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Paenibacillus dendritiformis Bacterial Colony Growth Depends on Surfactant but Not on Bacterial Motion▽†

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Most research on growing bacterial colonies on agar plates has concerned the effect of genetic or morphotype variation. Some studies have indicated that there is a correlation between microscopic bacterial motion and macroscopic colonial expansion, especially for swarming strains, but no measurements have been obtained for a single strain to relate the microscopic scale to the macroscopic scale. We examined here a single strain (*Paenibacillus dendritiformis* type *T*; tip splitting) to determine both the macroscopic growth of colonies and the microscopic bacterial motion within the colonies. Our multiscale measurements for a variety of growth conditions revealed that motion on the microscopic scale and colonial growth are largely independent. Instead, the growth of the colony is strongly affected by the availability of a surfactant that reduces surface tension.

Bacteria are able to colonize many different surfaces through collective behavior such as swarming and biofilm formation. Studies of such behavior (10, 18, 26, 31) have revealed cooperative phenomena on both microscopic and colonial scales (4, 5, 7, 8, 20), including production of extracellular "lubricant-wetting" fluid for movement on medium and hard surfaces (19, 22, 25), chemical signaling such as quorum sensing and chemotactic signaling (1, 12, 27), and the secretion of inhibiting and killing factors (2, 9, 11, 14, 15, 17).

Research has suggested possible links between the microscopic behavior of a colony and the rate at which the colony expands (12, 23, 24, 29). For *Pseudomonas aeruginosa*, increased reversal rates for flagella lead to hyperswarming (a larger colony) (26). Similar flagellar modulation affects *Escherichia coli* (32); if the bacteria never tumble (flagella rotate only counterclockwise) or only tumble (flagella rotate only clockwise), the final colony is much smaller than a colony formed when the bacteria both swim and tumble. For *Rhizobium etli*, a correlation has been observed between microscopic swarming motion and expansion of the colony, and an acylhomoserine lactone molecule has been found to be a swarming regulator, as well as a biosurfactant that controls surface activity (12). These studies suggest that there is a correlation between microscopic activity and colonial expansion; however, a mutation may be pleiotropic, affecting both motility and surfactant production. Further, there may be additional, unidentified differences between mutant and wild-type strains. For example, the failure of *Bacillus subtilis* laboratory strains to swarm is caused by a mutation in a gene (*sfp*) needed for surfactin synthesis and a mutation(s) in an additional unknown gene(s) (21). Experiments that avoid this ambiguity by studying the response of a single strain exposed to changing physical environments have not been performed. Further, except for measurements of the size of an expanding colony as a function of time (3, 6), no detailed time development studies of a growing bacterial colony have been reported.

Here we exposed a single bacterial strain, *Paenibacillus dendritiformis* type *T* (tip splitting) (4), to different substrate hardnesses, nutrition levels, and surfactant concentrations to identify the parameters that determine colonial growth. *P. dendritiformis* is a gram-positive rod-shaped (4 μm by 1 μm) bacterium that swims on top of an agar gel in a thin layer (a few micrometers thick) of fluid, presumably secreted by the bacterial cells. The bacteria develop complex colonial (bush-like) branching patterns that are sensitive to small changes in the environment when the bacteria are grown on nutrient-limited surfaces (low peptone levels [approximately 1 g/liter]) (6). The colonies grow slowly (0.1 mm/h) so the microscopic motion can be followed with a microscope for about 10 min without moving the field of view. Also, this strain shows swarming-like microscopic motion where the bacteria move collectively in whirls and jets. This makes this bacterium well suited for studying simultaneously the development of a colony and the internal structure of branches. We constructed a novel setup to observe 10 growing *P. dendritiformis* colonies in each experiment, and complementary microscopic measurements were obtained for the velocity field of individual bacteria or small groups of cells within the colonies. Specifically, we measured the "bacterial speed," which was the average of the values for the velocity vectors for the bacteria in a region near the edge of a growing colony, and the "tip velocity," which was the speed of the moving growth front at the edge of a colony. We also quantified the collective bacterial motion within the colonies by computing spatial and temporal velocity autocorrelation functions.

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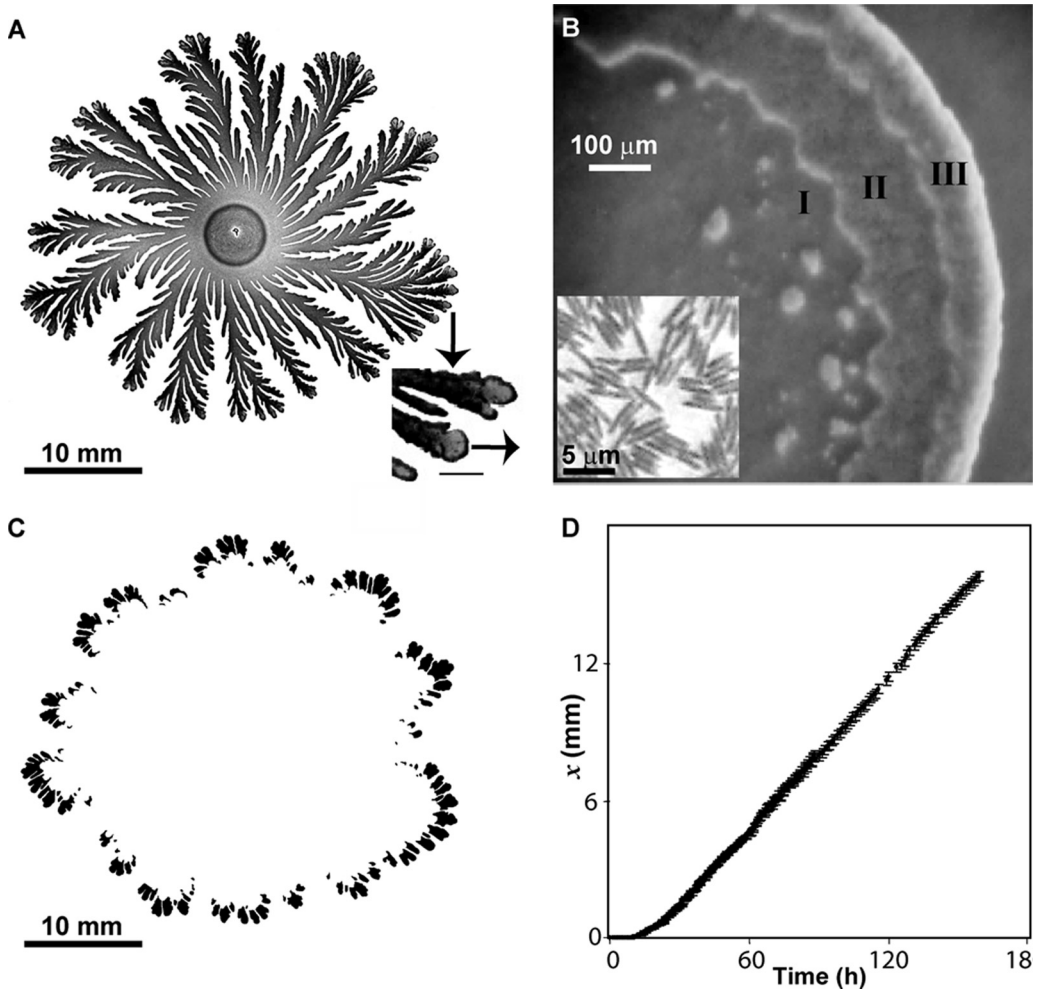

FIG. 1. *P. dendritiformis* bacterial colony grown on a 1.5% (wt/vol) agar gel with 2 g/liter peptone. (A) Colony 142 h after inoculation. The inset shows a close-up of the small region of the colony indicated by an arrow (bar = 1 mm). (B) Magnification of the region indicated by the arrow in panel A, showing three well-defined regions: region III, where the bacteria at the outer edge of the colony are in three layers and are very active; region II, where the bacteria are in two layers and are less active; and region I, where the bacteria are in a single layer and show little or no movement. The inset shows a higher magnification of region I, where individual bacteria can be resolved. (C) Colony growth occurs only near the tips (the colony edge), as determined by subtracting the image obtained at 130 h from the image obtained at 142 h. (D) The growth velocity (0.11 mm/h or 0.03 μm/s) is indicated by the slope of a plot of the position of the farthest edge of the colony (x) as a function of time. The error bars indicate the standard deviations for measurements for 10 colonies.

MATERIALS AND METHODS

Strain and growth media. *P. dendritiformis* type *T* (tip splitting) bacteria (2) were stored at −80°C in Luria broth (LB) (Sigma) with 25% (wt/vol) glycerol. A frozen stock was used to inoculate LB. After growing for 24 h at 30°C with shaking, the culture reached an optical density at 650 nm of 1.0, corresponding to approximately 1×10^8 bacteria/ml, as measured by counting colonies inoculated onto LB plates after culture dilution.

The peptone nutrient medium contained NaCl (5 g/liter), K_2HPO_4 (5 g/liter), and Bacto peptone (0 to 40 g/liter). Difco agar (Becton Dickinson) was added at concentrations of 0.7 to 1.7% (wt/vol). In some experiments a nonionic commercial surfactant, Brij 35 (Sigma), was added at various concentrations (0 to 0.0006%, wt/vol) to the agar medium prior to autoclaving. Twelve milliliters of dissolved agar was poured into each 8.8-cm-diameter petri dish, which was dried for 4 days at 25°C and 50% humidity until the weight of the plate decreased by 1 g to a final weight of about 25 g. This protocol ensured reproducible results.

Growth pattern experiments. The agar plates were inoculated by placing 5-μl droplets of the culture on the surface. The plates were mounted on a rotating stage inside a 1-m^3 chamber maintained at 30.0 ± 0.5°C and 90% ± 2% humidity (2). The rotating stage system allowed monitoring of the growth on 10 plates simultaneously. The stage was controlled by a stepper motor that stopped sequentially for each bacterial colony imaged. A rotation period of 1 h was short enough to capture the growth of the colony, the tips of which typically moved 0.5 mm/h (~0.1 μm/s). The reproducibility of positioning of the agar plates was ±15 μm; because of this excellent alignment, the (pixel by pixel) difference between two successive images yielded the growth in the colonial pattern during the time interval between the two images.

Images were obtained with a 10-megapixel Nikon D200 camera with a 60-mm lens, as described previously (2). For plates imaged only at a single time point, colonies were stained with 0.1% Coomassie brilliant blue to obtain images with higher contrast than images obtained using scattered light. The 20-ml stain solution consisted of 50% methanol, 40% water, 10% acetic acid (17.4 M), and 1 g/liter Coomassie brilliant blue and was poured onto each plate. This stain killed the bacteria but left the colonies blue on a pale agar background. A similar solution that lacked Coomassie brilliant blue was then used to destain the agar.

Microscopic measurements. An optical microscope (Olympus IX50) equipped with an LD 60× phase-contrast (PH2) objective lens was used to follow the microscopic motion. The microscope was placed in a temperature- and humidity-controlled environment. A digital camera captured the microscopic motion at a rate of 30 frames per s and a spatial resolution of 480 × 640 pixels. Images were taken for 30-s periods, which resulted in 900 images in a sequence.

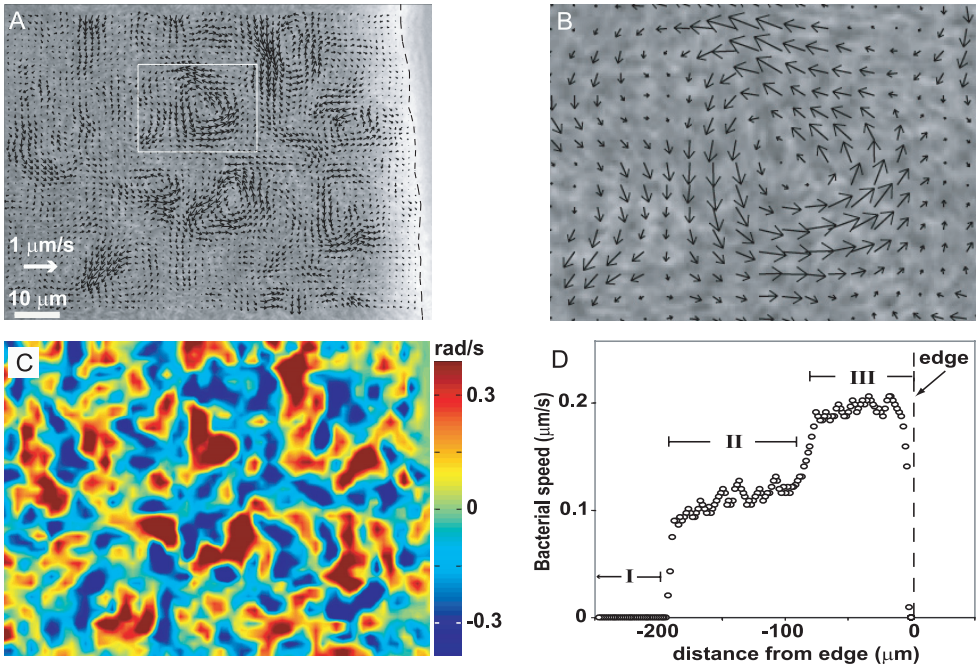

FIG. 2. Microscopic measurements of the speed of a *P. dendritiformis* bacterial colony grown on a 1.5% (wt/vol) agar gel with 2 g/liter peptone. (A) Phase-contrast microscopic image of region III near the colony's edge, which is indicated by the dashed line. Arrows indicate the local displacement for a time interval of 0.133 s. Note the vortices and the jets between them. (B) Enlarged image of the vortex in the box in panel A. (C) Vorticity field corresponding to the velocity field shown in panel A. Deep red (counterclockwise rotation) and blue (clockwise rotation) regions correspond to intense vortices. (D) Average bacterial speed as a function of distance from the colony edge, which is at position 0. Bacteria in each region have a distinct fairly uniform mean speed. The mean speed is calculated by averaging the speed versus distance along many lines parallel to the horizontal direction in panel A for about 1,000 frames for five different colonies. The standard deviation of five experiments is too small to be shown.

In order to quantify the bacterial motion at the microscopic level, we used particle image velocimetry (13, 16, 28, 30), which uses cross correlations over small interrogation regions between a pair of consecutive images to find particle displacements over known time intervals to determine two-dimensional local velocities [$\vec{V}(x,y)$]. The resulting velocities were interpolated to a uniform regular grid by cubic spline interpolation. The velocity data were used to compute vorticity (2 × local rotation rate), which is defined as the curl of the velocity field [$\vec{\omega}(x,y) = \nabla \times \vec{V}(x,y)$]. Velocity (and vorticity) fields were obtained on a 48-by-64 grid with a spatial resolution of 1.6 μm with 5% (and 10%) root mean square errors.

We calculated the spatiotemporal velocity correlation function for the microscopic velocity components V_x and V_y, which are components in the direction of colony expansion and perpendicular to the direction of expansion, respectively. The correlation function has the form,

$$C_i(\Delta x_0,\Delta y_0,\Delta t_0) = \frac{\langle v_i(x_0,y_0,t_0)v_i(x_0+\Delta x_0,y_0+\Delta y_0,t_0+\Delta t_0)\rangle}{\langle V_i(x_0,y_0,t_0)V_i(x_0,y_0,t_0)\rangle}$$

where $<\ldots>$ is average over both space (x_0, y_0) and time (t_0) and i is x or y. The spatial averaging is done for the entire region near (within ~100 μm) the edge of a growing tip. We call the velocity autocorrelation function $C_i(\Delta x_0, \Delta y_0, \Delta t_0)$ the "whirl correlation" to distinguish it from the relationship between the microscopic and macroscopic motions, which are referred to as "correlation."

RESULTS

We examined the effect of nutrient level and substrate rigidity on the growth of *P. dendritiformis* colonies and on the motion of the bacteria within the colonies.

Growth with an intermediate nutrient level (2 g/liter peptone) on a hard gel (1.5% [wt/vol] agar). During the first 18 h, the bacteria grew only inside the small inoculation circle. Then the colony started to expand, and a branched pattern developed (Fig. 1A). The velocity of the growing envelope (defined as a circle that just touched the fastest growing tips) was constant, as shown in Fig. 1D (2). By subtracting consecutive movie frames, we found that the growth of a colony is limited to an active zone at the tips of the branches; that is, branches grow forward and not to the side (Fig. 1C). Thus, a branch's width is already determined as it starts to grow. Regions only 300 μm back from the growing tips stop growing, even though there may be ample space available for growth on the side of a growing tip.

A close look near a colony's growing tip revealed that bacteria were in three well-defined regions (Fig. 1B). Region III was very close to the growing front and contained bacteria in three (and sometimes more) layers (z axis). The width of region III was typically 100 μm, and this width remained constant as a colony grew from less than 10 mm to more than 70 mm in diameter; this suggests that the number of active bacteria per unit area is fairly constant as a colony grows. Bacteria in region III were active, reproducing and moving with a collective swarming-like motion in whirls and jets, as shown in Movie S1 in the supplemental material. Snapshots of the velocity and vorticity fields are shown in Fig. 2A and 2C, respectively. The whirls and jets were ~10 μm long and lasted a few seconds.

Region II (width, about 200 μm) was slightly more interior and was composed of two layers of bacteria; these bacteria also moved in whirls and jets, but the speeds of the individual bacteria were about one-half those in region III (Fig. 2D). The

swimming speed of *P. dendritiformis* bacteria in rich liquid media is typically 2 μm/s, which is slow compared to the swimming speeds of other bacteria.

Region I, typically 300 μm or farther from a colony's front, contained bacteria in a single layer (Fig. 1B, inset); these bacteria hardly moved at all (Fig. 2D). The average size of bacteria in region I was 4.5 ± 0.5 μm, while the bacteria in regions II and III ranged from 3 to 8 μm long. This suggests that reproduction is limited to the regions with multiple layers. Region I contained spores, and the concentration of spores relative to active bacteria increased with increasing distance from a colony's edge.

Effect of nutrient level on macroscopic and microscopic motion. To explore environmental effects on correlations between microscopic motion and macroscopic motion, colonies were grown with various nutrient levels, keeping the hardness of the substrate constant (with 1.5% [wt/vol] agar). In each case the velocity of the growing envelope was observed to be constant as a function of time. The three distinct regions observed with 2 g/liter peptone (Fig. 1B) were also found with other nutrient levels. With a low initial peptone level, an increase in the peptone concentration resulted in an increase in the envelope velocity (Fig. 3A), indicating that the growth was food limited, but the tip velocity was maximum with 5 g/liter peptone, indicating that another factor limited the colony growth at levels higher than this level. However, the average speed of individual bacteria continued to increase with increasing peptone levels at concentrations greater than 5 g/liter, and with 12 g/liter the speed of the bacteria was three times the speed with 4 g/liter (Fig. 3B). Thus, in this range of concentrations the growth speed of a colony is clearly independent of the microscopic speed of the bacteria.

Similarly, for a softer gel (1.1% [wt/vol] agar), an increase in the peptone concentration from a small initial value resulted in an increase in the envelope velocity (Fig. 3A), indicating again that the growth is food limited. However, the tip velocity saturated in this case at a much higher peptone level (~20 g/liter). Interestingly, the increase in the average speed of the bacteria was saturated much sooner at a peptone level of 4 g/liter, while the tip grew faster with higher peptone levels. The striking contrast between the behavior with 1.1% (wt/vol) agar and the behavior with 1.5% (wt/vol) agar is summarized in the plot of tip velocity versus bacterial speed in Fig. 3B. Figure 3B shows dramatically that the colonial growth rate does not depend on the average speed of the individual bacteria. Rather, bacterial speed and the colonial growth rate behave like independent parameters, coupled at lower concentrations by the availability of food. Figure 3B shows in addition that there is a qualitative difference in the responses of the colonies to different agar concentrations. On soft agar, the average speed of the bacteria reaches a maximum, while hard agar limits the colonial growth.

Effect of the agar concentration on the macromotion and micromotion. To investigate the transition from conditions under which the maximal speed of the bacteria is limited to conditions under which the tip growth velocity is limited, we varied the agar concentration from 0.7% to 1.7% (wt/vol) while keeping the peptone level constant (for three different peptone levels, 1, 2, and 4 g/liter). Region III (where bacteria were in three layers) was larger for soft gels than for hard gels; for example, for a gel with 1.1% (wt/vol) agar, the width of region III was about 1 mm, compared to 0.1 mm for a gel with 1.5% (wt/vol) agar. With increasing agar concentrations, the tip velocity of colonies increased until it reached a maximum with an agar concentration of about 1.3% (wt/vol); with further increases in the agar concentration the tip velocity decreased rapidly (Fig. 4A). The average speed of individual bacteria showed a similar dependence on the agar concentration (Fig. 4B), but the maximum speed occurred at a lower agar concentration (about 1.0%, wt/vol) than the maximum tip velocity. The consequence of the different locations of the maxima as a function of agar concentration is illustrated by a graph of tip

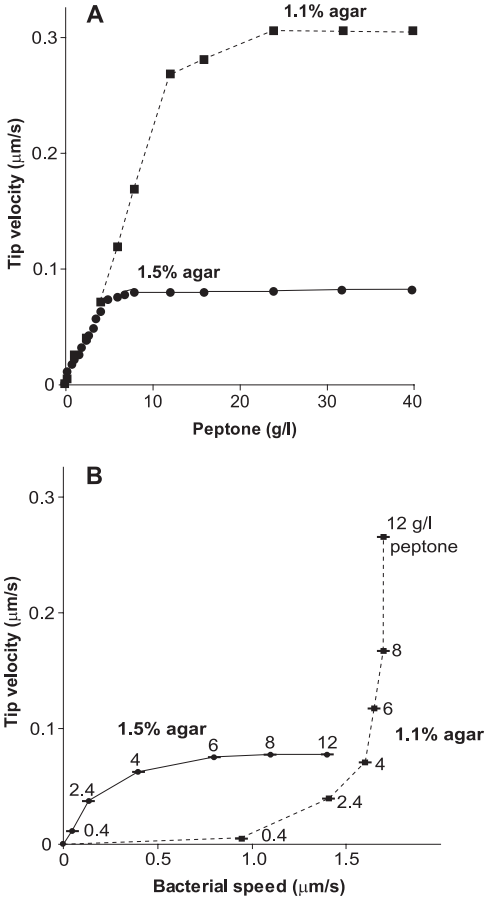

FIG. 3. Dependence of the colony growth rate on the nutrient concentration and on the average bacterial speed. (A) Velocity of the tip of a growing colony as a function of the peptone level for two agar concentrations. Each symbol indicates a constant velocity derived from the slope of the accumulated distance covered by the colony as a function of time, as shown in Fig. 1D. For both agar concentrations, increasing the peptone level initially increased the tip velocity. However, at some peptone level, the velocity value became saturated, indicating an additional bottleneck. The food-limited region extends farther for the lower agar concentration. (B) Tip velocity as a function of microscopic bacterial speed (in region III; see Fig. 1B) for different peptone levels (indicated for each point). For 1.5% (wt/vol) agar, there is a region where additional food significantly increases the microscopic bacterial speed (nutrient-limited regimen), but the tip velocity remains nearly the same. For 1.1% (wt/vol) agar, there is a region where additional food scarcely changes the microscopic bacterial speed (space-limited regimen), yet the tip velocity increases dramatically. The error bars, which in some cases are smaller than the symbols, indicate the standard deviations of three experiments.

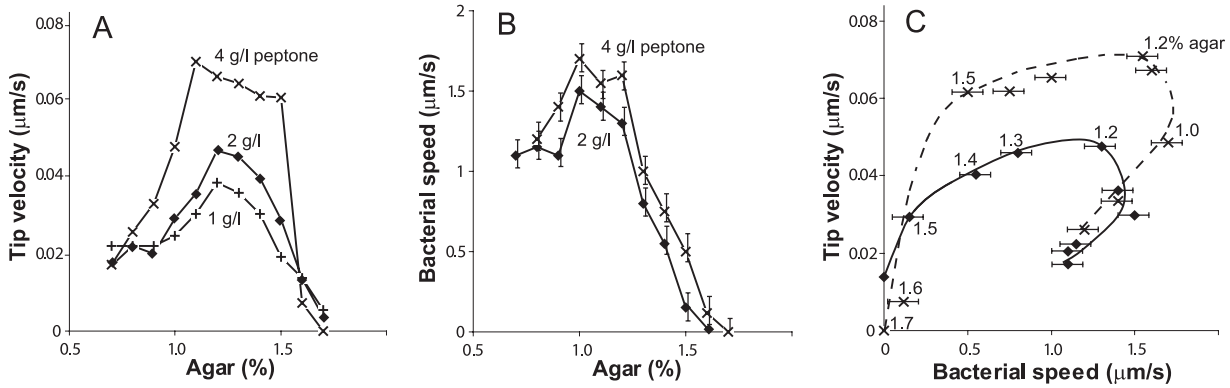

FIG. 4. Dependence of colony growth rate and bacterial speed on the agar concentration. (A) Tip velocity as a function of the agar concentration for 1, 2, and 4 g/liter peptone. Each value was determined from the slope of plots of x versus t, as shown in Fig. 1D. Colonies grow faster with agar concentrations around 1.3% (wt/vol) and more slowly on hard (>1.5% agar) or soft (<0.9%) gels. (B) Microscopic bacterial speed as a function of agar concentration for 2 and 4 g/liter peptone. The maximum mean speed of the bacteria is approximately 20 times the colony growth velocity. The bacterial speed is highest for agar concentrations around 1.1% (wt/vol) and rapidly drops to zero at concentrations near 1.6% (wt/vol). (C) Tip velocity as a function of microscopic bacterial speed for the agar concentrations indicated (in steps of 0.1% [wt/vol]) for 2 g/liter peptone (solid line) and 4 g/liter peptone (dashed line). In each graph the error bars (in some cases smaller than the symbol) indicate one standard deviation for three experiments.

velocity as a function of bacterial speed (Fig. 4C). These two parameters both respond to the changes in agar hardness but in slightly different ways, indicating again that the average bacterial speed does not drive colonial growth.

Whirls, jets, and collective micromotion. Since the tip motion seems not to be determined by the average speed of the bacteria, we investigated whether this motion depends instead on the collective motion of bacteria (i.e., whirls and jets [Fig. 2C]). The spatiotemporal whirl correlation was determined for the active region (region III), as described in Materials and Methods. The correlation functions for both V_x and V_y gave similar results; the function for V_x is shown in Fig. 5. A cut made through the center of the plot is shown in Fig. 5B; the correlation length was defined to be the width of the peak at a correlation of 0.5. The whirl correlation length increased approximately linearly with bacterial speed (Fig. 6A), but the relationship between the tip velocity and the whirl correlation length (Fig. 6B) was similar to the nonmonotonic relationship found between tip velocity and bacterial speed (Fig. 4C). Similar results were obtained if the whirl correlation length was defined as the distance between the antinodes. Thus, we concluded that the tip growth is also not driven by the collective motion of bacteria in whirls and jets.

Effect of surfactant on the motion. Another possible mechanism that could drive tip growth is a reduction in the surface tension caused by a surfactant, a mechanism that is independent of the microscopic motion of the bacteria and requires only the production of a surfactant. We examined this by adding a nonionic surfactant, Brij 35, when we prepared the gel. We found that the tip velocity increased by a factor of 3.5 when the surfactant concentration was increased from 0% to 0.0006% (wt/vol) (final concentrations), while the average speed of the bacteria remained essentially constant (Fig. 7). Further, the added surfactant did not affect the whirl correlation length. Brij added at various concentrations (0 to 0.0006%, wt/vol) to bacterial cultures grown in both poor and rich (LB) shaken liquid media did not affect the bacterial

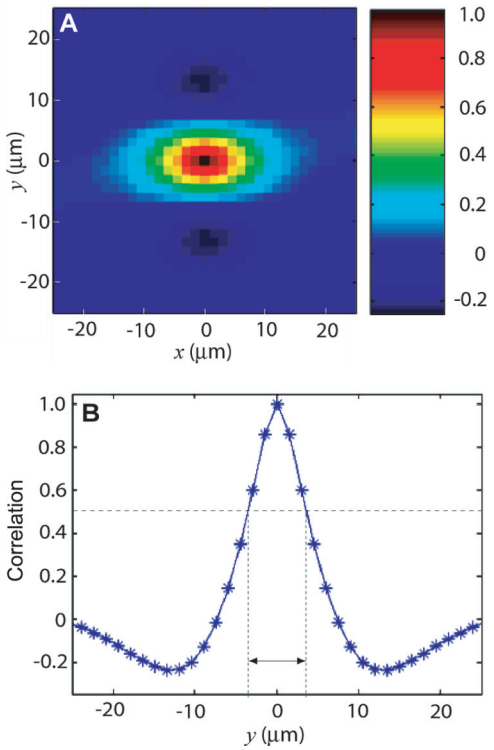

FIG. 5. Whirl correlation measurements for region III for a 1.5% (wt/vol) agar gel containing 2 g/liter peptone. (A) Two-dimensional correlation map of V_x (the velocity component along the propagation direction of the colony tip). The color bar indicates strong correlation (dark red) to anticorrelation (dark blue). Note the two nodes of anticorrelation above and below the center, which indicate the presence of vortices. (B) Vertical slice through the center of panel A. The dashed horizontal line crosses the curve at a correlation of 0.5; the segment on the horizontal axis (y) indicated by the arrow determines the whirl correlation length, about 7 μm in this case, as also shown in Fig. 6A.

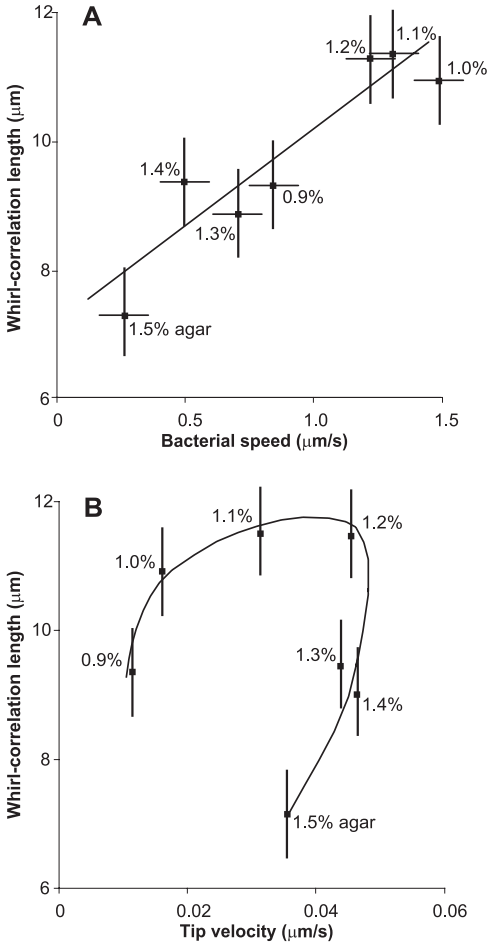

FIG. 6. Whirl correlation length measurements for bacteria grown with various agar concentrations and with 2 g/liter peptone. (A) The whirl correlation length increases monotonically with the microscopic bacterial speed. (B) Whirl correlation length variation with the tip velocity. The curve is similar to the curve in Fig. 4C.

growth. Together, these results suggest strongly that tip growth and thus colonial growth in *P. dendritiformis* type *T* (tip splitting) is controlled mainly by the production of surfactant molecules.

DISCUSSION

Rather than examining different bacterial strains, we studied a single strain that was exposed to various nutrient levels, agar hardnesses, and surfactant concentrations. The use of the same strain of bacteria eliminated possible effects of mutants other than the effects intended, so an observed correlation would not necessarily be a response to the intended change. Our experiments demonstrated that this was the case for the correlation between microscopic bacterial motion and macroscopic colony growth. Both of these parameters increase with the nutrient concentration at lower concentrations, suggesting that the microscopic motion drives the growth of the colony. Microscopic inspection of the growth front supports this idea as bacteria in the growth region show collective motion, forming whirls and jets near the growing tip of a colony. However, the speed of the bacteria and the speed of the colony growth level off at different nutrient concentrations, and therefore, the microscopic motion of the bacteria cannot be the origin of the colony growth. Experiments using different agar concentrations illustrated two extreme situations. For hard agar and high nutrient concentrations, bacterial speed increases with the nutrient concentration, while the tip velocity remains essentially the same. In contrast, for soft agar and high nutrient concentrations, the bacteria exhibit maximal speed, while the colony grows faster when the nutrient concentration increases. It is still possible that the collective motion of the bacteria instead of their average speed determines the colony growth. Pushing the interface forward may require the concerted effort of a group of bacteria, such as the observed jets and whirls. However, we have found that characteristic parameters of collective motion, such as the whirl correlation length or time, level off at different nutrient concentrations and, therefore, cannot be the driv-

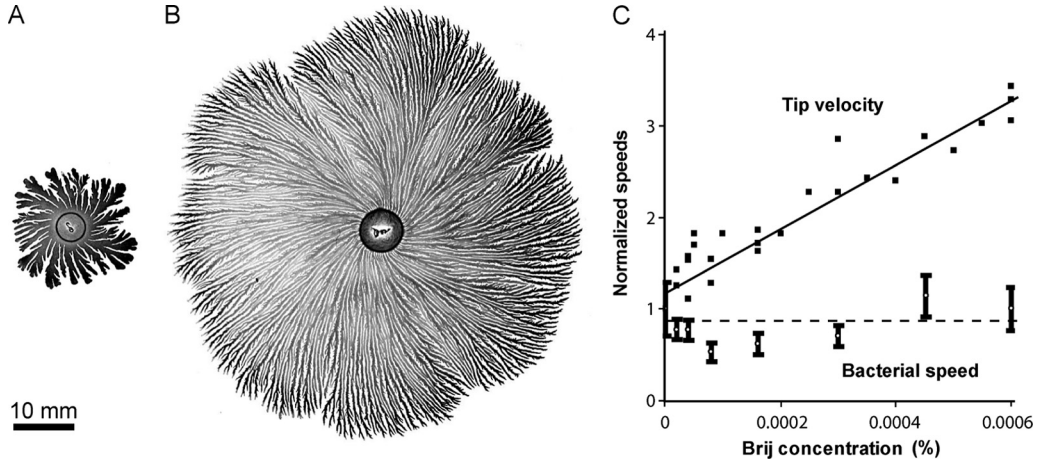

FIG. 7. Colony growth rate and bacterial speed dependence on surfactant concentration. Colonies grown for 80 h (A) without added surfactant and (B) with surfactant (0.0006% [wt/vol] Brij) added to the growth medium (1.5% [wt/vol] agar gels with 2 g/liter peptone). (C) Tip velocity and microscopic bacterial speed (each relative to the values with no added surfactant) as a function of surfactant concentration. Surfactant strongly affects the tip velocity but has little effect on the microscopic bacterial speed. The error bars indicate the differences between two experiments.

ing force for colonial growth. Thus, we concluded for *P. dendritiformis* type *T* (tip splitting) that the speed and pattern of bacterial motion and the overall colonial growth velocity are largely independent parameters. At a low nutrient concentration the bacterial speed and the colonial growth velocity both increase with the nutrient concentration, but at high nutrient concentrations the bacterial speed and colonial growth velocity are limited by different factors, which depend on the agar concentration.

If the bacterial motion is not the driving force for colonial growth, then what else determines it? The sharp interface is a result of the surface tension of the medium, which can be pushed forward either by pressure generated within the colony or by a reduction in the surface tension. Our last experiment suggests strongly that surfactant production determines colonial growth in *P. dendritiformis* type *T* (tip splitting), as increasing the surfactant concentration increased the colony growth speed but had little effect on bacterial motion.

In summary, our observations indicate that increased microscopic bacterial motion (i.e., increased average speed and increased collective motion in whirls and jets) does not in general lead to an increase in the growth rate of a bacterial colony, at least for *P. dendritiformis*. It is possible that our findings can also be applied to other bacteria that grow on surfaces; however, the generality of our finding remains to be determined since the relationship between microscopic motion and colonial growth has not been systematically studied for a single strain of any other bacteria.

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