild-type *P. putida* (PMS118S), (b) phenotypic variant *P. putida* (PMS118R), (c) wild-type *P. tolaasii* (PMS117S), (d) phenotypic variant *P. tolaasii* (PMS117R), (e) wild-type *P. putida* (PMS118S) showing fibrillar material. (f) High-power micrograph showing detail of fibrillar material associated with attached wild-type *P. putida* (PMS118S) cells. Arrows indicate fibrillar material connecting bacteria to each other and to the mycelial surface. Bars represent 10 μ m (a–d), 5 μ m (e) and 1 μ m (f).

Fig. 5. Transmission electron micrographs of wild-type *P. putida* (PMS118S) cells attached to *A. bisporus* hyphae. (a) After 30 min, (b) after 3 h, (c) after 3 h, showing TCH-positive staining material enveloping the bacterium and hyphae, and (d) after 24 h, showing build-up of amorphous material between the bacterium and hyphal surface (arrows). H, hypha; TCH, TCH-positive staining material. Bars represent 200 nm (a and b) and 100 nm (c and d).

after 3 h, but was most pronounced after 24 h. The amorphous material appeared to 'cement' bacteria firmly to the hyphal surface. TCH staining revealed this

material to be polysaccharide and it formed a continuum between the bacterium and the surface of the fungus (Fig. 5c, d). TCH-staining material was observed on the

Fig. 4. Scanning electron micrographs of *A. bisporus* mycelium colonized by *P. putida* and *P. tolaasii*. (a) Microcolonies of wild-type *P. putida* (PMS118S) attached to *A. bisporus* mycelium after 24 h in CMM and 48 h in distilled water. (b) Phenotypic variant *P. putida* (PMS118R) attached to *A. bisporus* mycelium after 24 h in CMM and 48 h in distilled water. (c) Wild-type *P. putida* (PMS118S) attached to *A. bisporus* mycelium after 72 h in CMM. (d) Phenotypic variant *P. putida* (PMS118R) attached to *A. bisporus* mycelium after 72 h in CMM. (e) Wild-type *P. tolaasii* (PMS117S) attached to *A. bisporus* mycelium after 24 h in CMM and 48 h in distilled water. (f) Phenotypic variant *P. tolaasii* (PMS117R) attached to *A. bisporus* mycelium after 24 h in CMM and 48 h in distilled water. Arrows indicate fibrillar and amorphous material connecting bacteria to each other and to the mycelial surface. Bars represent 5 µm (a–d and f), and 1 µm (e).

surface of bacteria and hyphae prior to inoculation of mycelium with bacteria.

Attachment of the phenotypic variant of *P. putida* is shown in Figs 6(a)–(d). The surface of this variant was particularly uneven and fibrillar rods, frequently longer than 100 nm, were observed protruding from the surface. TCH staining indicated that they were polysaccharide in nature (Fig. 6c). Fibrillar rods were also evident in the 24 h material and were more numerous and longer (up to 300 nm) than those seen after 3 h (Fig. 6d).

Short fibrillar rods were also seen protruding from the surface of wild-type *P. tolaasii* cells (Fig. 6e, f). The phenotypic variant of *P. tolaasii* was not seen under TEM, despite examination of five grids (approximately 10 sections per grid). This was indicative of the low number of cells which attached to the mycelium.

Discussion

A variety of complex interactions, including hydrophobic (Rosenberg & Kjelleberg, 1986) and electrostatic forces (Marshall *et al.*, 1971), are thought to account for attachment of bacteria to living surfaces. Several studies (Fletcher & Loeb, 1979; Lindahl *et al.*, 1981; Loosdrecht *et al.*, 1987) have shown a positive correlation between bacterial hydrophobicity and the ability of cells to colonize living surfaces. *P. putida* and *P. tolaasii* were able to adhere rapidly and firmly (that is, cells were not removed by washing) to the surface of *A. bisporus* mycelium, despite being relatively hydrophilic. This suggests that hydrophobic interactions may not play a significant role in the attachment process. Enhanced attachment of wild-type *P. putida* (PMS118S) in the presence of CaCl_2 indicates involvement of electrostatic forces (Marshall *et al.*, 1971; James *et al.*, 1985). Similar cation effects have been reported for adherence of *P. tolaasii* to barley roots (Nissen, 1973), adherence of *P. aeruginosa* to steel (Stanley, 1983), adherence of a marine pseudomonad to glass (Marshall *et al.*, 1971) and adherence of *P. fluorescens* to radish roots (James *et al.*, 1985). The presence of uneven polysaccharide polymers and fibrillar appendages on the surface of *P. putida* and *P. tolaasii* may be important in attachment, enabling these bacteria to overcome the repulsion energy barrier which results when two negatively charged surfaces are brought close together (Rutter & Vincent, 1980;

Fletcher, 1980) and achieve rapid, firm attachment to the hyphal surface. The build-up of polysaccharide material between the bacteria and hyphae may help to anchor the cells firmly to each other and to the hyphal surface (Marshall *et al.*, 1971; Fletcher, 1980).

Adhesive properties of bacteria are affected by a range of physiological and environmental conditions (Fletcher, 1980). The quantitative studies demonstrate the importance of conducting attachment assays under conditions which approximate the situation *in vivo*. Preece & Wong (1982) examined attachment of a range of bacteria to discs of mushroom cap mycelium after growth on nutrient agar and reported a percentage attachment value of 31% for *P. putida* and 74% for *P. tolaasii*. They concluded that the attachment process, in quantitative terms, differed markedly between pathogens and non-pathogens. The results from this study show that after growth on media more representative of the nutrient status of the casing layer, the difference between the percentage attachment of *P. putida* and *P. tolaasii* was not marked.

Results from the SEM study confirmed the findings of the quantitative attachment work. Fibrillar rods associated with the attached cells were also reported by Preece & Wong (1982), who found them joining *P. tolaasii* cells to each other, and to *A. bisporus* hyphae. Similar structures have been found anchoring *Agrobacterium* to plant cells (Matthysse, 1983; Graves *et al.*, 1988) and lactobacilli to chicken crop epithelium (Fuller & Brooker, 1980). Brooker & Fuller (1975) were able to show that the fibrillar material had the staining characteristics typical of carbohydrate material. Fibrillar-like material has also been observed linking *Rhizobium* cells to *Phytophthora megasperma* hyphae (Tu, 1979) and to *P. cinnamomi* (Malajczuk *et al.*, 1984) and anchoring *Azospirillum brasilense* Cd to plant roots (Bashan *et al.*, 1991).

Phenotypic variants of *P. putida* and of *P. tolaasii* were shown to migrate more rapidly toward *A. bisporus* mycelial exudate than the wild-type forms (Grewal & Rainey, 1991) and in the light of earlier studies (Rainey, 1989b) it has been suggested that the phenotypic variant, in each instance, is more suited to survival under nutrient-depleted conditions. This study has revealed further differences in the behaviour of phenotypic variants and wild-type cells. The differences were most

Fig. 6. Transmission electron micrographs of *P. putida* and *P. tolaasii* cells attached to *A. bisporus* hyphae. (a) Phenotypic variant *P. putida* (PMS118R) attached to *A. bisporus* hyphae after 30 min. Note contact between fibrillar appendages and surface of hypha (arrows). (b) Phenotypic variant *P. putida* (PMS118R) attached to *A. bisporus* hypha after 3 h. (c) Same as (b), but TCH stained. (d) Phenotypic variant *P. putida* (PMS118R) attached to *A. bisporus* hypha after 24 h. Note long fibrillar appendages (arrow). (e) Wild-type *P. tolaasii* (PMS117S) attached to *A. bisporus* hypha after 3 h and (f) after 24 h. Note effects of tolaasin (toxin) activity: plasma membrane pulled away from hyphal wall and loss of cell contents. H, hypha; PM, plasma membrane; TCH, TCH-positive staining material. Bars represent 100 nm (a–f).

pronounced in *P. tolaasii*, where the wild-type form consistently attached to mycelium in greater numbers than the phenotypic variant. In *P. putida* quantitative studies revealed little difference between the ability of the two forms to attach, but a striking difference in behaviour became evident after *P. putida* was incubated with mycelium for a prolonged period under nutrient-depleted conditions. Bashan *et al.* (1991) have also described differences in attachment behaviour of two forms of *Azospirillum brasilense* Cd and suggested that cyst forms are a response to water-stressed conditions.

The different attachment behaviour shown by the phenotypic variants highlights the pleiotropic nature of phenotypic variation. While the ecological significance of this behaviour remains to be fully elucidated, the ability of these pseudomonads to produce cell-types which differ in their ability to attach to surfaces is likely to extend the range of habitats in which they can persist and confer a survival advantage upon the species.

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