Surface motility in *Pseudomonas* sp. DSS73 is required for efficient biological containment of the root-pathogenic microfungi *Rhizoctonia solani* and *Pythium ultimum*

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*Pseudomonas* sp. DSS73 was isolated from the rhizoplane of sugar beet seedlings. This strain exhibits antagonism towards the root-pathogenic microfungi *Pythium ultimum* and *Rhizoctonia solani*. Production of the cyclic lipopeptide amphisin in combination with expression of flagella enables the growing bacterial culture to move readily over the surface of laboratory media. Amphisin is a new member of a group of dual-functioning compounds such as tensin, viscosin and viscosinamid that display both biosurfactant and antifungal properties. The ability of DSS73 to efficiently contain root-pathogenic microfungi is shown to arise from amphisin-dependent surface translocation and growth by which the bacterium can lay siege to the fungi. The synergistic effects of surface motility and synthesis of a battery of antifungal compounds efficiently contain and terminate growth of the microfungi.

**INTRODUCTION**

Damping-off disease in sugar beet seedlings is caused by a number of soil-borne plant pathogens, including the basidiomycete *Rhizoctonia solani* and the oomycete *Pythium ultimum*. To protect crops against soil-borne diseases in general, seeds are commonly treated with fungicides. Since fungicides may affect human health and the environment and since pathogens can develop resistance to fungicides, bacterial inoculants exhibiting antagonism against plant-pathogenic micro-organisms are receiving increased attention as environmentally friendly alternatives to the use of chemical pesticides. One group of bacteria that shows great promise with respect to protecting plant roots from fungal-induced diseases is that containing the fluorescent *Pseudomonas* spp. The biocontrol activity of these strains is usually caused by the synthesis of one or more antifungal factors, which include such diverse compounds as hydrogen cyanide (Voisard *et al.*, 1989), siderophores, pterines, pyrroles (Shanahan *et al.*, 1992), phenazines (Thomashow & Weller, 1988), phloroglucinols (Shanahan *et al.*, 1992), peptides (Nielsen *et al.*, 1999, 2000; Thrane *et al.*, 2000), proteases and chitinases (Nielsen *et al.*, 1998; Dowling & O’Gara, 1994). In fluorescent *Pseudomonas* strains, the biosynthesis of antifungal compounds is regulated by a cascade of endogenous signals, which is channelled through a sensor-kinase and response regulator encoded by *gacAS* (Corbell & Loper, 1995; Gaffney *et al.*, 1994; Laville *et al.*, 1992), sigma factors encoded by *rpoS* (Sarniguet *et al.*, 1995) and *rpoD* (Schnider *et al.*, 1995), and quorum-sensing systems (Pierson *et al.*, 1998).

Among the great variety of antifungal metabolites produced by fluorescent *Pseudomonas* spp., our attention has been focused on the cyclic lipopeptides (Kochi *et al.*, 1951; Nielsen *et al.*, 1998, 1999, 2000, 2002). In addition, there are several examples of a close correlation between the production of cyclic lipopeptides and the ability to carry out surface motility on low-agar nutrient plates (Nielsen *et al.*, 2002). One well-studied phenomenon regarding bacterial surface translocation and the involvement of cyclic lipopeptides, such as serrawettins and rubiwettins, is the swarming motility of *Serratia* spp. (Matsuyama *et al.*, 1990, 1992; Lindum *et al.*, 1998). The swarming cells are found almost exclusively in a motile layer present at the perimeter of the expanding colony. This motile layer forms a bacterial biofilm, which precedes the bulk of bacterial mass. *Serratia* swarm cells are generally longer and more...
flagellated than their counterparts propagated in liquid media (the swimmer cells) and move within an encasement of a self-produced ‘slime’ that surrounds the colony (see review by Eberl et al., 1999). The morphology of swarm cells combined with the production of biosurfactants as well as the extraction of water from the underlying medium is believed to reduce the surface friction which in turn conditions the surface and as a consequence enables rapid expansion of the growing bacterial culture (Matusyama et al., 1990, 1992; Bees et al., 2000). The surfactant synthesized by Pseudomonas sp. DSS73 has been designated amphibin. The complete structural elucidation of amphibin has revealed that it is a cyclic lipopeptide consisting of an 11 amino acid cyclic peptide that is linked at the N-terminal end to β-hydroxydecanoyl (Sørensen et al., 2001).

In the rhizosphere, the potential benefits of bacterial motility include increased efficiency in nutrient acquisition, avoidance of toxic substances, ability to translocate to preferred hosts and access to optimal colonization sites within them (Turnbull et al., 2001). Since root-pathogenic microfungi appear to be highly motile in the rhizosphere, we speculated that the surface motility of root-associated biocontrol bacteria might play an important role in the effective protection of plant roots against microfungal attack. In this study, we characterized the surface motility phenomenon exhibited by the Pseudomonas biocontrol strain DSS73. Here, we present in vitro data implicating surface motility as an important factor for ensuring efficient impediment of the spreading mycelium of two plant-pathogenic microfungi that are known to induce damping-off disease in sugar beet seedlings.

### METHODS

**Strains, plasmids and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. The root-pathogenic microfungi employed as test fungi in biocontrol assays were the basidiomycete R. solani (strain 92009; Danisco Seed, Holeby, Denmark) and the oomycete Pythium ultimum (strain 92001; Danisco Seed) (Wolffhechel et al., 1992), both capable of inducing damping-off disease in sugar beet. Both fungi were maintained as described by Nielsen et al. (1998). The basic growth medium used was ABT minimal medium [AB minimal medium (Clark & Maaløe, 1967) containing 2:5 mg thiamin 1⁻¹] supplemented with different carbon sources (see below). When appropriate, the medium was supplemented with the following amounts of antibiotics: 100 μg ampicillin ml⁻¹, 25 μg kanamycin ml⁻¹ and 10 μg tetracycline ml⁻¹ for Pseudomonas strains, and 25 μg kanamycin ml⁻¹ for Serratia liquefaciens.

**Sugar beet seed extract.** This was prepared by incubating 50 g granulated sugar beet seeds (cv. ‘Madison’; Danisco Seed) with 200 ml ABT minimal medium on a horizontal shaker operating at 200 r.p.m. for 3 h at 37 °C. Next, the extract was successively filtered through cloth, Whatman paper no. 1 (Millipore), Whatman paper no. 3 (Millipore), a 0.8 μm pore size syringe filter (Sartorius) and a 0.2 μm pore size syringe filter (Sartorius AG) to produce a sterile sugar beet seed extract.

**Surface motility assays.** These were carried out on ABT minimal medium plates supplemented with 0.4 % (w/w) glucose and 0.4 % (w/w) Casamino acids (Difco) and solidified with 0.6 % Bacto Agar (Difco). In the following, these are referred to as the standard surface motility plates. When appropriate, the medium was supplemented with other carbon sources (see below) and different agar concentrations. The media were poured as 25 ml aliquots into 9 cm diameter Petri dishes and the resulting surface motility plates were dried for 2 h at 20 °C prior to inoculation with 1 μl culture droplets (harvested and washed twice with 1 ml of 0.9 % NaCl) of wild-type DSS73, DSS73-15C2 (AmsY⁻) or DSS73-12H8 (GacS⁻) carrying the plasmid pEMH97 (this plasmid carries the gacS gene of Pseudomonas syringae pv. syringae). Following 20 h incubation at 20 °C, all surface motility plates were visually inspected and the surface motility phenotypes of the respective strains were recorded. All assays were done in triplicate and images of the representative surface motility phenotypes were captured using a Kodak DC240 digital camera. The resulting images were processed using Photoshop 5.5. To obtain bacterial inocula, strains were cultivated in liquid ABT medium containing 0.4 % glucose (w/w) and 0.4 % Casamino acids (w/w) at 20 °C for 24 h. Subsequently, 1 ml aliquots were harvested by centrifugation (2 min at 10,000 g) and washed twice with 1 ml of 0.9 % NaCl.

### Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant genotype/characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSS73 Pseudomonas sp.</td>
<td>Wild-type; amphisin producer</td>
<td>Nielsen et al. (2002)</td>
</tr>
<tr>
<td>DSS73-15C2 Pseudomonas sp. (AmsY⁻)</td>
<td>amsY mutant derived from DSS73; amphisin-deficient; Km’</td>
<td>Koch et al. (2002)</td>
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<tr>
<td>DSS73-12H8 Pseudomonas sp. (GacS⁻)</td>
<td>gacS mutant derived from DSS73; amphisin-deficient; Km’</td>
<td>Koch et al. (2002)</td>
</tr>
<tr>
<td>S. liquefaciens MG1</td>
<td>Wild-type; serrawettin W2 producer</td>
<td>Givskov et al. (1988)</td>
</tr>
<tr>
<td>S. liquefaciens PL10</td>
<td>swrA swrI double mutant derived from MG1; serrawettin W2-deficient; Km’, Strep’</td>
<td>Lindum et al. (1998)</td>
</tr>
<tr>
<td>Plasmid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pLAFR3</td>
<td>Cloning vector; Tc’</td>
<td>Staskawicz et al. (1987)</td>
</tr>
<tr>
<td>pEMH97</td>
<td>pLAFR3 encoding gacS isolated from Pseudomonas syringae pv. syringae; Tc’</td>
<td>Hrabak &amp; Willis (1992)</td>
</tr>
</tbody>
</table>
**Surfactant complementation assays.** Culture droplets (1 μl) were inoculated onto standard surface motility plates (see above) supplemented with 0, 1-25, 2-5, 5 or 10 μg ml⁻¹ of amphisin (a cyclic lipopeptide isolated from *Pseudomonas* sp. DS73) (Sørensen et al., 2001; Nielsen et al., 2002), tensin (a cyclic lipopeptide isolated from *Pseudomonas fluorescens* DR54) (Nielsen et al., 1999), serrawettin W2 (a cyclic lipopeptide isolated from *S. liquefaciens* MG1 (Lindum et al., 1998), NP40 (Nonidet P40, an artificial surfactant/non-ionic detergent; Sigma) or Triton X-100 (a detergent; Sigma). Following 24 h incubation at 20 °C, all plates were visually inspected and the surface motility phenotypes of the respective strains were recorded. All complementation assays were repeated three times and digital images of the representative surface motility phenotypes of the respective strains were captured and processed as described in the ‘Surface motility assays’ section of Methods.

**Nutritional requirements for surface motility.** Aliquots (25 ml) of ABT minimal medium containing 0·6 % Bacto agar and either 0·4 % (w/w) glucose and 0·4 % (w/w) Casamino acids, 0·4 % (w/w) Casamino acids and 2 % Bacto agar (Difco), or 0·1 % D-xylose or 50 % sugar beet extract were poured into sterile Petri dishes. The plates were dried for 2 h at 20 °C and then inoculated with 1 μl culture droplets. Following 20 h incubation at 20 °C, all plates were visually inspected for surface motility. Since all of the strains employed in the experiment grew slowly on plates containing d-xylose as carbon source, their ability to perform surface motility on d-xylose plates was re-evaluated following 7 days incubation at 20 °C. All strains were tested three times for their ability to perform surface motility on the plates described above; based on these triplicates a rough estimate of the maximal expansion velocity was obtained by measuring the increase in colony radius as a function of time.

**Biocontrol assays.** To compare the abilities of strains DS73, DS73-15C2 (AmsY'), DS73-12H8 (GacS') and DS73-12H8 (GacS') carrying pEMH97 to limit the growth of the root-pathogenic microfungi *R. solani* and *Pythium ultimum*, the relevant bacterial and fungal strains were co-cultivated on standard surface motility plates and on nutrient agar plates composed of ABT minimal medium containing 0·4 % (w/w) glucose, 0·4 % (w/w) Casamino acids and 2 % Bacto agar (Difco). A 1 μl pre-culture of each bacterial strain was inoculated 2·5 cm away from the center of an autoclaved agar plug containing mycelium of either *R. solani* or *Pythium ultimum*, and the plates were incubated at 20 °C. As a control, agar plugs containing mycelium of *R. solani* and *Pythium ultimum* were also inoculated alone onto the same types of nutrient plates. Following 24 and 48 h co-cultivation, all plates were visually inspected and the ability of each bacterial strain to inhibit mycelium spreading was evaluated. All biocontrol assays and appropriate controls were done in triplicate and digital images of the representative biocontrol phenotypes were captured and processed as described in the ‘Surface motility assays’ section of Methods. To address the viability of the microfungi contained by strain DS73 on standard surface motility plates (see Fig. 4A as an example) agar plugs were withdrawn from the outer edge of the mycelium and from the area close to the original fungal inoculation site. These plugs were transferred to nutrient plates composed of ABT minimal medium containing 0·4 % (w/w) glucose, 0·4 % (w/w) Casamino acids, 2 % Bacto agar and 10 μg tetracycline ml⁻¹ (tetracycline was added to prevent the growth of biocontrol strain DS73). As positive controls, agar plugs containing non-challenged mycelium of *R. solani* and *Pythium ultimum* were inoculated onto the same types of nutrient plates. Following 3 days incubation at 20 °C, the plates were visually inspected for growth of the respective fungus. All viability assays were repeated three times.

**Electron microscopy.** Bacteria from the centre and the leading edge of a moving colony were analysed with respect to their cell morphology and presence of flagella using electron microscopy. Bacteria from the centre or the margin of three different surface motile colonies of strain DS73 were gently scraped off with a toothpick and subsequently suspended in a drop of water mounted on top of a carbon-coated copper grid (mesh: 200) (Electron Microscopy Sciences). Next, the six grids were washed once with distilled water and stained with 1 % uranyl acetate. The negatively stained cells were visualized with a transmission electron microscope (Phillips TEM, CM 100) equipped with a SIS Megaview II digital camera system; the resulting electron micrographs were processed using the ANALYSIS software package (SIS). From the resulting electron micrographs, mean cell size and mean number of flagella were estimated.

**RESULTS**

**Surface motility studies of *Pseudomonas* sp. DS73**

In a recent screening for novel sugar beet protective strains, *Pseudomonas* sp. DS73 was isolated from the rhizoplane of sugar beet seedlings as a biosurfactant-producing strain that exhibited antagonism towards the root-pathogenic microfungi *Pythium ultimum* and *R. solani* (Nielsen et al., 2002). This study revealed that the ability to synthesize biosurfactants is a quite common property in root-associated, fluorescent *Pseudomonas* spp. Furthermore, many of the strains were found to form expanding colonies on semi-solid laboratory media. This suggests a connection between biosurfactant production and the ability to carry out surface motility (Nielsen et al., 2002). We decided to investigate conditions affecting the surface motility of strain DS73. Within the first 12–16 h of incubation, DS73 formed a morphologically normal colony with a diameter of approximately 2–4 mm on standard surface motility plates. However, as cell density increased further, small peninsulas of cells were eventually generated at the colony perimeter and were observed to move vertically away from the colony. These peninsulas never increased to more than 3 mm in length and 1 mm in width, as the free space between neighbouring peninsulas was continuously filled in by residual growth. As a result, the expanding colony exhibited small ‘tongue’-like structures of biofilms at its perimeter. Once this phenomenon had been triggered, the diameter of the DS73 colony was observed to expand by 8 mm h⁻¹, rendering the strain capable of covering the entire surface of a 9 cm Petri dish within 24 h after inoculation (Fig. 1). Microscopic inspection revealed that cells at the perimeter of the colony formed a highly motile swarm, and neither single cells nor small groups of cells were ever observed to escape/leave the dense colony. This motility pattern is very similar to the swarming motility observed with *S. liquefaciens* (Eberl et al., 1996, 1999). Since the hallmark of true swarming motility is considered to involve the differentiation of cells into an elongated, multinucleated and profusely flagellated form (Harshey, 1994), we decided to clarify if surface contact induced differentiation in strain DS73. Electron microscopy revealed that bacteria isolated from the edge of the expanding colony were on average approximately 2 μm long and possessed four to six polar
flagella, whereas cells from the centre of the colony were approximately 1 μm long and possessed just one polar flagellum (Fig. 2). We therefore decided to refer to the phenomenon as surface motility rather than swarming motility.

Propagation of strain DSS73 on a series of nutrient plates with increasing agar contents revealed that the expansion velocity declined when the agar content exceeded 0.7%. At 1% agar, the expansion velocity of the colony was drastically reduced (data not shown). In the presence of 1.2% agar, surface motility was completely abolished. This gave rise to colonies that were morphologically indistinguishable from colonies propagated on nutrient plates containing 1.5 and 2% agar (data not shown). In comparison, Proteus mirabilis (Allison & Hughes, 1991) and Vibrio parahaemolyticus (McCarter & Silverman, 1990) have been demonstrated to swarm at agar concentrations up to 2%.

Amphisin production is required for surface motility of strain DSS73

The amphisin synthesized by strain DSS73 might promote or enable surface translocation. In a recent study by Koch et al. (2002), two surfactant-negative mutants, DSS73-15C2 (AmSY<sup>-</sup>) and DSS73-12H8 (GacS<sup>-</sup>), carrying mutations in <i>amsY</i> (encoding the putative amphisin synthetase) and <i>gacS</i>, respectively, were isolated. These two mutants were subsequently demonstrated to be defective in the synthesis of amphisin. When stab inoculated on nutrient plates solidified with 0.3% agar, the swimming motility phenotypes of DSS73-15C2 (AmSY<sup>-</sup>) and DSS73-12H8 (GacS<sup>-</sup>) were indistinguishable from the swimming motility phenotype exhibited by the parent wild-type strain, DSS73 (data not shown). This indicates the presence of functional flagella and a fully operational chemotaxis apparatus in both of the amphisin-deficient mutants. DSS73-15C2 (AmSY<sup>-</sup>) and DSS73-12H8 (GacS<sup>-</sup>) failed to carry out surface motility on 0.6% agar plates unless the medium was supplemented with 2.5 μg amphisin ml<sup>-1</sup> (Fig. 1B, C, E and F). This clearly demonstrated that amphisin is the surface-tension-reducing compound that enables strain DSS73 to move over the agar surface (Fig. 1A). In addition, surface motility could be restored in the <i>gacS</i> mutant upon introduction of pEMH97 encoding the heterologous wild-type <i>gacS</i> gene from <i>Pseudomonas syringae</i> pv. syringae (Hrabak & Willis, 1992), supporting the finding of Koch et al. (2002) that amphisin synthesis is regulated by <i>gacS</i> (compare Fig. 1C and D).

**Fig. 1.** Surface motility phenotypes of <i>Pseudomonas</i> sp. DSS73 and mutant derivatives. (A) DSS73, (B) DSS73-15C2 (AmSY<sup>-</sup>), (C) DSS73-12H8 (GacS<sup>-</sup>) and (D) DSS73-12H8 (GacS<sup>-</sup>) carrying pEMH97 were cultivated on ABT/glucose/Casamino acids plates solidified with 0.6% agar (standard surface motility plates). (E) DSS73-15C2 (AmSY<sup>-</sup>) and (F) DSS73-12H8 (GacS<sup>-</sup>) were cultivated on ABT/glucose/Casamino acids plates containing 2.5 μg amphisin (ml medium)<sup>-1</sup> and solidified with 0.6% agar. All plates were inoculated with 1 μl of an overnight culture of the respective strain; digital images were captured following 20 h incubation at 20°C.
Surface motility of the two mutants, DSS73-15C2 (AmsY\textsuperscript{2}) and DSS73-12H8 (GacS\textsuperscript{2}), was also restored by exogenous tensin (2\textsuperscript{5} \textmu g ml\textsuperscript{-1}), viscosinamid (2\textsuperscript{5} \textmu g ml\textsuperscript{-1}) or serrawettin W2 (2\textsuperscript{5} \textmu g ml\textsuperscript{-1}) whereas the addition of synthetic surfactants such as NP40 and Triton X-100 failed to promote surface motility (Table 2). NP40 and Triton X-100 restored swarming motility in the surfactant-deficient S. liquefaciens strain PL10 (Table 2).

**Table 2. Surface motility in the presence of different surfactants**

<table>
<thead>
<tr>
<th>Surfactant*</th>
<th>Surface motility of strain†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DSS73</td>
</tr>
<tr>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>Amphisin‡</td>
<td>+</td>
</tr>
<tr>
<td>Tensin‡</td>
<td>+</td>
</tr>
<tr>
<td>Viscosinamid‡</td>
<td>+</td>
</tr>
<tr>
<td>Serrawettin W2‡</td>
<td>+</td>
</tr>
<tr>
<td>NP40§</td>
<td>+</td>
</tr>
<tr>
<td>Triton X-100§</td>
<td>+</td>
</tr>
</tbody>
</table>

*Surfactant added to ABT minimal medium supplemented with 0·4 % glucose, 0·4 % Casamino acids and 0·6 % agar prior to casting.
†Surface motility was evaluated following 24 h incubation at 20 °C; +, surface motility; –, no surface motility.
‡Final concentration of surfactant in the plates was 2·5 \textmu g ml\textsuperscript{-1}.
§Final concentration of surfactant in the plates was 10 \textmu g ml\textsuperscript{-1}.

Surface motility studies of *Pseudomonas* sp. DSS73

**Fig. 2.** Morphology of *Pseudomonas* sp. DSS73 cells isolated from a swarming colony cultivated on nutrient plates solidified with 0·6 % agar. (A) Electron micrograph of DSS73 taken directly from the centre of a swarming colony. At the centre, cells were approximately 1 \mu m and were observed to express just one polar flagellum. (B) Electron micrograph of DSS73 taken directly from the edge of a surface-motile colony.

**Nutritional requirements of surface motility in strain DSS73**

Unlike most surface-motile bacteria, the surface motility of strain DSS73 did not depend on the presence of Casamino acids, peptone or any single amino acid in the growth medium (Table 3). The only nutritional requirement of the bacterium for it to carry out rapid surface motility was a mineral medium containing an easily metabolizable carbon source such as glucose. However, it is noteworthy that a significantly higher colony expansion velocity was obtained for strain DSS73 in the presence of Casamino acids (the sole carbon source). D-Xylose (a sugar compound often encountered in root exudates) turned out to be a very poor carbon source for strain DSS73, as it took 3–4 days for the strain to form a normal-sized colony (Table 3). Nevertheless, surface motility was eventually triggered at day 4 of incubation, and within the next six days the colony reached the edge of the plate. Instead of the usual confluent spreading, strain DSS73 was observed to move in dendrites when propagated on D-xylose plates. Whether this changed colony morphology in DSS73 was the result of increased surface tension due to dehydration of the plate during the long incubation or was dictated by the D-xylose is not known.

Recently it was reported that amphisin production on glucose-supplemented minimal medium is increased sixfold upon the addition of a sugar beet seed extract to the medium (Koch et al., 2002). Interestingly, plates supplemented with sugar beet seed extract as the sole carbon source enabled strain DSS73 to traverse the standard surface motility plate with a speed indistinguishable from that recorded on Casamino acids (Table 3).

**Antifungal properties of strain DSS73 are regulated by GacS**

The ability of strain DSS73 to inhibit the growth of the root-pathogenic microfungi *Pythium ultimum* and *R. solani* is believed to originate from the production and excretion of amphisin, chitinases, proteases and hydrogen cyanide by the bacterium (Nielsen et al., 2002; Koch et al., 2002). Interestingly, the production of these antifungal metabolites requires a functional *gacS* gene whereas only the production of amphisin seems to be impaired in the *amsY* mutant (Koch et al., 2002). To characterize the antifungal properties of DSS73 in further detail, we compared the abilities of strains...
DSS73, DSS73-15C2 (AmsY<sup>2</sup>), DSS73-12H8 (GacS<sup>2</sup>) and DSS73-12H8 (GacS<sup>2</sup>) carrying pEMH97 to inhibit the growth of <i>R. solani</i> and <i>Pythium ultimum</i>. Following co-cultivation of strain DSS73 and <i>R. solani</i> on 2 % agar plates, a mycelium-deficient zone developed around the bacterial colony, confirming the antifungal properties of DSS73 (Fig. 3A). A similar result was obtained when the mycelium of <i>R. solani</i> approached the bacterial colony of the <i>amsY</i> mutant, DSS73-15C2 (AmsY<sup>2</sup>) (Fig. 3B). In striking contrast, <i>R. solani</i> appeared unaffected by the presence of the <i>gacS</i> mutant DSS73-12H8 (GacS<sup>2</sup>) when co-cultivated under identical conditions. Indeed, the mycelium of the fungus was eventually seen to cover the colony formed by DSS73-12H8 (GacS<sup>−</sup>), signifying a complete absence of antifungal metabolites in this strain (Fig. 3C). However, upon provision of the wild-type <i>gacS</i> gene product from <i>Pseudomonas syringae</i> pv. <i>syringae</i>, present on pEMH97, to DSS73-12H8 (GacS<sup>−</sup>) a mycelium-deficient zone was maintained around the bacterial colony, demonstrating that the antifungal phenotype had been restored (Fig. 3D). As similar observations were made when <i>Pythium ultimum</i> was challenged with strains DSS73 (AmsY<sup>−</sup>), DSS73-15C2 (GacS<sup>−</sup>), DSS73-12H8 and DSS73-12H8 (GacS<sup>−</sup>) carrying pEMH97 (data not shown), these results clearly show that synthesis of antifungal metabolites in DSS73 requires the presence of a functional <i>gacS</i> gene. This strongly suggests that expression of the antifungal properties of strain DSS73 is controlled by a <i>gacS</i>/<i>gacA</i>-encoded two-component system similar to other <i>Pseudomonas</i> spp. (Chancey et al., 1999; Laville et al., 1992; Gaffney et al., 1994; Pfender et al., 1994; Corbell & Loper, 1995; Schmidli-Sacherer et al., 1997).

### Table 3. Surface motility in the presence of different carbon sources

<table>
<thead>
<tr>
<th>Carbon source*</th>
<th>Surface motility of strain†</th>
<th>DSS73</th>
<th>DSS73-15C2 (AmsY&lt;sup&gt;−&lt;/sup&gt;)</th>
<th>DSS73-12H8 (GacS&lt;sup&gt;−&lt;/sup&gt;)</th>
<th>MG1</th>
<th>PL10</th>
</tr>
</thead>
<tbody>
<tr>
<td>None‡</td>
<td>No growth</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Glucose and Casamino acids‡</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Casamino acids‡</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Glucose‡</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>D-Xylose§</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Extract of sugar beet seeds‡</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
</tbody>
</table>

*Carbon source added to plates containing ABT minimal medium and 0·6 % agar.
†−, Surface immobile; +, maximal colony expansion velocity <2 mm h<sup>−1</sup>; ++, maximal colony expansion velocity 2–4 mm h<sup>−1</sup>; ++++, maximal colony expansion velocity >4 mm h<sup>−1</sup>.
‡Evaluation of surface motility was done following 20 h incubation at 20 °C.
§Due to slow growth of all strains tested, evaluation of surface motility was done following 7 days incubation at 20 °C.

**Surface motility promotes containment of microfungi**

Although strain DSS73 clearly exhibited antagonism towards <i>R. solani</i> and <i>Pythium ultimum</i> on 2 % agar nutrient plates (Fig. 3A, and data not shown), both microfungi eventually ended up occupying as much as 98 % of the available surface. We envisaged that this outcome could be reversed if both organisms were propagated on a medium that sustained bacterial surface motility. To confirm this, we evaluated the abilities of strains DSS73, DSS73-15C2 (AmsY<sup>−</sup>) and DSS73-12H8 (GacS<sup>−</sup>) to impede mycelium growth of <i>R. solani</i> and <i>Pythium ultimum</i> on 2 % agar plates (data not shown). These results clearly show that synthesis of antifungal metabolites in DSS73 requires the presence of a functional <i>gacS</i> gene. This strongly suggests that expression of the antifungal properties of strain DSS73 is controlled by a <i>gacS</i>/<i>gacA</i>-encoded two-component system similar to other <i>Pseudomonas</i> spp. (Chancey et al., 1999; Laville et al., 1992; Gaffney et al., 1994; Pfender et al., 1994; Corbell & Loper, 1995; Schmidli-Sacherer et al., 1997).
spreading of both *R. solani* and *Pythium ultimum* on standard surface motility plates. As expected, the antifungal properties of the non-surface-motile strains DSS73-15C2 (AmsY<sup>−</sup>) and DSS73-12H8 (GacS<sup>−</sup>) did not change (compare Fig. 3B with Fig. 4B and Fig. 3C with Fig. 4C). *R. solani* readily colonized 98 and 100 %, respectively, of the nutrient plate surface within 60 h of co-cultivation. In striking contrast, mycelium spreading of *R. solani* was severely hampered when it was co-cultivated with the surface-motile strain DSS73 (Fig. 4A). Once the DSS73 colony started to expand, contact between the preceding bacterial biofilm and the fungus was achieved within a few hours. Microscopic inspection revealed that upon contact, the biofilm was partly dissolved as planktonic bacteria moved along the hyphae by swimming motility while the areas between the hyphae were filled in by the biofilm swarm (not shown). In this way DSS73 rapidly traversed the fungal mycelium and the fungus was completely surrounded by the expanding bacterial colony. Apparently, due to the spatial limitation, the fungus seemed to be unable to expand. Furthermore, the trapped *R. solani* was no longer able to maintain mycelium growth (Fig. 4A). The activity of strain DSS73 seemed to be lethal to the fungus, as the fungus failed to resume mycelium growth when transferred to fresh nutrient plates (data not shown). Supporting the assumption that GacS/GacA is a key regulator of biocontrol in DSS73, almost identical observations were made when *R. solani* was challenged with strain DSS73-12H8 (GacS<sup>−</sup>) carrying pEMH97 on standard surface motility plates. In accordance with the restoration of both surface motility and synthesis of antifungal metabolites, the presence of a functional gacS product *in trans* clearly enabled the strain to impede mycelium spreading of the fungus (Fig. 4D).

Cultivation of *R. solani* on standard surface motility plates in which the growth medium had been supplemented with varying amounts of amphisin revealed that fungal growth was not significantly affected until a concentration of approximately 10 μg amphisin (ml medium)<sup>−1</sup> was reached (data not shown). In contrast, 2·5 μg amphisin (ml medium)<sup>−1</sup> was the minimum concentration required to fully restore surface motility of the *amsY* and *gacS* mutants (see Table 3). Therefore, it was possible to clarify whether spatial limitations alone or in combination with synthesis of antifungal agents were responsible for the observed efficient containment of the fungus. To address this question, *R. solani* was challenged with DSS73-12H8 (GacS<sup>−</sup>) and DSS73-15C2 (AmsY<sup>−</sup>), respectively, on standard surface motility plates supplemented with 2·5 μg amphisin ml<sup>−1</sup>. Under these conditions, the ability of the *amsY* mutant to block mycelium spreading was restored (compare Fig. 4B and E). Once surrounded by the bacterial

![Figure 4](http://mic.sgmjournals.org)
colony, *R. solani* was never observed to resume growth, not even following prolonged incubation. A somewhat different scenario emerged following co-cultivation of *R. solani* with the gacS mutant on standard surface motility plates supplemented with 2.5 µg amphisin ml⁻¹. Although fully capable of moving over the surface of the agar, strain DSS73-12H8 (GacS⁻) completely failed to stop the spreading mycelium. Even when completely surrounded by the bacterial colony, *R. solani* was still observed to maintain mycelium propagation into the agar beneath the DSS73-12H8 (GacS⁻) culture. Similar results were obtained by replacing *R. solani* with *Pythium ultimum* in all of the experiments described in this section (data not shown). This strongly suggests that the fungus had been effectively contained by strain DSS73 as a result of a combination of spatial limitations and a cocktail of antifungal agents.

**DISCUSSION**

The fluorescent *Pseudomonas* sp. DSS73 was originally isolated from the rhizoplane of sugar beet seedlings as a biosurfactant-producing strain capable of inhibiting the growth of the root-pathogenic microfungi *R. solani* and *Pythium ultimum* (Sørensen et al., 2001; Nielsen et al., 2002). This strain displayed a convincing surface motility phenotype when propagated on nutrient plates containing 0.4–1% agar. A study by Nielsen et al. (2002) revealed that the ability to produce biosurfactants is a quite common phenotype in root-associated fluorescent *Pseudomonas* spp., suggesting that surface motility *in vivo* requires the production of biosurfactants. At the macroscopic level, strain DSS73 predominantly gave rise to a concentric, confluent, moving colony that was distinctly different from the dendrite colony pattern that arose during surface translocation of *Pseudomonas aeruginosa* (Rashid & Kornberg, 2000; Köhler et al., 2000) and *Pseudomonas syringae* pv. syringae (Kinscherf & Willis, 1999). With strain DSS73, cells located at the edge of the expanding colony moved rapidly in swirls that closely resembled the swarming pattern reported for cells at the rim of swarming colonies of *S. liquefaciens* (Eberl et al., 1996, 1999). Since planktonic cells were never observed, the surface motility of strain DSS73 is likely to originate from the multicellular behaviour of the cells present in the motile biofilm part of the expanding colony. However, surface translocation of strain DSS73 was clearly not accompanied by any substantial increase in flagellation and cell elongation, and was therefore almost indistinguishable from surface-motile colonies of *Pseudomonas aeruginosa* (Rashid & Kornberg, 2000; Köhler et al., 2000). Our genetic analysis indicated that the surface motility of strain DSS73 occurs within the framework of the GacS/GacA-controlled regulon, as has been reported for *Pseudomonas syringae* (Kinscherf & Willis, 1999), and is not controlled by quorum sensing, as reported for *S. liquefaciens* (Eberl et al., 1996; Lindum et al., 1998), *Pseudomonas aeruginosa* (Köhler et al., 2000) and *Burkholderia cepacia* (Huber et al., 2001). Strain DSS73 failed to activate any of our N-acyl-L-lhomoserine lactone (AHL) sensors, which covered the entire range of known AHL signals (data not shown). In fact, during the screening for and characterization of sugar-beet-root-associated, biosurfactant-producing *Pseudomonas* spp., only three out of 80 surfactant-producing isolates appeared capable of synthesizing AHL signal molecules.

Similar to *Serratia* spp., the ability of *Pseudomonas* sp. DSS73 to move over the agar surface was clearly attributed to its production of an extracellular biosurfactant, since the presence of purified amphisin in the growth medium restored this phenotype to the *ansY* and *gacS* mutants. In contrast to *S. liquefaciens*, which is highly promiscuous with respect to accepting surfactants for swarming motility (Lindum et al., 1998; Eberl et al., 1999), amphisin and other closely related cyclic lipopeptides are prerequisites for the surface motility of strain DSS73. This suggests that not only surface tension reduction but also additional physical/chemical properties of these closely related cyclic biosurfactants are crucial for the surface motility of DSS73.

Fungal growth was inhibited on agar plates supplemented with purified amphisin at a minimum concentration of 10 µg ml⁻¹, which is four times the concentration required to efficiently promote bacterial surface motility. Therefore, amphisin can be considered a dual-functioning compound. For fungal antagonism, however, DSS73 relies not on amphisin alone. It produces a battery of antagonistic agents such as proteases, chitinases and hydrogen cyanide, all of which are controlled by the GacS/GacA system (Koch et al., 2002). Our results clearly demonstrate that the efficient elimination of competing fungi is due to the synergistic effect of surface motility and synthesis of antifungal agents. Without these factors the bacteria were unable to completely contain the fungi.

Our data also suggest that surfactant activity is a more important property than the antimicrobial action of amphisin. However, the artificial nature of the experimental set-up should be kept in mind. If the hardness of the surface *in vivo* prevents the bacterial culture moving over the surface, the antimicrobial activity of amphisin may be of greater value to the producer. With a MIC of 10 µg ml⁻¹, amphisin clearly retains its potency as an antimicrobial compound.

Expression of the total antimicrobial cocktail (including motility) produced by fluorescent *Pseudomonas* spp. is controlled by the GacS/GacA system. Therefore, only a single signal is required to mount the entire protective arsenal of the bacteria. In a recent study, it was demonstrated that synthesis of amphisin was increased approximately sixfold when strain DSS73 was cultivated in the presence of a sugar beet seed extract (Koch et al., 2002). Since the growth rate of the organism was unaffected, this result indicates the presence of a signal compound in the seed extract. The structure of this putative signal compound has not yet been elucidated; however, experiments have revealed that signal transduction leading to increased transcription of *ansY*...
clearly requires a functional gacS/gacA system (Koch et al., 2002).

The apparent interplay between strain DSS73 and the sugar beet seed might be considered as being mutually beneficial. Initially, the sugar beet seed supplies the bacteria with nutrients and releases signals that induce production of amphisin. We speculate that this enables a culture of DSS73 to efficiently expand on the seed surface, but in addition it also recruits the entire protective arsenal of the bacterium. In the environment, multicellular behaviour is considered an advantage for bacteria. Previous work by Eberl et al. (1997) and Mallory et al. (1983) strongly suggests that the formation of flocks offers bacteria protection against grazing protozoa. Similarly, in the cause of infection, Pseudomonas aeruginosa grows in biofilms that protect it from the action of leukocytes (Hoiby et al., 2001; Costerton et al., 1999). In this latter case, control over surface motility, biofilm architecture and the formation of a cocktail of virulence factors is integrated by quorum sensing. It is tempting to speculate that biocontrol bacteria, which coordinate their effort and work in a flock, are more successful in their interactions with other microbes and higher eukaryotes than their planktonic counterparts.

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REFERENCES


