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Pseudomonad Swarming Motility Is Restricted to a Narrow Range of High Matric Water Potentials

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Using a novel experimental system that allows control of the matric potential of an agar slab, we explored the hydration conditions under which swarming motility is possible. If there is recognition that this physical parameter is a key determinant of swarming, it is usually neither controlled nor measured rigorously but only manipulated through proxies, namely, the agar concentration and the drying time of "soft" agar plates (swarming plates). We contend that this not only obscures the biophysical mechanisms underlying swarming but also impedes a full assessment of its clinical and environmental significances. Our results indicate that swarming motility is restricted to a narrow range of high matric water potentials in the three pseudomonads tested (*Pseudomonas* sp. DSS73, *Pseudomonas syringae* B728a, and *Pseudomonas aeruginosa* PA14). The threshold below which no swarming was observed was about −0.45 kPa for the first and about −0.1 kPa for the latter two. Above the threshold, the expansion rate of DSS73 swarms increased exponentially with the matric potential. Mutants deficient in surfactant production were totally or partially unable to expand rapidly on the surface of the agar slab. Our results thus suggest that swarming motility in pseudomonads is restricted to (micro)sites where ambient humidity is very high (relative humidity of >99.99%). The spatiotemporal occurrence of such sites is limited in many types of terrestrial environments.

Swarming, a type of motility by which cells rapidly and collectively colonize a wet solid surface, has received considerable attention owing to the impressive patterns it generates and to its link to virulence in many pathogens (14). The last few decades have seen great progress in the description of the genetic bases of this mechanism (29, 32, 35). The underlying biophysics has, however, remained relatively obscure until very recently, when some clever experimental systems (34, 37) and microscopic techniques (5, 31, 36) provided important insights into the nature of swarming.

It is well established that swarming requires the presence of a thin liquid film to allow flagellum rotation (5, 31) and/or the overcoming of drag and viscous forces (8). This film has been observed microscopically, and its thickness has been recently estimated (37). The origin of this liquid film is debated, however, and it is thought to originate from the excretion of biosurfactant(s) and/or osmolyte(s) (4, 37).

In any case, the ability for bacteria to swarm and thus, presumably, generate fluid films at the surface of solid culture medium has been shown to be strongly dependent on the agar concentration. Most Gram-negative organisms optimally swarm at agar concentrations of 4 to 7 g liter^{-1}, although swarming has been reported to occur at concentrations as high as 15 g liter^{-1} (28). Swarming is notoriously affected by small variations in the drying time of the so-called swarming plates, to the point that standardization efforts have been attempted (30). Presumably, agar concentration and plate drying time directly affect the energy state of water at the surface of the solid medium. In the field of physics, the rigorous expression of this energy state is as water potential, an equivalent to the water activity used in classical microbiology (24). At the surface of the hydrogel that constitutes a solid culture medium, the main (additive) components of the water potential are the osmotic and matric potentials. The agar, which is not a solute but the gelling agent, is expected to contribute mostly to the matric potential, albeit in an ill-defined manner. A recent paper describes how the relative humidity (RH; directly convertible into water potential) over an agar gel decreases with increasing agar concentrations (12). Unfortunately, no data are available for concentrations lower than 20 g liter^{-1}, which was found to generate a water potential of about −126 kPa. To our knowledge, this value would be the best available estimate of the lower bound of the water potential range supporting swarming. This bound is, however, underestimated, as swarming in many bacteria typically requires agar concentrations lower than 10 g liter^{-1} (14). We argue that to understand the biophysical basis of swarming motility and predict its occurrence in a host or in the environment, a rigorous measure of the matric potential range enabling this type of motility is needed. We attempted this determination for three strains belonging to the genus *Pseudomonas*, using agar slabs placed in a system allowing full control of the matric potential down to values close to water saturation (i.e., 0 kPa or 100% RH).

MATERIALS AND METHODS

Strains and media. Three pseudomonad strains and their isogenic mutants, deficient in biosurfactant synthesis, were studied. The wild-type strains were *Pseudomonas syringae* B728a, *Pseudomonas aeruginosa* PA14, and *Pseudomonas* sp. DSS73. This last strain can be tentatively affiliated with the *Pseudomonas fluorescens* species based on the 16S sequence (GenBank accession number GQ334363) and phenotypic observations (20). These strains thus encompass three species and have been isolated from different environments (B728a from the bean phyllosphere [18], PA14 from human patient skin [26], and DSS73 from the sugar beet rhizosphere [20]). These strains were compared with the following mutants deficient in biosurfactant production: B728a Δ*syfA* (3), PA14 Δ*rhlA* (17), and

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DSS73 Δ*amsY* (16). The B728a and DSS73 strains were constitutively expressing *gfp* (2, 19).

All strains were maintained on FAB solid mineral medium (11) supplemented with 5 mM citrate. For the agar slab porous surface model (PSM) experiments (see below), SWM medium was used for B728a (15), modified M9 medium was used for PA14 (29), and FAB with 5 mM citrate was used for DSS73, as these media successfully induced swarming of their respective strains in standard swarming assays.

Agar slab PSM. We have previously described the porous surface model (PSM), which allows growth of cells on the surface of a porous ceramic plate under a controlled matric potential (6). In this system, the matric potential is directly imposed on the bacteria by setting the height of the hanging column of liquid medium that connects the ceramic plate to a bottle serving as the medium reservoir. Here, we have added an agar slab on the top of the ceramic plate so that its inoculated surface can be subjected to prescribed matric potentials. The agar slabs were obtained by pouring 5.5 ml of freshly autoclaved agar medium, as described above, into the lid of a small plastic petri dish (diameter, 40 mm; height, 12.5 mm; Phoenix Biomedical Products, Mississauga, Canada). The gel was left to dry for 5 min under a laminar flow bench before being transferred upside down onto the ceramic plate of a preautoclaved PSM, its reservoir filled with 200 ml of the appropriate liquid medium. While swarming medium typically contains less than 7 g agar liter^{-1}, we used a concentration of 15 g agar (Fluka; Sigma-Aldrich) liter^{-1} in the slab, unless noted otherwise. This provides sufficient strength to the gel for it to be easily transferred onto the PSM.

Inoculation. A suction of 0.7 kPa was applied to the agar slab at least 20 min before inoculation. The inoculum was prepared by scraping cells from a 24-h-old plate before suspending them in sterile saline solution and adjusting the suspension optical density at 600 nm (OD_{600}) to about 1. Seven microliters of this suspension was then carefully pipetted into the depression in the center of the slab (about 4 mm in diameter) which results from the presence of a protuberance in the wall of the lid of the petri dish used to cast the slab.

After inoculation, the agar slab PSM was capped with the lid of a 55-mm-diameter petri dish to maintain the system as aseptic while allowing visual inspection of the agar surface. A picture of the full system is presented as supporting information (see Fig. S1 in the supplemental material).

Incubation and swarming observations. Incubation was carried out at room temperature. During the first 15 h, the agar slabs were always incubated at −0.7 kPa, and then the matric potential was modified and the slabs were checked for the presence of swarms. The occurrence of swarming was either assessed visually and documented with a digital camera or, for DSS73 and its derivative, followed using a Leica MZ16 FA epifluorescent stereomicroscope equipped for green fluorescent protein (GFP) detection and fitted with a charge-coupled device (CCD) camera.

Experimental design and data analysis. For DSS73 strains, successive images of a colony (or swarm) were measured using Image Pro Plus (version 5.1) to estimate its radial expansion rate. From each image, the average colony radius was calculated by taking the square root of the value of the swarm surface area divided by π, even though swarms were not always strictly circular and often presented tendrils. The expansion rate of a colony at a given matric potential was estimated by linear regression on at least 4 successive data points. Each rate determination was performed on separate colonies on separate PSMs. No systematic replication was performed; instead, we covered a range of matric potentials to be able to perform accurate regression analysis of the expansion rate against the matric potential. The regression analysis was carried out in Sigma Plot (version 11).

For B728a and PA14, no measurements were performed and our qualitative description of the swarm dynamics relied on a minimum of three replicate agar slab PSMs.

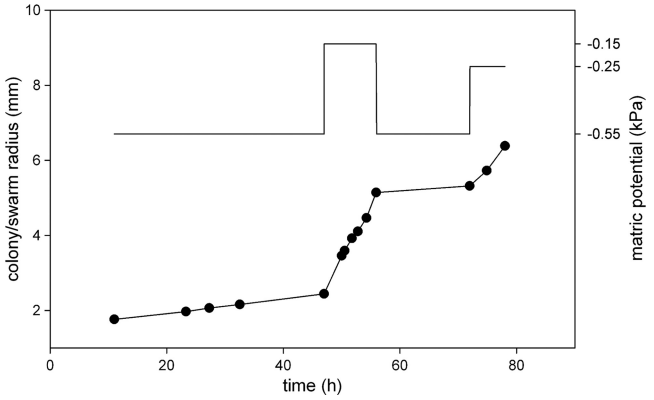

FIG 1 Spatial dynamic of a DSS73 colony (bottom curve, left axis) as affected by the matric potential imposed by the PSM (top curve, right axis). Note that the actual changes in matric potential experienced by the colony are more gradual than shown on the top graph because the equilibration between the agar slab and the PSM is not immediate.

RESULTS

Physical validation of the agar slab PSM system. We first explored the response of the agar slab to variations in matric potential in the absence of inoculated organisms by imposing cycles of matric potentials from −0.25 to −1.5 kPa. We observed changes in volume (not measured) and in weight (by about 9% between the two extremes) in the slab as water was drawn in or out when the matric potential was varied. Note that after a change of matric potential, the weight took several hours to stabilize, as expected for hydrogels submitted to drying or wetting (21). We thus concluded that the PSM was successful at imposing a prescribed matric potential to the agar slab.

Swarming of DSS73. For a given matric potential, the radii of DSS73 colonies increased quasilinearly with time (Fig. 1), a finding which allowed us to derive expansion rates by linear regression. The expansion rate of DSS73 colonies was affected by the matric potential (Fig. 1 and 2), while such an influence was absent for the DSS73 *amsY* mutant (Fig. 2, slope of linear regression not statistically different from 0 [$P = 0.954$]).

While DSS73 and the DSS73 *amsY* mutant presented similar expansion rates (in the 4- to 20-μm h^{-1} range) for matric potentials more negative than −0.5 kPa, under moister conditions, the wild type displayed faster expansion than the mutant and developed tendrils (Fig. 2). This indicated that the water potential threshold for the initiation of swarming for this strain was about −0.4 kPa. Above this threshold, the radial expansion rate of DSS73 swarms increased exponentially with the matric potential, as illustrated by the nonlinear regression presented in Fig. 2. The fitted model (radial expansion = 1,543 × exp[7.8 × matric potential], where "exp" indicates *e* raised to the power of the value in brackets and both parameters are significantly different from 0 with a *P* value of <0.0001) explained 85% of the variance of the data. The transition from slow colony expansion to swarming upon modification of the matric potential was relatively fast (typically less than 20 min) and fully reversible (Fig. 1).

To verify that our results were independent of the initial agar concentration of the agar slab, we measured the expansion rate of DSS73 at several matric potentials on slabs containing 9 or 20 g agar liter^{-1} instead of 15. As the matric potential thresholds for swarming initiation were similar irrespective of the initial agar

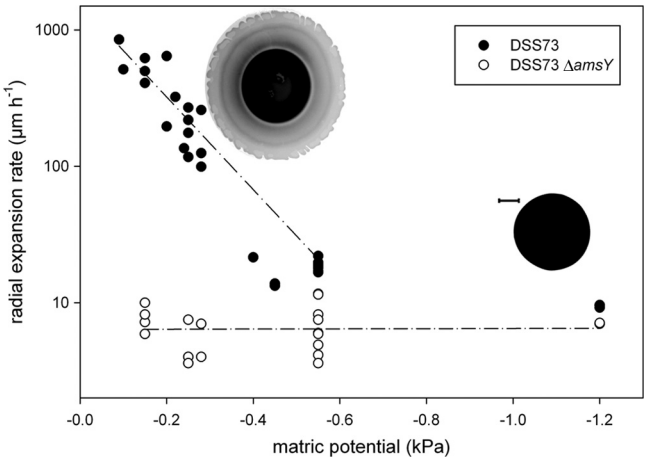

FIG 2 Radial expansion rates of DSS73 and DSS73 Δ*amsY*, an isogenic mutant that is unable to produce surfactant, as affected by the matric potential imposed via the PSM. The dashed lines illustrate a fitting of the data by regression (linear regression for the mutant; exponential regression for the wild type, after excluding data points outside the swarming range). Inset are representative inverted images of a typical colony (right) and swarm (top left), as observed with epifluorescence microscopy. Bar, 1 mm. The contrast of the images has been digitally altered to make the tendrils visible.

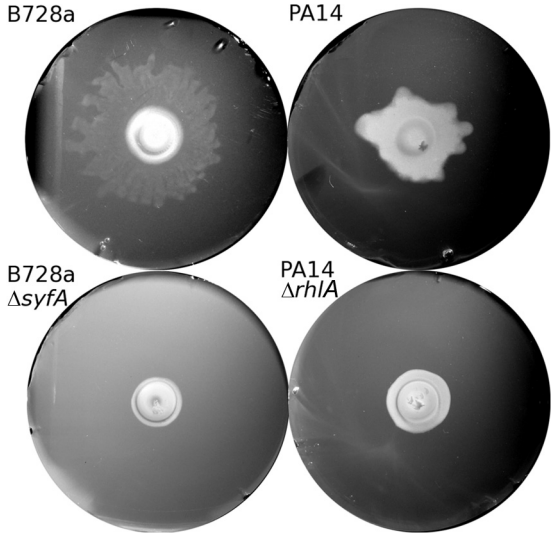

FIG 3 Representative photographs of colonies or swarms on agar slabs maintained at about −0.02 kPa after the initial 15 h of incubation at −0.7 kPa. The pictures on the left were acquired after 9 h of incubation, and those on the right were acquired after 23 h. The agar slabs are 40 mm in diameter. The contrast of the images has been improved digitally.

concentration (see Fig. S2 in the supplemental material), we conclude that the imposed matric potential is the main controlling factor in our experimental system. We note, however, that the slabs with 9 g agar liter^{-1} presented lower expansion rates (parameter a of the exponential regression $y = a \times e^{-b \times x}$ was estimated at 659 [standard deviation {SD}, 88], which is significantly different from that obtained for 15 g agar liter^{-1}, 1,543 [SD, 244]; $P < 0.05$). We hypothesize that this is due to the minor surface irregularities often visible on the fragile 9-g agar liter^{-1} slabs which can originate from their transfer onto the PSM system and/or from dehydration when subjected to the initial −0.7-kPa incubation.

Swarming of PA14 and B728a. PA14 and B728a swarmed only under extremely wet conditions, i.e., for matric potentials higher than −0.1 kPa (RH > 99.99992%). We determined the threshold for tendril formation for both strains at about −0.07 kPa, but it was with some uncertainty because our experimental system has a precision of ±0.02 kPa. The mutant strains did not present tendrils (Fig. 3). Recognizing the challenge associated with accurately prescribing matric potentials in this very high range, we did not attempt measurements of expansion rates for these strains. We did, however, confirm qualitatively that, as for DSS73, the expansion speed of the swarms increased with the matric potential.

DISCUSSION

Our agar slab PSM system allowed us to measure for the first time the wetness range that supports swarming on an agar surface. These ranges were not identical for the 3 strains, with DSS73 being able to swarm under slightly drier conditions than the two other strains. This difference is consistent with the behavior of the strains on standard swarming plates. Indeed, although PA14 and B728a typically favor "soft" swarming plates (4 to 6 g agar liter^{-1} [15, 29]), DSS73 is capable of rapid swarming on plates solidified with as much as 10 to 12 g agar liter^{-1}, as illustrated in Fig. S3 in the supplemental material. The superior swarming ability of DSS73 can probably be explained by the very strong surface tension reduction activity of amphisin, the cyclic lipopeptide it produces (20). Despite these interstrain differences, swarming was shown to require very moist conditions (i.e., very close to 100% RH) in all organisms considered here. Such extremely humid conditions have a limited occurrence, both spatially and temporally, in most nonaquatic bacterial habitats, such as the phyllosphere or the rhizosphere. At these high matric potentials, swimming motility is typically possible—and efficient—in soils (9, 33) and sand (7) matrixes, on the ceramic surface of the PSM (8), and presumably in grooves present on most plant leaves (1). This points to the fact that the agar surface presents physical characteristics that hinder microbial motion more than many environmental surfaces. We speculate that such characteristics may include viscosity or the absence of marked microtopography, which would limit the thickness of surficial liquid film at a given matric potential (22). It is possible that swarming has evolved to permit bacterial motion on surfaces that share these unfavorable characteristics. Such surfaces might be found in animal or vegetal tissues since swarming has been shown to be important for seed and straw colonization by DSS73 (19). Alternatively, one can hypothesize that swarming has mainly evolved to permit the spatial structuring of biofilms fully immersed in liquid. Swarming has indeed been demonstrated to be integral to the development of mature *P. aeruginosa* biofilms in flow cells (23, 27). Another open question is that of the mechanisms underlying the sensitivity of swarming to modest changes of humidity that were quantified here and highlighted previously (e.g., references 13 and 30). The rapidity and reversibility of the transition from swarming to nonswarming states upon variations in matric potential, as illustrated in Fig. 1, are reminiscent of those observed for the *Pseudomonas putida* KT2440 transition from swimming to nonswimming states (8). It is thus tempting to suggest that they similarly are merely manifestations of the physical pinning (or release) of the cells on (or from) the gel surface as a result of variations in liquid film thickness dictated by the prescribed matric potentials (8). However, we cannot rule out

that these transitions result from some active behavior and that the cells are able to sense minute variations in hydration conditions and adapt their transcriptional activity accordingly. Indeed, it has recently been reported that the swarm edge in *P. aeruginosa* presents much higher *rhlA* expression on soft agar (0.4%) than on slightly harder agar (0.6%) (13). This raises the question of the sensing mechanism that triggers this transition, especially considering that these variations in matric potential (less than 1 kPa) are minor compared to the total water potential experienced by the colony in our system, which is dominated by the osmotic potential of the culture medium (typically more negative than −300 kPa [24] and not affected by operating the PSM).

From a methodological point of view, the agar slab PSM system presents several advantages over the standard swarming plates. First, it allows the creation of constant, reproducible, and rigorously characterized moisture conditions which swarming plates fail to yield, jeopardizing the interlaboratory reproducibility of this standard assay (see, for example, the controversies in references 25 and 10). Particularly problematic is the drying of the swarming plates, either performed on purpose after plate pouring (30) or happening uncontrollably during subsequent incubation. Even the use of humidity-controlled chambers for incubation does not guarantee that the organisms are truly exposed to a known matric potential because the humidity of the air phase of the swarming plates will not equilibrate rapidly with that of the chamber. Such an absence of equilibrium must have happened in the work of Hamze and collaborators (10), who incubated their swarming plates (7 g agar liter^{-1}) in a chamber set at either 40 or 70% RH (−115 and −45 MPa, respectively), values unlikely to sustain swarming or even continued growth. The second benefit of the agar slab PSM is that it permits subjecting swarms to variations of ambient moisture conditions while maintaining online microscopic observability. This holds promise for identifying the genetic determinants and, possibly, the sensing machinery contributing to swarming by exploring the transcriptional dynamic as it is affected by hydration conditions by using *gfp* bioreporters, as in reference 13.

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