Branched swarming patterns on a synthetic medium formed by wild-type *Bacillus subtilis* strain 3610: detection of different cellular morphologies and constellations of cells as the complex architecture develops

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After optimizing the conditions, including nutrients and temperature, swarming of *Bacillus subtilis* 3610 was obtained on a synthetic, fully defined medium. The swarms formed highly branched (dendritic) patterns, generated by successive waves of moving cells. A detailed microscopic *in situ* analysis of swarms 1 and 2 revealed varied cell morphologies and a remarkable series of events, with cells assembling into different 'structures', as the architecture of the swarm developed. Long filamentous cells begin to form before the onset of the first swarming (11 h) and are again observed at later stages in the interior of individual mature dendrites. Swarm 2, detected at 18–22 h, is accompanied by the rapid movement of a wave of dispersed (non-filamentous) cells. Subsequently at the forward edge of this swarm, individual cells begin to cluster together, gradually forming *de novo* the shape of a dendrite tip with progressive lengthening of this new structure ‘backwards’ towards the swarm centre. In both swarms 1 and 2, after the initial clustering of cells, there is the progressive appearance of a spreading monolayer of rafts (4–5 non-filamented cells, neatly aligned). The alternative possible roles of the rafts in the development of the swarm are discussed.

INTRODUCTION

*Bacillus subtilis*, inoculated from a central point, is able rapidly to colonize large areas of nutrient agar (0·6–1·5 %) by a coordinated, symmetrical migration or swarming over the surface (Ben-Jacob et al., 1994; Matsushita, 1997; Mendelson & Salhi, 1996; see also Shapiro, 1995). Swarming may take several forms, including the production of highly branched, fractal patterns (Matsuyama et al., 1989; Matsuyama & Matsushita, 1992; Vicsek, 1992). Such patterns also occur in the branching architecture of the lung and in structures formed by non-living systems, for example snow crystals. Swarming in *B. subtilis* is reported to require the presence of flagella (Ohgiwari et al., 1992), although flagella-independent swarming has also been described recently (Kinsinger et al., 2003). An antibiotic surfactant, whose synthesis is subject to control by quorum sensing (Solomon et al., 1995; Solomon & Grossman, 1996), has also been implicated in swarming in this organism (Kearns & Losick, 2003; Bees et al., 2000; see also Wakita et al., 1998). However, opposing results have been obtained concerning the importance of surfactin in swarming (Mendelson & Salhi, 1996; Dixit et al., 2002). In fact, the mechanism of all aspects of swarming, including the production and nature of presumed 'swarmer' cells, as found in various Gram-negative bacteria, remains poorly understood in *B. subtilis*.

In order to analyse the effect of swarming on the interplay between nutrient concentration and percentage of agar, most previous studies have used complex medium with laboratory strains of *B. subtilis*, in particular strain 168, grown on peptone (Ben-Jacob et al., 1994; Matsushita, 1997), beef extract/tryptose (Mendelson & Salhi, 1996) and LB (Dixit et al., 2002). These studies revealed different swarming patterns, from amorphous, largely confluent growth zones, through relatively simple radial branching, to highly complex, densely branched structures. Patterns with pronounced chirality, or repeating concentric zones, were also observed. Whilst the concentration of nutrients influenced patterns, the agar concentration appeared most important in determining bacterial swarming per se (Mendelson & Salhi, 1996), leading to the conclusion that swarming requires the extraction of water from the agar or reduction of surface friction, processes in which surfactants presumably play a role (Matsuyama et al., 1989; Matsuyama & Matsushita, 2001; Bees et al., 2000).
Although there has been speculation on the possible role of different physical factors, nutrient gradients, quorum sensing or chemotactic signalling (Ben-Jacob et al., 1994; Matsushita, 1997; Mendelson & Salhi, 1996; Harsey, 1994; Belas, 1997; Rauprich et al., 1996; Bees et al., 2000) in the control of bacterial swarming, the precise mechanism(s) by which the bacteria sense a suitable surface to be colonized, and in a co-ordinated way then occupy that surface by seemingly different strategies, remains a mystery.

In this study, we sought to establish conditions that would facilitate the systematic analysis of the nature of the activity of B. subtilis that constitutes the swarming process through branching patterns. This involved the use of a non-laboratory strain, 3610 (a natural wild-type), grown under controlled conditions. As we report below, strain 3610 forms a wide range of swarming patterns under different conditions, including so-called fractal branching (Matsuyama et al., 1989). We have established optimal conditions for reproducible swarming patterns with radial branching, and for the first time on a completely synthetic medium. At least two waves of swarming were analysed microscopically and by time-lapse photography. Importantly, we used *in situ* phase-contrast microscopy, to document a number of surprising and novel features in the development of the swarm colony. These included long filaments and rafts of cells (near normal length) detected in different locations in the developing swarm colony, and following an abrupt outward migration of many cells, new swarm 2 dendrites assembled de novo from nascent tips, beyond the edge of swarm 1, which then elongated backwards *towards* the colony centre. In an analysis limited to growth on LB-agar, an independent study by Kearns & Losick (2003), carried out whilst this work was in progress, has recently described a form of swarming of strain 3610, in which hyperflagellated cells associated in rafts were detected in samples extracted from the swarming edge. Under these conditions no distinguishable architectural features, such as branching, were observable, and no *in situ* analysis was carried out.

**METHODS**

**Strains and media.** Strain 3610, a natural wild-type, was obtained from the Bacillus subtilis Genetic Stock Center. *B. subtilis* cultures were grown in LB (tryptose 10 g l\(^{-1}\), yeast extract 5 g l\(^{-1}\), NaCl 10 g l\(^{-1}\)), TY (tryptose 10 g l\(^{-1}\), yeast extract 5 g l\(^{-1}\), NaCl 5 g l\(^{-1}\)), Columbia (Difco), peptone (5 g l\(^{-1}\)) or the synthetic B-medium (Antelmann et al., 1997) which contains 15 mM (NH\(_4\))\(_2\)SO\(_4\), 8 mM MgSO\(_4\), 27 mM KCl, 7 mM sodium citrate, 50 mM Tris/\(\text{HCl}\) (pH 7.5) supplemented with 0.6 mM KH\(_2\)PO\(_4\), 2 mM CaCl\(_2\), 1 mM FeSO\(_4\), 10 mM MnSO\(_4\), 4.5 mM glutamic acid, 780 mM tryptophan, 860 mM lysine and 0.2% (w/v) glucose. All plates were prepared by supplementing the medium with 0.7% or the required concentration of Bacto agar.

**Swarming experiments.** Ten millilitres of the medium to be used in a final swarm plate was inoculated with a single colony and shaken overnight at 37 °C. The culture was diluted to OD\(_{570}\) = 0.1 and grown until OD\(_{570}\) ~ 0.2. This procedure was repeated twice and finally the culture was grown to time \(t_0\) (4 h after exit from exponential phase), the OD\(_{570}\) was measured and the culture diluted to OD\(_{570}\) ~ 0.01. Two microlitres (~10\(^{6}\) c.f.u.) of this diluted culture were placed at the centre of an agar plate with the appropriate medium and incubated at 30 °C (relative humidity 40% saturation), or other temperatures as indicated in the text, for the requisite time. The inoculum formed a spot, diameter 4 mm. Plates (10 cm diameter) containing 25 ml agar medium were prepared 1 h before final inoculation and dried open for 15 min in a laminar-flow chamber. In some experiments, 15 cm diameter plates with 75 ml agar medium, prepared similarly, were also used. For measuring swarming rates, the diameter of the swarm on duplicate plates was measured each hour after swarming commenced. Measurements from replicate plates were essentially identical.

**Microscope observations and time-lapse video.** The photographs of the developing swarm were taken *in situ* with an Axiosplan (Zeiss) microscope equipped with a camera (Princeton Instruments). Bacteria were observed on the plates using Neofluar Zeiss objectives \(\times\) 1-25, \(\times\) 5, \(\times\) 20 and \(\times\) 40. Images were captured (by computer) using the IP Lab Spectrum (Princeton Instruments) software version 4.2, exported as TIFF files and adjusted with Adobe Photoshop software. Plates were removed from the incubator at intervals and examined for a minimal time to allow any delay in further swarming; alternatively several replicate plates inoculated at the same time were employed, discarding the plate after microscope examination. Replicate swarm plates were highly reproducible in both the timing and pattern of the swarming colony.

Time-lapse video films of swarming colonies were produced using a Proscope digital camera with a Computar zoom TV 8-5 mm lens. The camera was positioned above the Petri dish and the light source below it in a constant-temperature environment. Images were recorded with USB Shot software and adjusted with the Adobe Photoshop program.

**RESULTS**

**The natural isolate, strain 3610, swarms on all media tested, with a wide variety of swarming patterns**

Strain 3610 was found to swarm on all the media tested, but as shown in Fig. 1, the swarming patterns varied markedly. Thus, different branching forms were produced with, in some cases, evidence of repeated concentric zones. Swarming was however relatively slow on most media, as shown in Fig. 1(A–C), where 6–10 days was required to observe the full swarming pattern. Notably, however, in complete contrast, strain 3610 swarmed rapidly and extensively on the synthetic B-medium (0.7% agar in this case), forming thick central branches, which merged into highly complex, intricately branched structures usually extending to the edge of the plate in 24 h. It may be noted that, when such a swarm is observed on X-Gal plates, the entire swarm remains completely white. This indicates the presence of only the Lac\(^-\) 3610 strain and therefore no Lac\(^+\) bacteria, typical of Type 1 bacilli, noted previously as swarming ‘contaminants’ of laboratory strains of *B. subtilis* under some conditions (Rudner et al., 1998). Especially rapid swarming, reaching the edge of the plate in 8 h, was observed on LB (yeast extract) medium, accompanied by distinct concentric zones visible in most experiments (Fig. 1D). Branching was much less evident or even absent
on LB, as reported recently by Kearns & Losick (2003), when compared with B-medium.

**Effect of the agar concentration, temperature and inoculum size on 3610 swarming on B-medium**

In order to better characterize the conditions for swarming in strain 3610, several factors were examined. The results showed, first, that the extent of swarming and, to a degree, the swarming pattern (intensity of branching) were dependent on the agar concentration, with swarming usually observed at up to 1%, but abolished at 1-25% (data not shown). For optimal results, 0-7% agar was adopted as the standard for most subsequent experiments.

The effect of the size of the inoculum was investigated over the range of 50 to $5 \times 10^4$ c.f.u. Swarm colonies were obtained in all cases, with the onset of swarming from the central inoculation point clearly later when fewer cells were used. For example, with an inoculum of 50 c.f.u., the delay was close to 24 h (data not shown). Under the standard inoculum conditions finally adopted ($10^4$ c.f.u.), swarming was first visible (at 30°C) after 11–12 h, that is, approximately 7 generations, based on a doubling time of 100 min in cultures grown in liquid B-medium.

Finally, the effect of growth temperature (on B-medium) on the rate of swarming was examined over the range 22–42°C. Periodic rounds of swarming appeared to occur under all conditions, although this was less obvious at high temperatures. This may be due to the constraints of the 9 cm plate when swarming is accelerated, and the masking or blurring of the transitions from one swarm to another by the more rapid growth of the bacteria at the higher temperatures. Temperature also had some effect on the pattern of branching, with particularly dense and intricate branching at 37 and 42°C, and a central zone of thicker branches increasingly obvious at lower temperatures (see photographs in Fig. 2, and also the higher magnification, Fig. 1E). The expansion of the colony appeared to increase linearly over most of the time-course at all temperatures.

The rate of swarming was then calculated for each temperature and the overall effect of temperature on the rate of swarming determined. As shown in Fig. 2 (upper panel), the rate of expansion of the colony increased with temperature. However, between 30 and 34°C there is a break in the curve, which may indicate an abrupt change in the physiology of the cells or the physical properties of the agar surface in this temperature range.
The swarming pattern is not dependent upon the presence of glucose or amino acids

The standard B-medium contains glucose as the carbon source and is also supplemented by three amino acids: lysine, glutamate and tryptophan. First we compared swarming (on 0-7% agar) with glucose or glycerol as carbon source. As expected with the cells growing more slowly on glycerol, swarming was slower but the final results were essentially the same (data not shown). This is in contrast to swarming in *E. coli* (Harshey & Matsuyama, 1994) or *Pseudomonas aeruginosa* (Kohler et al., 2000), which is abolished by growth on glycerol. In the presence or absence of tryptophan or lysine, the swarming rate and patterns obtained were indistinguishable. In the absence of glutamate, which resulted in an increase of the doubling time of cultures in liquid medium (120 min compared with 100 min at 30°C), the rate of swarming appeared to be reduced. Moreover, supplementation of the basic B-medium simply by glutamate was sufficient to restore the growth rate and the rate of swarming to that obtained with all three amino acids present (all data not shown). Therefore, under these conditions, a supply of amino acids in the medium does not appear to be specifically required for swarming. We also noted that *B. subtilis* grows in the full B-medium without glucose, presumably using the amino acids as carbon source (generation time 285 min at 30°C in liquid medium). In the absence of any added sugar (data not shown), the same swarming pattern was still observed, although developing much more slowly.

The swarming zone on B-medium is preceded by a bacteria-free area bounded by a narrow ‘ring’

Swarming colonies were examined with the Proscope digital camera or by phase-contrast microscopy. As shown in Fig. 3, when colonies are photographed in reflected light,
it is possible to see that the swarm zone is always preceded by a transparent zone devoid of bacteria and delimited several millimetres from the swarm by a narrow ring, approximately 1 mm wide (see also Wakita et al., 1998). We suggest that this zone, and the ring surrounding the swarm, may contain the wetting agent surfactin (a lipopeptide: Nakano et al., 1992), secreted by the bacteria to aid in some way their translocation across the surface. Our view that this zone contains surfactin is based on our analysis of several other B. subtilis strains and their ability to swarm on LB-agar, where surfactin is not essential for swarming. This analysis demonstrated a strict correlation between the presence of surfactin and the ability to produce the ring preceding the swarm. Moreover a drop of surfactin (Sigma) placed at the centre of an agar plate produces an identical spreading ring in the absence of bacteria (unpublished data).

Swarming involves distinct waves and the formation of concentric zones

Using time-lapse photography (see Methods), we next examined in more detail the development of a swarm colony of B. subtilis 3610 over 36 h on 0·7% B-agar inoculated with 5 × 10^4 c.f.u. Several features may be noted. Swarming expansion from the central spot was first observed after 11 h. The swarming process in this experiment is characterized by a first phase of 11–18 h (illustrated at 18 h in Fig. 3A) in which the colony expands through elongation and subsequent growth (consolidation) of deeply but relatively sparsely branched dendrites (usually 25–30), emanating from the central point of inoculation. As shown in Fig. 4, two upper left panels (arrowed), the second wave of swarming was first observed at 21 h. This is accompanied by an overlapping multiplication of the cells and an increased frequency of branching. This wave then progressively expanded the colony to 7·5 cm diameter in a maximum of 2 h. A third swarm was detected at 26 h (arrowed in Fig. 4, bottom right panel), expanding the colony to 9 cm in the next hour. Between 32 and 33 h, a final, but limited swarm of 2·5 mm was observed, taking the final diameter of the colony to 9·5 cm (not shown). We have so far observed final colonies with at least four concentric zones, especially when large 15 cm plates were used, as employed in the experiment in Fig. 4.

As illustrated in Fig. 4 each wave of migration of the bacteria is accompanied by the ‘filling in’ and thickening of the dendrites by multiplication of the cells. The termination of at least swarm 1 under these conditions is sometimes accompanied by swelling of the ends of the dendrites to form floret-like structures (see Fig. 3A and Fig. 4 marked by the long arrow in the upper central panel). Finally, when the swarming process is completed, the colony can continue to ‘expand’ by apparently less organized growth and division of the cells in situ at the periphery of the colony. The rapid spreading of the bacteria over the agar through the specialized process of swarming is therefore not necessarily limited by the ability to grow but by some other factor. Thus, in some experiments even with 0·7% agar, swarming (but not growth) ceased before the edge of the plate was reached.

Successive swarming waves were not limited to the dendritic patterns observed on B-medium, but were also evident when swarming on LB was monitored, especially at intermediate times, 5–15 h (data not shown), but the trace of them was not always visible in the ‘mature’ swarm after 24 h.

Filamentous cells and many multicellular ‘rafts’ are present at different stages of the swarming process

Swarm 1. The leading region of a swarm colony on B-medium at 30 °C was examined at different times in situ, without disturbing the structure of the colony, by low-magnification microscopy and the Proscope digital camera (see Methods). First, we observed that within 3–4 generations (4–6 h) division is blocked in a proportion of the cells, with the consequent appearance of long filamentous cells already at 11–12 h, in particular detectable in the rim of the central colony (Fig. 5C). The first indication of the onset of swarming itself (swarm 1) was detected at 10–11 h with the appearance of the ring of presumed ‘surfactin’ around the colony, followed 1–2 h later by the initiation of dendrite formation (see below).

A more detailed microscope analysis was then carried out at 11–12 h coinciding with initiation of the first swarm. In particular, the tips of the dendrites at the swarm edge were examined directly on the swarm plate. Fig. 5(A, B) shows the first swellings emerging from the edge of the central area of inoculation between 11 and 12 h, when the number of c.f.u. present is estimated to be 3 × 10^6, assuming no lag in growth after inoculation. At this time the area covered by the original inoculum (~4 mm diameter) is incompletely covered with bacteria and distinct micro-colonies are still detectable (Fig. 5A). The initial swellings from the rim of the growing colony, whose appearance signals the onset of swarm 1, subsequently elongate, in as yet an unknown way, into long branching dendrites, typical of the first swarm stage on B-medium (see Figs 1, 3 and 4). The nascent dendrite or bud (Fig. 5B) is in fact loosely packed with cells, with initially no ‘rafts’ (see below) detectable, whilst surprisingly the rim of the central colony itself, at the base of the bud, contains many filamentous cells (Fig. 5C). In fact, as the new dendrite develops during the next hour, many small groups of aligned cells (near normal size) or rafts do appear (easily visible at higher magnification, see Figs 6 and 8), forming a monolayer particularly around the tip (in the region arrowed in Fig. 5D).

Interestingly, we have observed that strain 168, which fails to make swarming patterns on B-medium, also forms filaments in the central colony and initiates the production
of buds, which eventually become bordered by a palisade of the characteristic rafts. However, the process is blocked at this point and no swarming takes place (unpublished data).

At 21–22 h, which coincides with the completion of the first swarm and the beginning of the second swarm (Fig. 4, upper left panel), the mature dendrites of swarm 1 were also examined in situ; representative results are shown in Fig. 6(A). Each dendrite is bordered along its length by a wide monolayer formed by a palisade of rafts, each containing 4–5 cells side by side, with in many cases the poles at one end rather precisely aligned (Figs 6B, 6C and 8B). The multilayered centre of these dendrites is covered by many filamentous cells, some of which also congregate in rafts, where the internal region of the dendrite merges into the palisade (Fig. 6C).

**Swarm 2.** In many experiments with strain 3610, the initiation of swarm 2 displayed the surprising characteristics shown most clearly in Fig. 3(B). Thus, between 18 and 21 h into the swarm, and only possible to observe by reflected light, the edge of the second swarm rather abruptly appears more than 1 cm beyond the edge of swarm 1, with the intervening region seemingly lacking any cellular structures visible by eye without magnification. However, phase-contrast microscopy shows that this...
region in fact contains a thinly dispersed population of cells, mostly of normal size distribution. In contrast, the visible edge of the swarm, in particular after 21–22 h, appears to be constituted by cells assembling or coalescing de novo into new dendrites, commencing at the tips and progressively assembling backwards towards the colony centre. This is shown in Fig. 7.

At slightly later stages, as shown in Fig. 8, many rafts, forming a monolayer around the tip of dendrites, were observed at the edge of swarm 2 after 22 h. When these rafts first form at the tips, the palisade is initially narrow and the region internal to it contains loosely packed cells with few rafts present. The cells composing the rafts showed no movement, whilst the internal region displayed some random tumbling of individual cells or the swirling of groups of cells. As shown in Fig. 8(B, C) the older tips contain more closely packed rafts forming a wider palisade, but these are still present as a monolayer. This monolayer of rafts in fact appears to spread, ultimately perhaps forming the entire under surface of the new swarm 2 dendrite.

**DISCUSSION**

Bacterial swarming involves the colonization of large areas of a surface also suitable for growth, for example nutrient agar. Swarming occurs by rapid translocation over the surface, with speeds up to 1 cm h⁻¹, as found also in this study. The most detailed studies have been carried out with *P. mirabilis*, where specific ‘swarmers’, filamentous hyperflagellated cells, associated in raft-like structures, have been identified. *P. mirabilis* swarming is characterized by a control switch to form the differentiated

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**Fig. 5.** Analysis of the initiation of swarming from the central inoculation spot. A plate of B-medium was inoculated with strain 3610 and incubated at 30 °C as described in Methods; this was analysed *in situ* between 11 and 12 h after inoculation, using a Zeiss Axioplan microscope. A, emerging ‘buds’ at 11 h (magnification ×15-6) with individual micro-colonies still visible (confirmed as such at higher magnification – not shown). B, a bud at higher magnification ( × 62-5). C, higher magnification of the boxed region from A, showing the edge of the inoculation spot with a bud emerging at right (magnification ×250). This panel also shows the presence of filamentous cells (arrowed). The filamentous cells often form characteristic ‘swirls’ of cells but these are difficult to photograph because many cells are piled over each other. D, a more advanced bud with a monolayer of rafts (between the arrows) clearly visible at the tip (confirmed at higher magnification, not shown), as the population density increases in the interior of the bud (magnification ×250).
swarmer cells, followed by their co-ordinated movement, as a symmetrical wave of cells outwards. Subsequently, a second switch triggers de-differentiation, followed by multiplication of the cells in situ before the cycle repeats at characteristically constant intervals (Belas, 1997). Current thinking suggests that neither chemotaxis nor nutritional stress is specifically involved in triggering the swarming waves of bacteria. Rather, changes in population density, facilitated by quorum-sensing mechanisms and/or cell-to-cell contacts, signal the onset of different phases (Rauprich et al., 1996).

In B. subtilis, in which the swarming process has been little studied so far, the swarms appear in different forms, from highly intricate branching to carpet-like coverage of the surface, depending on the conditions. However, the mechanisms that trigger swarming behaviour or the formation of different patterns, including dendritic branching, remain completely unknown. Secretion of the protease Epr was reported to be required for swarming of the laboratory strain 168 on LB (Dixit et al., 2002). The presence of both flagella and a detergent-like lipopeptide (surfactin), secreted by the bacteria and subject to regulation by quorum sensing in planktonic culture (Solomon et al., 1995), has also been implicated in swarming in this organism (Ohgiwari et al., 1992; Wakita et al., 1998). Some reports, however, have indicated that swarming can occur independently of either flagella or surfactin (Dixit et al., 2002; Kinsinger et al., 2003; Mendelson & Salhi, 1996). In our studies with the laboratory strain 168 (Trp<sup>−</sup>), the bacteria, although SrfA<sup>−</sup>, still swarmed efficiently on LB agar, albeit less rapidly than strain 3610. Swarming, however, was apparently dependent on flagella since a hag mutant failed to swarm at all on LB. Notably, however, on the synthetic B-medium, strain 168 only swarmed in the presence of surfactin (all unpublished data), indicating that this is absolutely essential for the swarming of this strain under these conditions.

Rafts of hyperflagellated cells of close to normal size distribution, in contrast to the filamentous P. mirabilis swarmer cells, were detected at the swarm edge of strain 3610 on LB (Kearns & Losick, 2003) and a similar grouping of cells into such rafts was observed on B-medium in our in situ analysis. However, the localization of the rafts in this study suggested a role in maintaining the structure of the dendrites, in addition to any possible role they may have in swarming movement per se (see below).

In order to establish a more amenable system for the analysis of the regulation of this complex process of co-ordinated behavioural activity, we considered it important to employ a defined medium under optimized conditions, together with a natural wild-type strain, 3610. This strain has not so far been subjected to extensive culture in the laboratory, which can lead, in particular, to the inactivation of dispensable, so-called social functions (Velicer et al., 1998; Branda et al., 2001). In addition, the development of the swarm colony was examined in situ, without disrupting the organization of the swarming colony, in our attempts to define some of the important stages in the process under these conditions, including any morphological changes to the cells.

The results obtained in this study indicated a surprisingly
complex series of events when 3610 was examined on the synthetic medium. The events include, at the very initial stage of swarm 1, the appearance of bud-like structures at the edge of the inoculation point, composed of a loose association of normal-sized cells. More strikingly, the initiation of swarm 2 is accompanied by rapid migration of individual, non-filamentous cells 1–2 cm beyond the end of swarm 1. These cells then begin to form de novo the tips of new dendrites which subsequently appear to assemble backwards towards the colony centre. Other notable features include the formation of rafts of cells at the edges of dendrites and filamentous cells in the centre of dendrites. Our analysis also detected an expanding, bacteria-free zone of material preceding the swarm front throughout the swarming process. We suggest this material to be the biosurfactant surfactin, but further studies are required to confirm its nature and its possible role.

In the wild-type strain, the initial buds of swarm 1 in the edge of the central colony develop into the observed dendrites, although how this occurs remains to be analysed. The elongating dendrites then branch in some way into unexpectedly complex patterns. After 18 h, the more mature dendrites, thickened by multiplication of cells, are seen to be bordered by a wide monolayer zone of randomly arranged, closely packed rafts, enclosing a central

Fig. 7. Steps in the formation of the new swarm 2 dendrites at the swarm front (18–19 h). A, at the swarm front, dendrites begin to assemble de novo from the tip (magnification ×80); B, early stage in assembly, loosely packed rafts begin to appear in a newly forming tip (magnification ×250); C, a later-stage tip, formation of more closely packed rafts spreading inwards from the tip (towards the colony centre) (magnification ×250); D, E, behind the tip, at a later stage of formation of a dendrite as it proceeds to elongate towards the colony centre, still lacking a regular edge (D), and the interior of the forming dendrite (E), still with large spaces, devoid of cells (magnification ×250, ×500, respectively).
multilayered area, containing many filamentous cells. Thus, in some remarkable but ordered way, the division cycle of cells in very close proximity in the swarm community is regulated differentially. Concerning the rafts, we have so far only been able to observe a palisade at the edges of the mature dendrites. However, from analysis of the way the nascent dendrites of swarm 2 develop, we suggest that the entire lower surface of each dendrite, abutting onto the agar surface, may initially be laid down as a complete monolayer of such rafts. The tightly packed nature of the uniformly sized rafts and their persistence as a monolayer at least at the edges of dendrites suggests, intriguingly, that growth may have been arrested in these cells. Future studies will address these points. Finally, in some experiments, the swarm 1 dendrites were observed at the end of a period of swarming to terminate at the tips as even more complex, floret-like formations (see Fig. 3A), whose function and detailed structure remain to be analysed.

On the B-medium, the manifestation of the second swarm provides a further surprisingly complex behaviour. In this case, an organized structure, corresponding to the disconnected tips of new dendrites, was first detected at least 1–2 cm outward from the edge of the first swarm (see Fig. 3B), with the intervening region containing no organized structures, simply an area filled with a low-density population of individual cells. Subsequently, although the precise order of events needs to be confirmed, the new full-length dendrites appeared to form through progressive ‘coalescence’ or aggregation of cells, commencing from the tips and continuing to assemble towards the colony centre (shown in Fig. 7A). This presumably occurs by recruiting cells from the intervening region (i.e. between the boundary of swarm 1 and the edge of the new swarm 2). Further studies are also now required to establish the nature of the switch or signalling mechanism(s) that gives rise to the second swarm and the subsequent ability to assemble into the new structures.

As in the very earliest stage of development of swarm 1, in the initial formation of the tips of the swarm 2 dendrites no rafts were detected. However, a monolayer of rafts soon appears in the nascent swarm 2 tips, which progressively widens and spreads backwards towards the colony centre as assembly proceeds. Whilst the cells internal to this palisade continued to move actively in random directions – until the increasing population density seemed to preclude this – the rafts were quite immobile relative to each other, even at the earliest stages. In their studies of 3610 swarming on LB-agar, Kearns & Losick (2003) observed similar rafts of cells on LB-agar, which they showed were hyperflagellated. However, since the samples were removed from the swarm edge for microscopy the actual location of these rafts in the swarm colony was not established in that study. Rafts of hyperflagellated cells suggest that such groups collaborate to promote movement over the surface, similar to the role of filamentous swarmers in \textit{P. mirabilis}. In fact our observations of the positioning of the rafts around the entire borders of dendrites, and apparently on the lower surface of dendrites, could indicate another role for rafts, not mutually exclusive, in maintaining the integrity of these branching structures. On the other hand, on LB medium, as the first swarm of cells of strain 3610 emerges from the colony centre, we have detected (unpublished data) very large groups of rafts that are, quite dramatically, all aligned in the same direction, exactly as described previously for \textit{P. mirabilis} swarmers (Harshey, 1994). Nevertheless, in relation to the complex structures that we observe on the synthetic B-medium, a role for the rafts or the large bundles of filamentous cells in driving the rapid movement of the swarm front and the elongation of the dendrites is not yet clear.
Considerable complexity in behaviour and in the concomitant structural organization of the \textit{B. subtilis} swarming colony has been revealed in this study. This is presumably orchestrated at different stages by a variety of molecular switches responding to signalling mechanisms that should now be amenable to genetic and biochemical analysis. We note, however, that if quorum sensing is important, for example, in controlling processes such as inhibition of cell division or surfactin synthesis (which appears to be associated with the initiation of swarm 1), then this occurs whilst separate micro-colonies containing only hundreds to thousands of bacteria are still detectable at the site of the original inoculum. Therefore, in contrast to conditions in liquid cultures, quorum sensing may be manifest at extremely low cell densities, facilitated perhaps by the close packing of a very small number of cells.

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REFERENCES


