Single-cell analysis *in situ* in a *Bacillus subtilis* swarming community identifies distinct spatially separated subpopulations differentially expressing *hag* (flagellin), including specialized swimmers

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The non-domesticated *Bacillus subtilis* strain 3610 displays, over a wide range of humidity, hyper-branched, dendritic, swarming-like migration on a minimal agar medium. At high (70 %) humidity, the laboratory strain 168 *sfp*⁺ (producing surfactin) behaves very similarly, although this strain carries a frameshift mutation in *swrA*, which another group has shown under their conditions (which include low humidity) is essential for swarming. We reconcile these different results by demonstrating that, while *swrA* is essential for dendritic migration at low humidity (30–40 %), it is dispensable at high humidity. Dendritic migration (flagella- and surfactin-dependent) of strains 168 *sfp*⁺ *swrA* and 3610 involves elongation of dendrites for several hours as a monolayer of cells in a thin fluid film. This enabled us to determine *in situ* the spatiotemporal pattern of expression of some key players in migration as dendrites develop, using *gfp* transcriptional fusions for *hag* (encoding flagellin), *comA* (regulation of surfactin synthesis) as well as *eps* (exopolysaccharide synthesis). Quantitative (single-cell) analysis of *hag* expression *in situ* revealed three spatially separated subpopulations or cell types: (i) networks of chains arising early in the mother colony (MC), expressing *eps* but not *hag*; (ii) largely immobile cells in dendrite stems expressing intermediate levels of *hag*; and (iii) a subpopulation of cells with several distinctive features, including very low *comA* expression but hyper-expression of *hag* (and flagella). These specialized cells emerge from the MC to spearhead the terminal 1 mm of dendrite tips as swirling and streaming packs, a major characteristic of swarming migration. We discuss a model for this swarming process, emphasizing the importance of population density and of the complementary roles of packs of swimmers driving dendrite extension, while non-mobile cells in the stems extend dendrites by multiplication.

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Abbreviations: a.u., arbitrary units; MC, mother colony.

Supplementary Methods, eight supplementary figures and three supplementary movie files are available with the online version of this paper.

INTRODUCTION

The formation of bacterial communities, such as *Bacillus subtilis* colonies, was shown recently, while this work was in progress, to occur through a developmental-like program, associated with the presence of different cell types (see Vlamakis *et al.*, 2008; Lemon *et al.*, 2008; Lpez & Kolter, 2010). Another form of community swarming migration over a surface, apparently occurring in thin fluid films, requires flagella, a surfactant and the production of specialized swimmer cells (Harshey, 2003; Darnton *et al.*, 2010).

Studies of swarming to date have primarily involved Gram-negative bacteria on rich media or minimal media fortified with Casamino acids. Most studies have concerned the migration of *Proteus mirabilis* as waves of confluent expansion over hard agar (2 %), involving coordinated movement of very large clusters of aligned swimmers – extremely long, profusely flagellated filamentous cells – at the swarm front (Bisset, 1973; Fraser & Hughes, 1999). *Serratia liquefaciens* swimmers, however, appear to be only moderately elongated, although profusely flagellated (Eberl *et al.*, 1999). In contrast, in *Escherichia coli* and *Salmonella typhimurium* (Darnton *et al.*, 2010; Turner *et al.*, 2010; Harshey, 2003), migration is restricted to softer agar (around 0.5 %), and involves smaller clusters (packs) of swimmers that are only moderately elongated and flagellated (two- to threefold in each case). In *Pseudomonas aeruginosa*, swarming takes the form of unbranched dendrites or tendrils, rather than confluent fields, and swimmers are around twofold larger than vegetative cells, with two flagella rather than one (Rashid & Kornberg, 2000; Caiazza *et al.*, 2005). Finally, particularly in *E. coli*, coordinated movement appears to be achieved through alignment of around five cells into groups or packs (McCarter, 2010, and the Rowland Institute at Harvard website cited therein).

The most studied example of swarming in Gram-positive bacteria is that of *B. subtilis*. Swarming is limited to 0.7–1 % agar, and is absolutely dependent upon the presence of flagella (Kearns & Losick, 2003; Julkowska *et al.*, 2005; Hamze *et al.*, 2009) and the production of surfactin, which spreads 1–2 mm ahead of the advancing swarm front (Kearns & Losick, 2003; Julkowska *et al.*, 2004, 2005; Debois *et al.*, 2008). Two types of swarming-like migration by *B. subtilis* have been described. On Luria broth (LB) agar at low humidity and 37 °C (primarily analysed with non-domesticated strains such as 3610), migration proceeds as an expanding confluent field, headed by small groups of bacteria, apparently with little change in individual size (Kearns & Losick, 2003; Julkowska *et al.*, 2004). In contrast, we have described the formation of hyper-branched dendritic patterns by strain 3610 on the minimal B medium at both high and low humidity, which develop through a series of well-defined stages (Julkowska *et al.*, 2004). We have also observed very similar behaviour for the *sfp*⁺ derivative of the laboratory strain 168 (able to produce surfactin) at high humidity, although the cells fail to swarm reproducibly at low humidity. This strain in fact carries a mutation in *swrA* (Kearns *et al.*, 2004; Hamze *et al.*, 2009), a gene of unknown function implicated in activation of transcription of the large *fla/che* operon (Kearns & Losick, 2006). *swrA* has been reported (apparently at low humidity) to be essential for swarming migration on rich medium (Kearns *et al.*, 2004). Recently, Patrick & Kearns (2009) have also reported that strain 168 *sfp*⁺ *swrA* fails to show surface migration on B medium under their conditions. Those authors, from their results, claim that dendritic migration of strain 168 *sfp*⁺ *swrA* at

70 % humidity can only be explained by ‘sporadic’ *in situ* reversion to *swrA*⁺, or, surprisingly, they imply that dendritic migration involves a form of swimming in surface water and not swarming. Unfortunately, they did not control humidity in their studies and therefore failed adequately to test its effect on the migration of strain 168 *sfp*⁺ *swrA*. One objective of this study was to reconcile these apparently contradictory results. We have done this by confirming that *swrA* is required for migration at low humidity, but is dispensable at high humidity, without reversion to *swrA*⁺. We also show that both 168 *sfp*⁺ *swrA* and an *swrA*⁺ derivative produce very similar dendritic migration at high humidity. In addition, we present several lines of evidence to show that dendritic migration of the laboratory strain 168 *sfp*⁺ *swrA*, like the migration of *swrA*⁺ strains, does not involve swimming of individuals in a large volume of surface water, but instead is like classical swarming in being restricted to a thin film.

Dendritic migration of strain 168 *sfp*⁺ *swrA* involves distinct morphological and genetically defined stages at high humidity (Julkowska *et al.*, 2004, 2005; Hamze *et al.*, 2009). Thus, following the first visible indication of initiation of migration, a spreading zone of surfactin emanating from the mother colony (MC; the site of inoculation), several small bud-like structures burst outwards from the edge of the MC. Importantly, a characteristic feature of dendritic migration is an advancing monolayer of cells that form the developing buds and primary dendrites up to about 1.5 cm in length, before the switch to multilayering and finally the formation of hyper-branched fractal-like patterns (Julkowska *et al.*, 2004, 2005; Hamze *et al.*, 2009). This form of community development is of interest to mathematicians and physicists studying pattern formation (Marrocco *et al.*, 2010), while the initial several hours of development of the swarm as a monolayer provide a highly convenient system for molecular geneticists to analyse gene expression *in situ* at the single-cell level.

Since the development of the dendritic community of *B. subtilis* *sfp*⁺ *swrA* involves distinct stages, we anticipated that this should reflect differential expression of swarming genes. The major objective in this study was therefore to examine whether the development of dendrites was accompanied by spatiotemporal changes in gene expression and the appearance of distinctive cell types, with the primary focus on the *hag* gene (encoding flagellin). In addition, we hoped to define better the properties of swimmers, which so far are poorly characterized in *B. subtilis*. By measuring the pattern of expression from the *hag* promoter of an ectopic copy of a *hag*–*gfp* fusion, we were able to identify three distinct subpopulations of cells that differ in the level of *hag* expression: (i) long chains close to the edge of the MC with no detectable expression of *hag*; (ii) largely non-mobile cells in dendrite stems, with intermediate levels of *hag* expression; and (iii) a unimodal population of highly mobile cells, with hyper-expression of *hag* and other distinctive characteristics. These cells are first detected in pre-dendrite buds before forming the extreme 1 mm at the tip of elongating

dendrites. Finally, we discuss the relative roles of the different cell types in the development of the swarm as dendrites elongate, with the high population density at dendrite tips playing a crucial role in the observed collective movement of the specialized swimmers.

METHODS

Strains and growth conditions. Bacterial strains used in this study are listed in Table 1. Details of strain construction in this study are shown in the Supplementary Methods. Bacteria were grown with aeration at 37 °C in synthetic B medium, composed of (final concentrations): 15 mM (NH₄)₂SO₄, 8 mM MgSO₄·7H₂O, 27 mM KCl, 7 mM sodium citrate·2H₂O, 50 mM Tris/HCl, pH 7.5, 2 mM CaCl₂·2H₂O, 1 µM FeSO₄·7H₂O, 10 µM MnSO₄·4H₂O, 0.6 mM KH₂PO₄, 4.5 mM glutamic acid, 860 µM lysine, 784 µM tryptophan and 0.5% glucose (Antelmann *et al.*, 1997). For the *thr* auxotrophs, both liquid and solid media were always supplemented with 1 mM threonine. Antibiotics were added to plates at the following final concentrations: chloramphenicol, 5 µg ml⁻¹; spectinomycin, 100 µg ml⁻¹; erythromycin, 1 µg ml⁻¹; lyncomycin, 12.5 µg ml⁻¹.

Conditions for dendritic swarming experiments. For swarming on B medium, 9 cm swarm plates containing 25 ml medium (0.7% Bacto agar) were prepared 1 h before inoculation and dried with lids open for 5 min in a laminar flow hood. Plates could be dried for up to 15 min without affecting initial swarming behaviour. However, the longer periods of drying could result in the arrest of swarming, particularly in the laboratory strain, before reaching the edge of the plates, which normally took around 20–24 h. Cultures for inoculation were prepared in 10 ml B medium inoculated from a single colony on an LB agar plate and shaken overnight at 37 °C. The culture was diluted to OD₅₇₀ ~0.1 and grown at 37 °C to OD₅₇₀ ~0.2. This

procedure was repeated twice and finally the culture was grown to T₄ (4 h after the transition from exponential growth). The culture was diluted, and 10⁴ bacteria (2 µl) were placed at the centre of a swarm plate and incubated at 30 °C (usually at a relative humidity of 70%) for the times indicated below, or until the dendrites were approximately 1.5 cm in length (~16 h). It was important to pay careful attention to the level of humidity in order to obtain robust, sustained swarming on B medium. In order to achieve this, we used an incubator that allowed precise control of temperature and humidity (Climacell 111). This protocol then ensured reproducible timing of the initiation of migration and pattern formation, but avoided any residual free water on the agar surface. Thus, a drop of water placed on these swarm plates did not spread (Banaha *et al.*, 2009). Note that about 11 h of growth of the central MC occurred before eight to 12 monolayered primary dendrites radiated outwards from the edge of the MC at a constant speed of approximately 3.5 mm h⁻¹ (1 µm s⁻¹) at 30 °C (Julkowska *et al.*, 2004).

Images of swarm plates were taken with an Epson 1600 Pro scanner at 300 dpi, or where otherwise indicated at 1200 dpi.

Isolation of an *swrA*⁺ revertant of strain 168 *sfp*⁺. Swarm plates of strain 168 *sfp*⁺ *swrA* (OMG 930) were prepared as above and incubated at 30–40% humidity. A swarm plate after 24 h, showing some dendritic migration but prematurely arrested after approximately 1.5–2 cm, was left at room temperature for 3 days at about 30% humidity. From the additional dendrites that finally arose from the edge of the arrested zone, some cells were removed and purified to single colonies, and the DNA was sequenced, as indicated below, to identify an *swrA*⁺ revertant, SSB 2026.

Imaging and single-cell analysis: stereomicroscopy, phase-contrast and fluorescence microscopy. Cells at magnification × 150 were examined *in situ* with a stereomicroscope (Zeiss Lumar), or with different objectives appropriate to a phase-contrast/fluor-

Table 1. Strains and plasmids used in this study

All the OMG strains (and SSB 2019, 2026) were derived from the laboratory strain 168 *trp* C2 *swrA*, which was restored to *sfp*⁺ by insertion into *amyE* or into *thrC*. OMG 991 is derived from the non-domesticated wild-type strain 3610.

Strain or plasmid	Genotype	Reference, source or construction
Strains		
3610	Natural isolate	<i>Bacillus</i> Genetic Stock Center collection
168	<i>trpC2 swrA sfp</i>	European <i>B. subtilis</i> Genome Consortium
OMG 900	<i>trpC2 swrA amyE::sfp⁺ cat</i>	Julkowska <i>et al.</i> (2005)
OMG 930	<i>trpC2 swrA thrC::sfp⁺ erm</i>	This work
SSB 2026	<i>trpC2 swrA⁺ thrC::sfp⁺ erm</i>	This work
OMG 954	<i>trpC2 swrA thrC::sfp⁺ erm Δhag::cat</i>	Hamze <i>et al.</i> (2009)
OMG 982	<i>trpC2 swrA thrC::sfp⁺ erm P_{R_{hag}}-gfpmut3 spc</i>	This work
OMG 984	<i>trpC2 swrA thrC::sfp⁺ erm amyE::P_{comA}-gfpmut3 spc</i>	This work
OMG 991 (3610)	<i>amyE::P_{hag}-gfpmut3 spc</i>	This work
OMG 992	<i>trpC2 swrA thrC::sfp⁺ erm amyE::P_{hag}-gfpmut3 spc</i>	This work
SSB 2019	<i>trpC2 swrA thrC::sfp⁺ erm amyE::P_{eps-yfp} spc</i>	Derived from strain HV1046 (168 <i>amyE::P_{eps-yfp} spc</i>), a gift from H. Vlamakis and R. Kolter
Plasmids		
pOMG 808	PDG 1663 <i>thrC::sfp</i>	This work; derived from pDG 1663 (Guérout-Fleury <i>et al.</i> , 1996) with <i>sfp</i> from pSV <i>sfp</i> (Nakano <i>et al.</i> , 1992)
pOMG 810	PDL30 <i>amyE::gfpmut3-ter</i>	This work; derived from pDL30 (Lynch <i>et al.</i> , 2001)
pOMG 812	PDL30 <i>amyE::P_{comA}-gfpmut3-ter</i>	This work
pOMG 816	PDL30 <i>amyE::P_{hag}-gfpmut3-ter</i>	This work
pOMG 829	PDL30 <i>amyE::P_{R_{hag}}-gfpmut3-ter</i>	This work

escence microscope (Zeiss AxioImager M1). Both microscopes were fitted with an AxioCam camera (Zeiss). In the Lumar instrument, GFP was excited at 450–490 nm and the fluorescence collected in the range 500–550 nm (filter set 38 HE eGFP, Zeiss). In the fluorescence microscope, excitation wavelengths were 450–490 nm and emission wavelengths were 515–565 nm (filter set 10, Zeiss). A range of low-magnification air objectives ($\times 1.25$, 5, 20 and 40) were used initially to establish the perspective of images before selecting an appropriate field for analysis at high magnification. This was carried out as required with a $\times 63$ air or a $\times 100$ oil objective (numerical aperture=1.3). For the latter, regions of dendrites were observed with a microscope coverslip placed gently over the required portion of the swarm. This ensured minimal disturbance of the sample with the displacement at most of only a few cells from the edge of a dendrite.

To measure relative levels of fluorescence intensity in monolayered images resulting from $P_{hag-gfp}$ expression along the dendrite (semiquantitative analysis), images were captured using AxioVision software (release 4.6.3) and false-coloured. ImageJ software (Abramoff *et al.*, 2004) was then used to calculate and format relative fluorescence intensities. It is important to note that for a highly expressed gene such as *hag*, swarms examined in the stereomicroscope were inoculated with 50 % of cells expressing *hag-gfp* and 50 % of the otherwise identical strain not expressing *hag-gfp*. At the low magnification employed with the stereomicroscope, this avoids any overlap of fluorescence signals from neighbouring cells, especially where a strong promoter is involved and in high population density regions, giving high fluorescence intensities that might be attributed spuriously to single cells by the software.

For the quantitative analysis of $P_{hag-gfp}$ expression along the dendrite with the microscope, images were captured using AxioVision software (release 4.6.3), false-coloured, and analysed with background subtraction, using AutoMeasure associated with the AxioVision software. The results were expressed in three ways: at the single-cell level (scatter plot), as fluorescence intensity per unit surface area (see Fig. 4b), as the mean fluorescence intensity of an entire field of cells (Fig. 4c), or as the fluorescence distribution within the population (Figs 5 and 6), where the single-cell dataset was fractionated to identify the number of cells in successive increments of 100 or 200 fluorescence units, as indicated below. The experiments, repeated three times, with one dendrite analysed from three different swarms, gave very similar results. For all quantitative single-cell measurements, between 500 and 900 cells per field were measured. Control experiments, in which cells were scraped from dendrites and dispersed at low density in buffer for fluorescence measurements, confirmed the existence of a gradient along dendrites, with tip cells hyper-expressing *hag-gfp*. Other control experiments also demonstrated that replicate fields in the same region in dendrites gave very similar results. This supports the notion that the dendrite population, except at the tips, is stable, with relatively little mixing, and maintains a constant population density.

Measurement of population density and length distribution along dendrites. For measuring the population density when dendrites were approximately 1 or 1.5 cm and still monolayered, cells were examined *in situ* by an AxioImager M1 microscope (Zeiss), using a $\times 40$ Neofluar objective. Images were captured using AxioVision software (release 4.6.3), and the population density was calculated using ImageJ software. The results were confirmed manually, with at least 3000 cells counted in duplicate or triplicate fields. Note that cells containing a clear septum were counted as two cells. As controls, several replicate fields sampled in close proximity were shown to give virtually identical counts. In order to obtain sharp images of otherwise hyper-mobile cells *in situ* in the tip region, swarms were placed under the microscope without covers for 5 min to reduce mobility.

DNA sequence analysis. Samples were transferred by toothpick from different locations (MC, base and tip of dendrites) of 3–4 cm diameter swarms on B medium plates of strain 168 *sfp*⁺ *swrA* directly onto 50 μ l of the complete PCR mixture (containing KOD polymerase, Novagen). The entire *swrA* gene and flanking regions (1.8 kb) were amplified (25 cycles) with oligonucleotides HP1433 (5'-ATATCTC-GAGCGTACCCTGTCAATGTGATTACG-3') and HP1436 (5'-ATG-TACTAGTCTTCAGTGATAATCAGCAAACCTAG-3'). The amplified product was then purified (NucleoSpin Plasmid kit, Macherey-Nagel) and sequenced. Three experiments were carried out on different days, and in each case a dendrite was sampled at the locations indicated in the text from each of three swarm plates. The potential *swrA* revertant cells picked from dendrites developing at room temperature and low humidity were purified to single colonies, sampled for PCR and sequenced as above. The wild-type *swrA* gene in the non-domesticated strain 3610 was sequenced from cultures grown in liquid B medium.

RESULTS

Dendritic migration of the laboratory strain 168 *sfp*⁺ requires *swrA*⁺ at low but not at high humidity

The non-domesticated strain of *B. subtilis*, 3610, showed robust dendritic migration on the synthetic B medium over a wide range of humidity. The laboratory strain 168, when restored to *sfp*⁺ (necessary for surfactin production), formed very similar branched dendritic patterns at 70 % humidity (Supplementary Fig. S1), but failed to migrate reproducibly at low humidity (Julkowska *et al.*, 2004, 2005; Hamze *et al.*, 2009). This surface migration, as we showed previously, absolutely requires flagella and a surfactant, surfactin (Supplementary Fig. S2), i.e. the classical requirements for swarming migration (Harshey, 2003). In fact, strain 168 carries a mutation in *swrA* (Kearns *et al.*, 2004; Hamze *et al.*, 2009) which has been shown to be required for swarming on LB (Kearns *et al.*, 2004) and recently on the minimal B medium (Patrick & Kearns, 2009). Those authors, however, used an experimental protocol quite different from that employed here, including very dry conditions. We confirmed (Supplementary Fig. S3) that 168 *sfp*⁺ *swrA* (OMG 930) failed to migrate or that migration arrested prematurely at 30–40 % humidity.

In order to address further the question of the role of *swrA* in dendritic migration, we isolated an *swrA*⁺ revertant of strain 168 *sfp*⁺ under strong selective conditions, i.e. extensive incubation around 30 % humidity (see Methods). The migratory behaviour of this revertant strain, 168 *sfp*⁺ *swrA*⁺ (SSB 2026), was compared at both 30–40 and 70 % humidity with that of the parental 168 *sfp*⁺ *swrA* strain. Fig. 1 shows that the *swrA* mutant, as expected, failed to migrate at low humidity, while the revertant migrated robustly, further evidence that *swrA* is required for migration at low but not at high humidity. At 70 % humidity, the initiation of dendritic migration is normally preceded by the appearance of the surfactin zone just before the emergence of pre-dendrite buds at around 11 h post-inoculation. For both the *swrA* and the *swrA*⁺ strain, surfactin production and dendrite emergence were

Fig. 1. Dendritic migration of strain 168 *sfp*⁺ requires *swrA*⁺ at low but not at high humidity. B medium swarm plates (0.7 % agar) were inoculated at the centre with 10⁴ early stationary phase (T₄) cells of strains 168 *sfp*⁺ *swrA* (OMG 930) or 168 *sfp*⁺ *swrA*⁺ (SSB 2026). For details of the preparation of swarm plates and the inoculum, see Methods. Plates were incubated at 30 °C and 30–40 % (a, b) or 70 % relative humidity (c, d). Images were obtained after 22 h (a, c) and 40 h (b, d) by scanning (see Methods). Arrows in (a) and (c) indicate the limit of the expanding surfactin zone, approximately 2 mm ahead of the advancing dendrite tips, not visible in these photographs. At 70 % humidity, the number of initial dendrites in the mutant tended to be variable in the absence of *swrA* (compare with Supplementary Figs S1 and S2).

observed at approximately the same time (data not shown). Moreover, development was still very similar at 22 h in the two strains (Fig. 1a, c). Finally, at 40 h, both strains showed overall similar robust patterns of migration (Fig. 1b, d). We note that in this experiment, the number of initial dendrites at 70 % humidity was lower in the *swrA* strain than in the *swrA*⁺ strain. However, this varied in different experiments, and, as shown in Supplementary Fig. S1, in some experiments the *swrA* strain produced a much higher number of dendrites.

Next, we analysed the status of the *swrA* gene during expansion of the community of 168 *sfp*⁺ *swrA* (OMG 930) at 70 % humidity. The tips of 1.5–2 cm dendrites were sampled from a total of nine dendrites from three different plates in separate experiments, and analysed by PCR and sequencing of the entire *swrA* gene (see Methods). Two additional samples were taken from MCs and from the base of two dendrites from different plates, and analysed similarly. As shown in Supplementary Fig. S4, the wild-type *swrA*⁺ gene was not detected in any of the samples and the frameshift mutation was still present in the 168 *sfp*⁺ cells, i.e. an insertion of a single base pair, AT, at codon 12, in a run of eight Ts in the *swrA* gene. This insertion is not found in the

non-domesticated strain 3610 (also shown in Supplementary Fig. S4). Thus, in our hands, robust dendritic migration of this strain is not accompanied by reversion of the *swrA* mutation, confirming that at 70 % humidity the *swrA* gene is dispensable.

Long septated chains of cells not expressing *hag* appear early and accumulate at the edge of the MC on swarm plates incubated at 70 % humidity

Following inoculation of swarm plates with strain 168 *sfp*⁺ *swrA*, cells grew for 10–11 h to form the MC, before the appearance of the outward-expanding zone of surfactin. We have observed in both the domesticated and non-domesticated strain (168 *sfp*⁺ *swrA* and 3610, respectively) that this early growth of the MC is accompanied by the appearance of long cell forms on both LB and B medium plates (Julkowska *et al.*, 2004; Hamze *et al.*, 2009). We now show that staining of such cells *in situ* in the MC with a membrane stain (see Supplementary Methods) shows that the long cell forms are septated (Supplementary Fig. S5). These cells therefore appear identical to *B. subtilis* chains that previously were first identified as a distinct

subpopulation present in liquid cultures. In such cells, the production and activity of the transcription factor σ^D is switched off, resulting in the absence of *hag* expression (flagellin) and of the expression of certain autolysins encoded by *lytABC* (Kearns & Losick, 2005; Chen *et al.*, 2009; Cozy & Kearns, 2010).

To examine *hag* expression in the chain forms, a derivative of 168 *sfp*⁺ *swrA* (OMG 992) was constructed that carries, in addition to the normal *hag* gene, a *P_{hag}-gfp* fusion inserted into the *amyE* locus (see Supplementary Methods). Examination of cells during the early stages of growth of the MC and subsequent migration confirmed that the long forms did not fluoresce, i.e. they did not express *hag*. Thus, Fig. 2(a, b) shows at 10 h post-inoculation the appearance of microcolonies and elongated non-fluorescent cells already present randomly distributed in the MC. By 12 h the long cells formed an extensive network, in particular at the edge of the MC (Fig. 2d). We note, however, that the chaining state in the swarm community is transient and has disappeared in older multilayered dendrites. Thus, when viewed at 24 h, the great majority of cells in the MC and along dendrites were of normal length and expressed *hag* (data not shown). Finally, we showed that the long forms expressed high levels of *eps* (exopolysaccharide production), with very low or background levels of expression in distal regions of dendrites, as revealed using an *eps-gfp* fusion in strain 168 *sfp*⁺ (Supplementary Fig. S6).

Single-cell quantitative analysis of *hag-gfp* expression *in situ* reveals a gradient along dendrites, increasing sharply at the tips

In anticipation that the regulation of flagella production is coupled in some way to the mechanism of elongation/migration of dendrites, we examined the spatial expression of *hag*, using 168 *sfp*⁺ *swrA* carrying the *P_{hag}-gfp* fusion (OMG 992). Dendritic migration from the multilayered MC was allowed to develop until dendrites extended up to 1.5 cm. This occurs essentially as a monolayer of cells, ideal for *in situ* analysis without disturbing the cells. For greater convenience and rapid acquisition of data, the initial experiments employed a stereomicroscope at a relatively low magnification ($\times 150$). In this way, semiquantitative measurements of the relative fluorescence intensity of cells could be generated rapidly from images taken *in situ*. Several images were obtained along different dendrites (1 or 1.5 cm) and fluorescence levels were analysed (see Methods). Typical results, shown in Fig. 3, indicated an apparent gradient of expression of *hag-gfp* along the dendrites, with a particularly marked increase towards the tip (Fig. 3, a1, a2, b). Similar results were consistently obtained with different dendrites on the same plate or with dendrites from swarms on different days. Moreover, a similar gradient of *hag* expression from a maximum at the tip was detected, as shown in Supplementary Fig. S7, for the non-domesticated strain 3610 *P_{hag}-gfp* (OMG 991; see Supplementary Methods for

Fig. 2. Long chains that accumulate early at the edge of the MC do not express *hag*. B medium swarm plates were prepared and inoculated, as in Fig. 1, in this case with strain 168 *sfp*⁺ *P_{hag}-gfp* (OMG 992). (a) Bright-field view of the MC *in situ* (10 h) with microcolonies beginning to develop. (b) Image taken *in situ* at 10 h in the fluorescence microscope at higher magnification, showing normal-sized fluorescent cells and longer, non-fluorescent cells (examples arrowed), already appearing within the MC. (c) At 11–12 h, small mono-layered buds emerge from the edge of the MC and large numbers of the long septated forms are seen accumulating in the region indicated between the arrows. (d) Fluorescent image at higher magnification of the region between the arrows in (c), with the long forms clearly non-fluorescent. Note the clear demarcation of normal-sized cells, which form a narrow zone at the extreme edge of the MC, from the network of long-chain forms, indicating clonal development of distinct cell types in close proximity.

Fig. 3. Semiquantitative analysis of *hag-gfp* fluorescence *in situ* along dendrites shows a gradient of expression, maximal at the tip. B medium swarm plates and cells were prepared as in Fig. 1, and plates were inoculated with the strains indicated below. Plates were incubated at 30 °C for approximately 16 h (producing 1.5 cm dendrites) or 14.5 h (1 cm dendrites). Images were taken *in situ* at different positions along a monolayered dendrite, as indicated in the cartoon at the top. (a1) Raw fluorescence images for a 1.5 cm dendrite after inoculation with 50 % 168 *sfp*⁺ *P_{hag}-gfp* cells and 50 % 168 *sfp*⁺ cells with no *gfp* fusion (OMG 992 and OMG 930, respectively). This avoids the overlap of fluorescence signals from neighbouring cells, and is necessary at the low magnification employed in the stereomicroscope when analysing highly expressed promoters. Note the apparently increased population density at the tip. (a2, b) Typical images of the *hag-gfp* swarms obtained *in situ* from a 1.5 cm and a 1 cm dendrite, respectively, analysed by ImageJ software (see Methods). The relative fluorescence intensity per cell is indicated by yellow maximum and blue minimum. (c) Expression along a dendrite after inoculation with a strain expressing *comA-gfp* (OMG 984). (d) Expression along a dendrite after inoculation with 50 % OMG 982 expressing *gfp* from a constitutive λ *P_R* promoter and 50 % OMG 930. The dimensions of the stereomicroscope fields analysed are indicated to the left of (d).

construction). In contrast to *hag-gfp*, the expression of an artificial gene construct of *gfp* inserted into the *amyE* locus and expressed from a modified (constitutive) *E. coli* λ *P_R* promoter (see Supplementary Methods) displayed no obvious gradient along the dendrite (Fig. 3d). Another control experiment involved a strain expressing a *comA-gfp* fusion to monitor the expression of a gene involved in regulation of surfactin production. Interestingly, this construct also revealed a gradient of expression, although the complete reverse of *hag*, being minimal at dendrite tips

(Fig. 3c). These results all support the conclusion that the expression of *hag* varies to form a specific gradient along dendrites.

In order to confirm quantitatively the gradient of *hag-gfp* expression with strain 168 *sfp*⁺ *swrA*, we measured fluorescence intensity in single cells from a series of images taken *in situ* along dendrites at high magnification. In such images, the great majority of cells in the monolayer were clearly separable (Fig. 4a), and typical results for a 1.5 cm

Fig. 4. Single-cell, quantitative analysis *in situ* along a dendrite reveals hyper-expression of P_{hag} -*gfp* in dendrite tips overlapping a region of high population density. B medium swarm plates were prepared and inoculated as in Fig. 1, but with strain 168 *sfp*⁺ P_{hag} -*gfp* (OMG 992). Plates were incubated until dendrites reached 1.5 cm (16 h). (a) Typical microscope field of the monolayer of cells (1.25 cm from the base in this example) obtained *in situ* with the fluorescence microscope ($\times 100$ objective). (b) Typical scatter plot of fluorescence intensity (a.u.) per unit surface area of cells versus cell length, in this case a field of cells in the tip region. (c) Mean fluorescence intensity values from images ($\times 40$ objective) of 500–900 cells obtained along the dendrite and plotted against dendrite length. Two individual replicate fields analysed in the adjacent regions at a particular position of the dendrite are represented by different symbols. The mean fluorescence intensity for each field was then calculated. (d) Population density along a dendrite determined from images ($\times 40$ objective) obtained *in situ*, counting at least 1000 cells in each of two adjacent fields at each position (see Methods).

dendrite, based on the analysis of 500–900 cells per field, are presented as a function of cell length in a scatter plot and in graphical form (Fig. 4b and c, respectively). The results of such single-cell measurements of *hag* expression *in situ* (expressed as the mean fluorescence intensity in cells per unit surface area) confirmed the presence of a shallow gradient of increasing expression of *hag*-*gfp*, from the base to close to the tip of the dendrite. However, the mean fluorescence within the last 1–1.2 mm at the extremity of the dendrite rose rapidly, giving a 2.5-fold increase compared with the base, with a maximum mean fluorescence intensity of 1850 arbitrary units (a.u.).

The results presented above, indicating sharply increased *hag* expression in dendrite tips, suggested the presence of hyper-flagellated cells. This was indeed confirmed by removal of cells from different positions along dendrites

and examination by electron microscopy (see Methods). Cells taken from the tips of dendrites had around 24 flagella, while cells from the base of dendrites had around 10–12 flagella (Supplementary Fig. S8).

A unimodal population expressing high levels of *hag*-*gfp* in dendrite tips overlaps a region of high population density of strain 168 *sfp*⁺ *swrA*

The raw fluorescence intensity data for *hag*-*gfp* expression shown, for example, in Fig. 4(b) were also analysed with respect to the distribution of fluorescence levels amongst the population in different regions along a 1.5 cm dendrite. The results presented in Fig. 5 show a wide range of *hag* expression in the population at the base of dendrites with respect to fluorescence levels. However, analysis along the

stem of the dendrite progressively revealed a more bimodal distribution. Finally, a unimodal population of cells with high levels of *hag* expression was detected within approximately the terminal 1.0 mm at the tip. Similar results were obtained from three independent swarms analysed on different days.

Microscope observations demonstrated that the cells in the extreme tip regions of dendrites were also characterized by their hyper-motility and apparent packing to a higher population density compared with the rest of the dendrite. This was confirmed by directly measuring the population density along 1.5 cm dendrites. Images were taken *in situ* in three dendrites from different swarms, with virtually identical results. Although the cells were distributed in the form of a loose irregular matrix along the stems of dendrites, the results in Fig. 4(d) show that the overall population density in the microscope fields was constant throughout most of the dendrite. This, together with the general immobility of these cells, also suggested that there was relatively little mixing within the dendrite stem. In contrast to the constant population density in the stem, the population density rose sharply within the terminal 1 mm, overlapping the region occupied by the hyper-mobile, high *hag*-expressing cells. The possible relationship between population density and the mobility of these cells will be discussed below.

A unimodal population of cells (swarmers) with hyper-expression of *hag-gfp* can already be detected in the 'pre-dendrite' buds

The observed increased *hag* expression that was confined to the extreme tips of dendrites, as described above, raised the question of whether *hag* hyper-expression might be essential for initiation and maintenance of swarming per se, or whether upregulation was only triggered during elongation of dendrites. To address this question, we investigated the properties of the cells in 'pre-dendrite' buds.

As the first indication of dendrite formation, buds emerged from the edge of the MC at around 11 h post-inoculation (see Fig. 2c). In buds, as in dendrite tips, the cells were seen to be packed to a high density, which was reduced in the proximal region (Fig. 6c). Using strain 168 *sfp*⁺ *swrA* carrying the *P_{hag}-gfp* fusion (OMG 992), a quantitative, single-cell analysis *in situ* was carried out from microscope images taken at different positions across an entire 1 mm pre-dendrite bud (Fig. 6a). This revealed high mean fluorescence intensities in all these cells, similar to that found in dendrite tips. Moreover, the distribution analysis of fluorescence intensity in the buds (Fig. 6b) also showed a unimodal population of cells, similar to that found in dendrite tips (compare with Fig. 5). Finally, we observed that the cells in the buds were also highly mobile (data not shown). We conclude that the cells in this subpopulation are required to lead the way out of the MC, resulting in bud formation. These specialized hyper-mobile cells, which we designate swarmers, then spearhead the developing dendrites, moving, as we show below, in packs of aligned cells.

B. subtilis swarmers move over swarm plates in a thin fluid film, and show coordinated movement of swirling and streaming packs

Swarmers in *P. mirabilis* are long filamentous cells and are abundantly flagellated (Fraser & Hughes, 1999), while their equivalents in *E. coli* and *Salmonella* (McCarter, 2010) are much less elongated, with only a moderate increase in flagella. Similarly, for *B. subtilis* on LB plates, cells at the swarm front are not filamentous (Kearns & Losick, 2003; Julkowska *et al.*, 2005). As shown in Fig. 7, a length distribution analysis from images taken along 1.5 cm dendrites (strain 168 *sfp*⁺ *swrA*) revealed a population at the base and along most of the stem of dendrites, with a length distributed around 2.5 µm. In contrast, at the tips, a large proportion of cells were smaller, with a mean length of around 2 µm, with a long tail of a distribution of longer cells similar to that found in the dendrite stem.

Fig. 5. A unimodal population of cells hyper-expressing maximum levels of *P_{hag}-gfp* localizes to dendrite tips. B medium swarm plates were prepared and inoculated as in Fig. 1, in this case with strain 168 *sfp*⁺ *P_{hag}-gfp* (OMG 992). Plates were incubated for 16 h, and single-cell measurement of fluorescence intensity (in a.u.) along a 1.5 cm dendrite *in situ* was carried out as in Fig. 4. The distribution of fluorescence intensity (increments of 100 a.u.) within the population was determined as described in Methods from images taken at different positions (indicated at the right). At least 1600 cells were measured for each position.

Fig. 6. Single-cell analysis *in situ* of P_{hag} -*gfp* expression across an emerging bud. B medium swarm plates were prepared and inoculated as in Fig. 1, in this case with strain 168 $sfp^+ P_{hag}$ -*gfp* (OMG 992). Plates were incubated for 11 h until emerging buds were seen at the edge of the MC. (a) Images were taken in the microscope ($\times 100$ objective) at four different locations from base to tip. Duplicate images (different symbols) were obtained at each position (400–700 cells analysed per field). (b) Mean fluorescence intensity in single cells measured from an entire field, as described in Fig. 4, was determined as a function of bud length. Then the distribution of fluorescence intensity (fractionated in increments of 200 a.u.) in the population across the bud was derived from single-cell measurements. (c) Raw fluorescence image of a 1 mm bud (dotted line indicates the limit of the MC) taken *in situ* at low magnification, illustrating the typical closer packing of the cells towards the leading region of the bud.

Patrick & Kearns (2009) inferred from their results that dendritic migration of the laboratory strain 168 $sfp^+ swrA$ at 70 % humidity might be explained by excess water exposed on the surface on the agar in swarm plates, facilitating swimming rather than swarming. However, several lines of evidence clearly preclude this interpretation. For example, a drop of water placed on a correctly prepared B medium swarm plate does not spread, showing the absence of surface water. In contrast, on insufficiently dried plates we observed that the bacteria simply dispersed and that dendritic patterns did not form.

Importantly, we have emphasized previously (Julkowska *et al.*, 2004, 2005; Hamze *et al.*, 2009) that dendritic migration involves translocation of cells over the surface as a monolayer. Close inspection of the monolayer at high magnification showed that the bacteria were restricted to a

single focal plane (see, for example, Supplementary Movie Files S1 and S2), demonstrating that they are confined to a thin (effectively 2D) film, considered to be a typical feature of swarming bacteria (Harshey, 2003; Darnton *et al.*, 2010). Finally, when we examined the monolayer of *B. subtilis* cells (168 $sfp^+ swrA$) at the extreme 1 mm of dendrite tips, time-lapse filming revealed coordinated movements, very similar to those recently described for *E. coli* swimmers. Thus, as illustrated in Supplementary Movie Files S1 and S2 (14 frames s^{-1}), the cells formed many short-lived rafts or packs composed of around three to eight aligned cells. As shown in Supplementary Movie File S3, when cells at dendrite tips were filmed at 40 frames s^{-1} , we saw highly mobile swirling packs and large streams of cells, behaviour resembling the ‘acrobatics’ shown by *E. coli* (Copeland *et al.*, 2010; Darnton *et al.*, 2010; Turner *et al.*, 2010; McCarter, 2010). Such behaviour has been shown to be

Fig. 7. Length distribution of cells along a 1.5 cm dendrite. B medium swarm plates were prepared and inoculated as in Fig. 1, but with strain 168 *sfp*⁺ *P_{hag}-gfp* (OMG 992), and the plates were incubated until dendrites reached 1.5 cm. The length distribution of the cells was determined at the different positions indicated along the dendrite from images taken in the microscope ($\times 100$ objective), using the AxioVision software (see Methods). At least 2000 cells were measured for each position, and the data shown represent the percentage of cells with increasing cell length in successive 0.25 μm increments. Similar results were also obtained with strain OMG 984.

characteristic of bacteria suspended in a thin fluid film (Sokolov *et al.*, 2007).

DISCUSSION

Dendritic migration of *B. subtilis* 168 *sfp*⁺ *swrA* at high (70%) humidity displays all the hallmarks of swarming

The previous contradictory reports (Julkowska *et al.*, 2004, 2005; Hamze *et al.*, 2009; Patrick & Kearns, 2009) on the ability of a laboratory strain of *B. subtilis* 168 *sfp*⁺ *swrA* to swarm are resolved here with the finding that while *swrA* is required for robust migration at low humidity (also found by Patrick & Kearns, 2009), this is not required at 70% humidity, as found previously (Hamze *et al.*, 2009). Moreover, at 70% humidity, the time course of migration

and subsequent pattern formation of both the *swrA* and *swrA*⁺ forms of 168 *sfp*⁺ were similar, and migration did not involve reversion to *swrA*⁺.

Early stages of dendritic migration involve the extension of stems and tips as a monolayer of cells, apparently within a thin fluid film, with movement limited to two dimensions. On the other hand, the amphiphilic surfactin molecules, as they spread ahead of the advancing cells, presumably partition at the air–agar interface. Consequently, we suggest that the cells move over the surface in a thin layer of fluid beneath the surfactant. Indeed, this has recently been reported for *E. coli* swimmers (Zhang *et al.*, 2010), although in that case the surfactant remains to be identified. We now also demonstrate (Supplementary Movie File S3) that 168 *sfp*⁺ *swrA* cells in dendrite tips move collectively as swirling and streaming packs. This behaviour is very similar to that of the *E. coli* swimmers

described in three recent studies, all employing 90–100 % humidity (Copeland *et al.*, 2010; Darnton *et al.*, 2010; Turner *et al.*, 2010; and see review by McCarter, 2010, and movie files accessible therein). Thus, dendritic migration for strain 168 *sfp*⁺ *swrA*, which, in addition, we have shown is dependent on flagella and surfactin (Julkowska *et al.*, 2005; Hamze *et al.*, 2009), quite clearly shows all the generally accepted characteristics of swarming.

Long-chain forms

In this study, our major objective was to focus on the temporal and particularly the spatial regulation of *hag* expression *in situ* in the developing community of the laboratory strain 168 *sfp*⁺. For this we took advantage of the persisting monolayer of cells that occurs from early bud (pre-dendrite) formation to dendrites up to 1.5 cm long. As a result, as discussed below with respect to *hag* expression, we identified three distinct cell types, temporally and spatially separated.

The long-chain forms could already be detected at 10 h, and accumulated in large numbers at the edge of the MC at around 12–16 h post-inoculation, as we have previously shown for the non-domesticated strain 3610 (Julkowska *et al.*, 2004). These cells expressed high levels of *eps* (extracellular polysaccharide), but had no detectable *hag* expression. These chain forms appeared identical to the subpopulation of chaining cells in cultures of *B. subtilis* observed at the centre of swarms on rich medium (Kearns & Losick, 2003, 2005; Julkowska *et al.*, 2005). The origin of these cells has been shown in liquid cultures to be dependent upon a bistable switch (Kearns & Losick, 2005; Cozy & Kearns, 2010), which ultimately regulates the level of the transcription factor σ^D , leading to repression of flagellar and autolysin genes in the chain forms. The SinI/SinR and Slr regulators also play a critical role in this switch, involving the upregulation of extracellular matrix production (Chu *et al.*, 2006, 2008; Kobayashi, 2008; Chai *et al.*, 2009; see also Chen *et al.*, 2009), as we also found here. Since bundling of such chain forms constitutes an early step in biofilm formation (Brandt *et al.*, 2001; Kearns *et al.*, 2005; Kobayashi, 2007), we assume therefore that such cells play a similar role in building the MC at the centre of the swarm community. However, we have not ruled out an additional role in the migration phase.

Non-swarmers, apparently physically immobilized

A rather heterogeneous population of cells was seen to constitute the great majority in dendrite stems. Further studies are required to establish the properties of these cells. However, in comparison with chain forms and swarmers, these cells differed with respect to length distribution and had intermediate levels of *hag* expression and correspondingly around 12 flagella, although they were nevertheless largely immobile. We suggest that this immobility is due to the fact that these cells, present at a relatively low density

compared with tip cells, are ‘clamped’ by the high capillary forces prevailing in thin films. Thus, these cells, if physically removed from stems and resuspended in medium, become mobile. This appears to rule out a mechanism of immobilization due to the binding of EpsE to the flagellar motor (see also Supplementary Fig. S6), which occurs in *B. subtilis* biofilms (Blair *et al.*, 2008). Interestingly, front-line cells at the extreme edge of tips were also normally static, but if individuals were reincorporated into the interior as the tip advanced, they immediately became mobile (Supplementary Movie Files S1–S3). Conversely, if mobile cells became incorporated into the front line of dendrite tips, they became immobile, but then regained mobility if reincorporated as dendrites advanced. All these findings argue strongly for a physical explanation of the observed immobility of cells in dendrite stems and at the extreme edge of tips, where capillary forces should be maximal. In contrast, in the interior of dendrite tips, the closely packed hyper-flagellated swarmers, benefiting from overlapping menisci, could be subject to reduced capillary forces and therefore able to remain mobile.

Specialized swarmers

A third, spatially distinct cell type in the swarm community in strain 168 *sfp*⁺ *swrA*, detected later than the chain forms, was identified as a unimodal, monolayered population at the extreme 1 mm of tips, and was present during dendrite elongation up to at least 1.5 cm (Fig. 5). These highly mobile cells packed to a high population density and displayed hyper-expression of the *hag* gene, with, conversely, minimal levels of *comA* and *eps* expression compared with cells in the base of the dendrites. Importantly, an apparently identical subpopulation of highly mobile cells, expressing high levels of *hag*, was identified in pre-dendrite buds (Fig. 6). Therefore, we conclude that these cells, which we designate as swarmers, first spearhead the emerging buds and then constitute the tips of the elongating dendrites. However, we have no indication as yet of the mechanism that triggers and then maintains, over several generations, the characteristic properties of these swarmers.

Differentiated swarmers of *P. mirabilis* are long, filamentous, multinucleate cells with extremely large numbers of flagella, perhaps explaining their ability to migrate over 2 % agar, which is not possible for other bacteria (Henrichsen, 1972; Rauprich *et al.*, 1996; Fraser & Hughes, 1999; Harshey, 2003). In contrast, other swarmers, for example those of *E. coli*, are much smaller and only moderately flagellated (McCarter, 2010). *B. subtilis* swarmers have been poorly characterized to date, and now we present several lines of evidence suggesting that they indeed constitute a distinctive class of cells. Thus, swarmers on B medium: (i) showed elevated *hag* expression, corresponding to an approximately twofold increase in flagella; (ii) were on average smaller than non-swarmers; (iii) showed low levels of *comA* (surfactin synthesis regulation) and *eps* expression compared with cells at the base of dendrites and in the MC;

(iv) packed to a high density in dendrite tips; and (v) showed coordinated movement, forming short-lived swirling packs (Supplementary Movie File S3). Thus, in contrast to swimmers from *P. mirabilis*, *B. subtilis* swimmers are more like those described for *E. coli*, i.e. relatively small and with only moderate increases in flagella. Interestingly, the unexpectedly small size of *B. subtilis* swimmers could be consistent with a reduced growth rate, reflecting the diversion of resources to increasing mobility, but this remains to be tested.

The importance of population density

The correlation between hyper-mobility and a high population density raises the question of whether these are directly linked through cause and effect. In fact, some recent physical and modelling studies concerning the dynamics of suspensions of self-propelled organisms and their capacity to display coordinated movement may provide answers to this question. Thus, high-frame-rate filming (30–100 frames s⁻¹) of flagellated bacteria, including laboratory strains of *B. subtilis*, has shown that batch-grown cells can display, specifically at high population densities in thin fluid films, a so-called cooperative or collective motion. This behaviour includes cells forming whirls and vortices capable of generating large hydrodynamic flows (Sokolov *et al.*, 2007; Aranson *et al.*, 2007; Tuval *et al.*, 2005). Notably, the population densities employed in such studies are apparently similar (around 50% coverage) to those seen here at dendrite tips as well as those in *E. coli* swarm fronts (Darnton *et al.*, 2010; McCarter, 2010). We suggest therefore that the high population density in dendrite tips is crucial in promoting pack formation and swirling activity.

A model for dendrite elongation

Finally, we suggest a model to explain some important features of swarming migration involving the elongation of dendrites, with a role for both swimmers and non-swimmers. The swimmers establish the radiating tracks, and this is facilitated by the modification of the agar surface by surfactin. Surfactin is known to increase the wettability of the agar (Leclère *et al.*, 2006), consistent with, for example, the observed induced swelling of the agar surface on swarm plates, which effectively reduces the surface agar concentration (Banaha *et al.*, 2009). The high population density in dendrite tips promotes the high-velocity collective motion of the swimmers, which in turn provides the physical force necessary to push the dendrite tips forward. On the other hand, we propose that the non-swimmers build the dendrite by growth and multiplication. Although the model still leaves much to be explained, not least the precise significance of hyper-flagellation, the switch that triggers differentiation into swimmers, the mechanism generating the high population density of swimmers, and the nature of the process ensuring radial outward expansion of dendrites and their subsequent branching, it does provide a basis for future experiments.

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