Identification of genes required for different stages of dendritic swarming in *Bacillus subtilis*, with a novel role for *phrC*

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Highly branched dendritic swarming of *B. subtilis* on synthetic B-medium involves a developmental-like process that is absolutely dependent on flagella and surfactin secretion. In order to identify new swarming genes, we targeted the two-component ComPA signalling pathway and associated global regulators. In liquid cultures, the histidine kinase ComP, and the response regulator ComA, respond to secreted pheromones ComX and CSF (encoded by *phrC*) in order to control production of surfactin synthases and ComS (competence regulator). In this study, for what is believed to be the first time, we established that distinct early stages of dendritic swarming can be clearly defined, and that they are amenable to genetic analysis. In a mutational analysis producing several mutants with distinctive phenotypes, we were able to assign the genes *sfp* (activation of surfactin synthases), *comA*, *abrB* and *codY* (global regulators), *hag* (flagellin), *mecA* and *yvzB* (*hag*-like), and *swrB* (motility), to the different swarming stages. Surprisingly, mutations in genes *comPX*, *comQ*, *comS*, *rapC* and *oppD*, which are normally indispensable for import of CSF, had only modest effects, if any, on swarming and surfactin production. Therefore, during dendritic swarming, surfactin synthesis is apparently subject to novel regulation that is largely independent of the ComXP pathway; we discuss possible alternative mechanisms for driving *srfABCD* transcription. We showed that the *phrC* mutant, largely independent of any effect on surfactin production, was also, nevertheless, blocked early in swarming, forming stunted dendrites, with abnormal dendrite initiation morphology. In a mixed swarm co-inoculated with *phrC* + *sfp* and *phrC* + (GFP), an apparently normal swarm was produced. In fact, while initiation of all dendrites was of the abnormal *phrC* type, these were predominantly populated by *sfp* cells, which migrated faster than the *phrC* cells. This and other results indicated a specific migration defect in the *phrC* mutant that could not be trans-complemented by CSF in a mixed swarm. CSF is the C-terminal pentapeptide of the surface-exposed PhrC pre-peptide and we propose that the residual PhrC 35 aa residue peptide anchored in the exterior of the cytoplasmic membrane has an apparently novel extracellular role in swarming.

**INTRODUCTION**

Bacteria growing on a surface form multicellular communities (Shapiro, 1998; Aguilar et al., 2007) and, in nature, such biofilms are responsible for many industrial and health problems. However, detailed analysis of the development of the multilayered biofilms at the level of gene expression presents many technical challenges. Recently, we have been studying another example of a bacterial community: the process of swarming in *Bacillus subtilis*. This is a cooperative process involving mass movement over a surface on a synthetic agar medium. This process has the great advantage that key stages in
development occur entirely as a monolayer (Julkowska et al., 2004, 2005).

Swarming of *B. subtilis* is absolutely dependent upon flagella, and, under most conditions, the production and secretion of surfactin (Kearns & Losick, 2003; Julkowska et al., 2004, 2005). Surfactin presumably reduces surface tension, friction or viscosity, or modifies the agar surface to maintain a depth of fluid that is sufficient for swarming. Surfactin is a cyclic lipopeptide (Peypoux et al., 1999) that is synthesized non-ribosomally, which spreads just ahead of the migrating bacteria throughout the swarming process (Julkowska et al., 2004, 2005; Debois et al., 2008). Efficient swarming on B-medium is manifest at agar concentrations of 0.7–1 %, but is abolished at 1.5 %, where the surface film of water is presumed to be insufficient for migration of the cells.

Our previous studies have shown that both the wild-type (WT) *B. subtilis* (undomesticated) strain 3610, and the *sfp* + derivative of the laboratory strain 168, form very similarly branched (dendritic) patterns of swarming on synthetic B-medium (Julkowska et al., 2004). Moreover, at the microscopic level, this process involves the appearance of morphologically distinct types of cells and complex aggregation patterns of bacteria, in both the WT and the *sfp* + derivative. Thus, dendritic swarming appears to be a multistage developmental-like process that is likely to be controlled by extracellular signalling mechanisms that should be amenable to genetic analysis. Swarming on Luria–Bertani (LB) medium, on the other hand, following an early transient dendritic phase, primarily involves a continuous multilayered advancing front. Swarming in *B. subtilis*, as observed for biofilm formation (Branda et al., 2005), in fact appears to be capable of developing along alternative pathways. Thus, we have observed that surfactin is not essential for swarming on a rich LB medium by the 168 strain used in this laboratory (Julkowska et al., 2005). This process is distinct from the phenomenon of surfactin-dependent, but flagella-independent, ‘spreading’ or ‘sliding’. This has been described recently by Fall and co-workers (Kinsinger et al., 2003, 2005) and occurs on agar that is less-concentrated than that employed in this study. Bacteria, in fact, are capable of surface translocation using a variety of mechanisms, and we follow the suggestion of Harshey (2003) that the term swarming be reserved for rapid cooperative movement, requiring flagella, over low-concentration agar (0.6–1.0 %).

The production of surfactin depends upon the expression of the *srf*ABCD operon, which is reported, at least in liquid cultures, to be subject to multiple levels of positive and negative regulation (see Cosby et al., 1998; Marahiel et al., 1993; Hamoen et al., 2003). However, expression of *srfA* also contributes to the regulation of competence, since *comS*, a factor required for activation of the competence regulon, is embedded within the *srf* operon (D’Souza et al., 1995; Hamoen et al., 1995). Regulation of *srfA* expression in liquid cultures includes an apparent quorum-sensing control via the two-component system ComPA, which responds primarily to the level of the secreted pheromone ComX, and, to a lesser extent, to a second pentapeptide pheromone, CSF (Hahn & Dubnau, 1991; Marahiel et al., 1993; Cosby & Zuber, 1997; Cosby et al., 1998; Schneider et al., 2002). The activation of the Srf synthases A, B and C depends upon the Sfp transferase encoded by the *sfp* gene located downstream of *srfABCD* (Nakano et al., 1988; Lambalot et al., 1996; Steller et al., 2004). Studies have shown that the laboratory strain 168 carries a frame-shift mutation in *sfp* (Nakano et al., 1992) and that this prevents or slows swarming on LB (Kearns & Losick, 2003; Julkowska et al., 2005).

In a previous comparative study, the laboratory strain 168 *trp sfp*, in contrast to the non-domesticated strain 3610, completely failed to swarm on B-medium (Julkowska et al., 2005). However, a complex dendritic pattern of rapid swarming on B-medium, very similar to that displayed by 3610, was obtained when 168 *trp sfp* was restored to *sfp* +. Interestingly, the 168 *sfp* strain (and strain 1085, see below) swarmed (flagella dependent) quite effectively on LB, albeit with some reduction in speed, and with different characteristics compared with the *sfp* + derivative (see Fig. 1b). The observed swarming of strain 168 *sfp* on LB may indicate that, on this rich medium, at least under our laboratory conditions, an alternative factor produced by the bacteria, or the nature of the surface fluid, is not limiting for swarming per se, and therefore surfactin is dispensable to some extent.

Studies by Kearns and colleagues (Kearns & Losick, 2003; Kearns et al., 2004) have identified *swrB* (motility), *swrC* and *efp* (encoding resistance to surfactin and a putative elongation factor, respectively), and *cheACDY*, as required for non-dendritic swarming of *B. subtilis sfp* + on LB. However, swarming was not dependent on *mcpAB*, *tipAB* or *mcpC*, which encode chemotactic receptors. The extracellular protease Epr has also been shown to be required for swarming on LB in an apparently *sfp* – strain 168 (Dixit et al., 2002; Connelly et al., 2004). In addition, *swrA*, implicated in the regulation of flagellin synthesis via activation of sigma D, has also been shown to be required for swarming on LB (Kearns et al., 2004; Calvio et al., 2005; Kearns & Losick, 2005).

This study concerns a mutational analysis of dendritic swarming of strain 168 *sfp* + on synthetic B-medium, and, to a lesser extent, swarming on LB. We examine the role in swarming of the two-component signal-transduction system ComPA and also some relevant global regulators that are known to control the production of the surfactin synthases in liquid cultures. We were able to link the function of several genes to distinct early stages in the swarming process on B-medium, including a novel role for *phrC*. We also found that regulation of surfactin production is apparently significantly different from that operating in planktonic cells.

**METHODS**

**Strains and growth conditions.** Bacterial strains used in this study are listed in Table 1. Bacteria were grown with aeration at...
37 °C in LB medium (10 g Difco peptone l\(^{-1}\), 5 g Difco yeast extract l\(^{-1}\), 5 g NaCl l\(^{-1}\), pH adjusted to 7.0), or in minimal B-medium composed of (all final concentrations) 15 mM (NH\(_4\))\(_2\)SO\(_4\), 8 mM MgSO\(_4\), 7H\(_2\)O, 27 mM KCl, 7 mM sodium citrate, 2H\(_2\)O, 50 mM Tris/HCl, pH 7.5; and 2 mM CaCl\(_2\), 2H\(_2\)O, 1 mM FeSO\(_4\), 7H\(_2\)O, 10 mM MnSO\(_4\), 4H\(_2\)O, 0.6 mM KH\(_2\)PO\(_4\), 4.5 mM glutamic acid, 862 μM lysine, 784 μM tryptophan and 0.5% glucose were added before use (Antelmann et al., 1997). Antibiotics were added to plates at the following final concentrations: 5 μg chloramphenicol ml\(^{-1}\), 5 μg kanamycin ml\(^{-1}\), 100 μg spectinomycin ml\(^{-1}\) and 5 μg erythromycin ml\(^{-1}\) plus 12.5 μg lycormycin ml\(^{-1}\).

### Table 1. Strains used in this study

All strains were constructed in the strain 168 background, unless otherwise indicated.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference, source or construction</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS 215 (3610)</td>
<td>ΔswrA::tet</td>
<td>Kearns et al. (2004)</td>
</tr>
<tr>
<td>PY 79</td>
<td>swrA sfp</td>
<td>Kearns et al. (2004)</td>
</tr>
<tr>
<td>OI 1085</td>
<td>hisH2 metC trp scp</td>
<td>Hanlon &amp; Ordal (1994)</td>
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<tr>
<td>168</td>
<td>trpC2 swrA sfp</td>
<td>European B. subtilis Genome Consortium</td>
</tr>
<tr>
<td>OMG 900</td>
<td>trpC2 swrA amyE::sfp(^+) cat</td>
<td>Derived from FS258 provided by L. Sonenshein</td>
</tr>
<tr>
<td>OMG 901</td>
<td>trpC2 swrA amyE::sfp(^+) cat codY::erm</td>
<td>Derived from BD 2356; Piazza et al. (1999)</td>
</tr>
<tr>
<td>OMG 903</td>
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<td>Derived from plasmid pMMN238, D’Souza et al. (1995)</td>
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<tr>
<td>OMG 905</td>
<td>trpC2 swrA amyE::sfp(^+) cat [comS (Ami)]</td>
<td>Derived from QB 4398; Msadek et al. (1991)</td>
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<td>OMG 906</td>
<td>trpC2 swrA amyE::sfp(^+) cat comQ::kan</td>
<td>Derived from OMG 911</td>
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<tr>
<td>OMG 910</td>
<td>trpC2 swrA amyE::sfp(^+) cat ΔphrC::erm</td>
<td>Derived from BAL 125; Lazazzera et al. (1999)</td>
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<td>OMG 911</td>
<td>trpC2 swrA sfp ΔphrC::erm</td>
<td>Derived from JMA 70; From A. Grossman</td>
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<tr>
<td>OMG 917</td>
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<td>Derived from JH 14116; Koide &amp; Hoch (1994)</td>
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<td>This work</td>
</tr>
<tr>
<td>OMG 934</td>
<td>trpC2 swrA amyE::sfp(^+) cat ΔcomK::kan</td>
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<td>This work</td>
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<tr>
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<tr>
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</tr>
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<td>OMG 982</td>
<td>trpC2 swrA thrC::sfp(^+) erm P(_{phrC}) gfpmut3 spc</td>
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Strain construction. *B. subtilis* strains OMG 901, 903, 905, 906, 910, 917, 919, 921, 925, 934, 953, 963, 965 and 966 were constructed by transformation of chromosomal DNA from different donor strains into the *sfp*<sup>+</sup> background, as presented in Table 1. Two sources of *sfp*<sup>+</sup> were used, with the gene introduced either in *amyE* or in *thrC*. The *comS* amber mutation was introduced into strain 168 *sfp*<sup>+</sup> to create OMG 905, essentially as described (D’Souza et al., 1995), using the plasmid pMMN238, which was kindly provided by Peter Zuber (OGI School of Science and Engineering, Oregon University, Beaverton, USA). The deletion of *yveZ* was constructed by cloning the two fragments flanking *yveZ* (a *Salt–BanHI* fragment carrying *yvkN*, and a *SacI–EcoRI* fragment carrying the uraA gene) on each side of a Cm<sup>R</sup> cassette for insertion into a derivative of plasmid pMTL20E, in order to create strain OMG 964. An identical strategy was used to create OMG 954 (*Δhag*). The details for the *P<sub> amyE</sub>+* construction are described below. A fusion of *rpmGB* to *gfp*mut3 (providing a GFP marker) was constructed as follows. A 413 bp DNA fragment containing the promoter and Shine–Dalgarno regions of *rpmGB* was amplified from the chromosome using the primers sigH-up (EcoRI) (5′-CTTGTAGTCTGGAGACCGGAGGCC-3′) and *L33-dn* (EcoRI) (5′-CTTGGATATGCTTAGACCTATAAAGG-3′). The amplified fragment was digested with EcoRI and EcoRV, and cloned into plasmid pDL30-*gfp*mut3-ter, the construction of which is described below. The resulting plasmid was then used to transform strain the OMG 930 strain by double crossing-over recombination, and integration into the *amyE* locus. In order to have a second GFP marker, we used a similar strategy to clone the *P<sub> thrC </sub>* promoter of the *Escherichia coli* phage λ fused to a synthetic Shine–Dalgarno in front of *gfp*mut3, thus creating OMG 982. Strain OMG 943 (*thrC::sfp<sup>+</sup>* *amyE::P<sub> thrC </sub>-lacZ*) was constructed by transformation of chromosomal DNA from strain BAL 125 (Lazzerza et al., 1999) into the OMG 930 background. The source of all other strains is indicated in Table 1.

Construction of *P<sub> amyE </sub>+*-orf Plasmid *P<sub> amyE </sub>+*-orf was cloned by building two fragments flanking *P<sub> amyE </sub>* (an *EcoRI–BanHI* fragment carrying nucleotides −903 to −450 relative to the *sfp* translation start point) and a *PstI–HindIII* fragment carrying nucleotides −49 to +512 relative to the *sfp* translation start point) into plasmid pUC19. A Km<sup>R</sup> cassette was inserted between these fragments. This plasmid was then used to transform strain OMG 900 by double crossing-over recombination, yielding strains OMG 936 and OMG 937.

Construction of pDL30-*gfp*mut3-ter and pDL30-*gfp*ASV-ter To construct the pDL30-*gfp*mut3-ter vector, a 495 bp DNA fragment containing the termination region *ter* was amplified according to Suel et al. (2006) using the primers Ter SalI EcoRV For (5′-GGTGGCTTACGATACACACACACACTCCATC-3′) and Ter HindIII Rev (5′-GGTGTCAAGTCCTGAGTACGGGGTCTCTCCATGATG-3′). The fragment was digested with *SalI* and *HindIII*, and cloned into the plasmid pDL30 to give plasmid *ter*. The *gfp*mut3 fragment was constructed by PCR amplification of the *gfp* gene region, using psB2018 (Qazi et al., 2001) as a template and the primers gfp EcoRV For (5′-GGAAAGGATACATGATTAGAATTAGGCCAGGAGAAC-3′) and gfp-ASV Rev Sall (5′-GGTGGTTCGATATTTTGGTAT-3′). The amplified fragment was cloned into plasmid pDL30-ter at the EcoRV and Sall restriction sites to yield pDL30-*gfp*ASV-ter.

The *gfpASV* fragment was constructed by PCR amplification of the *gfp* gene region, using psB2021 (Qazi et al., 2001) as a template and the primers gfp EcoRV For (5′-GGAAAGGATACATGATTAGAATTAGGCCAGGAGAAC-3′) and gfp-ASV Rev Sall (5′-GGTGGTTCGATATTTTGGTAT-3′). The amplified fragment was cloned into plasmid pDL30-ter at the EcoRV and Sall restriction sites to yield pDL30-*gfp*ASV-ter.

Conditions for swarming experiments. For swarming on LB, 9 cm swim plates containing 25 ml medium (0.7% agar) were prepared 1 h before inoculation and dried with lids open for 15 min in a laminar-flow hood. Cultures for the swim inoculum were prepared in 10 ml LB inoculated with a single colony and shaken overnight at 37°C. The culture was diluted to an OD<sub>570</sub> of approximately 0.1 and grown at 37°C until it reached an OD<sub>570</sub> of approximately 0.2. This procedure was repeated twice and finally the culture was grown to an OD<sub>570</sub> of 1.0. The culture was diluted and 10<sup>6</sup> bacteria (10 µl) were placed at the centre of a swarm plate, dried at room temperature (10 min in a laminar-flow hood) and incubated at 37°C (relative humidity at least 45%) for the requisite time. For swarming on B-medium, cells were pre-grown as described above for LB, but cells were finally allowed to grow until T4 (4 h after the transition from exponential growth). B-medium swim plates (0.7% agar) were prepared with drying restricted to 5 min before inoculation with 10<sup>6</sup> cells in 2 µl, and incubated, without further drying, at 30°C (relative humidity 60–70%). Careful attention to the level of humidity is important for efficient swarming on B-medium. The *P<sub> amyE </sub>+*-orf containing strains were grown on swim plates in the presence of 0.5% sucrose as sole carbon source and 1% xylose. Note that mutant swarms were incubated for varying times in order to identify the terminal phenotype.

Synergy, complementation and mixed swim experiments with B-medium. Cells were pre-grown as described above, and the two strains were then mixed in the proportions indicated in the text. Swarm plates were inoculated with the mixed population and incubated as described above. To determine the frequency of fluorescent and non-fluorescent cells, images were obtained in situ with a Zeiss fluorescence microscope (×100 objective), and cells were counted manually in different fields selected at random. The results from two independent experiments (counting 3000–6000 cells in each case) were combined to give a mean value.

Swimming/motility assays. These assays were carried out on B-medium and LB, containing 0.3% agar, by inoculating cells (pre-grown in the corresponding medium, as described above) in volumes of 2 and 10 µl, respectively, at the centre of plates, and incubating at 30 or 37°C for 30 or 15 h, respectively.

Flagella-staining procedure. Cells were grown in B-medium to an OD<sub>570</sub> of 1.0. Flagella staining was carried out as described by Kearns & Losick (2003) and examined with a ×100 objective.

Imaging. Photographs of swimming plates were taken at the indicated times, using an Epson 1600 Pro scanner at a resolution of 600 d.p.i. in transparent mode. In order to detect the surfactin ring, or at early stages in the swarming process (up to 16–17 h), when the bacteria normally form only a monolayer, plates were photographed with reflected light, using the UVP Image Store 5000 system equipped with Kaiser RT1 camera with Rainbow TV Zoom lens (8–48 mm) and two lamps (Kaiser RB5000). Details of swarm structures, or at an individual cell level, were obtained at different magnifications in situ by a Zeiss stereomicroscope (LUMAR) or a phase-contrast Zeiss AxiosImager M1 fluorescence microscope (×1.25, ×5, ×20 and ×40 Neofluor objectives); both microscopes were fitted with an AxioCam camera (Zeiss). In a few experiments, high-magnification analysis was carried out under oil (×100 objective), with a microscope slide placed gently over the required portion of the swarm, ensuring that disturbance was restricted to a very few cells at the edges of dendrites. Images were captured using Axiovision software (Rel. 4.6.3), and figures were prepared for publication using Adobe photoshop software (version 7.0) and Corel Draw X3.
RESULTS

**swrA status of the swarming strain 168 sfp**, and swarming on B-medium and LB

We note that Kearns et al. (2004) and Calvio et al. (2005) showed that, in addition to the mutation sfp, the 168 strain used in their laboratories carried a frame-shift mutation in swrA, a gene implicated in regulating sigma D synthesis on LB medium (Kearns & Losick, 2005). In fact, we have previously shown that, on the synthetic B-medium, swarming of the 168 sfp strain used in this laboratory (Kunst et al., 1997) could be restored, as shown in Fig. 1(a), simply by insertion of the wild-type sfp gene (thereby allowing surfactin production). Thus, it was important to establish the status of the swrA gene in this strain. Consequently, the entire swrA locus was sequenced, including approximately 100 bp on either side of the gene. The results confirmed, as described by Kearns et al. (2004) in their laboratory strain, that the swrA gene in our 168 strain contained an insertion of a single base pair (AT) at codon 12 in a run of eight ATs.

Kearns et al. (2004) also reported the appearance of spontaneous suppressors of swrA as flares at the edge of non-swarming colonies. Sequencing confirmed that these were revertants of the swrA frame-shift mutation, including swrA intragenic suppressors and deletions that fused sigD (controlling flagellin synthesis) to the upstream codY transcription unit. However, with B-medium swarms, we did not observe such revertant flares with the 168 sfp strain (Fig. 1a), and sequencing of swrA or the sigD region of our 168 strain failed to reveal any suppressors. Therefore, we conclude that swrA is dispensable for swarming on B-medium under the conditions used in this study. Moreover, the non-domesticated strain 3610 carrying the swrA mutation, kindly provided by Dan Kearns (Department of Biology, Bloomington, USA), displayed highly branched swarming patterns similar to those of the parental swrA strain on the synthetic B-medium (Fig. 1a). Similarly, strain PY79 swrA sfp+ and another 168-related strain 1085 sfp+ (presumably also swrA), as seen with our strain 168 sfp+, showed robust swarming on LB and B-medium. On the other hand, 3610 swrA showed substantially reduced swarming on LB (data not shown). Thus, in the conditions used in this study for swarming on LB, the requirement for swrA appears strain dependent.

As shown in the composite photograph of an early-stage swarm in Fig. 1(b), in contrast to the strain used by Kearns et al. (2004), strain 168 swrA sfp used in this laboratory swarmed on LB, albeit more slowly and with different macro-characteristics compared with the 168 swrA sfp+ strain (Julkowska et al., 2005). In particular, initial swarming of the sfp+ strain was accompanied by the formation of unbranched dendrites that ultimately merged to form a continuous front. In contrast, in the mutant strain lacking surfactin, swarming commenced along a continuous front, often involving two phases of varying duration (the two inner zones in Fig. 1b, left half), followed by the formation of a third zone that developed into relatively unbranched broad finger-like projections, often along a relatively uncoordinated front.

In the studies reported below, we first examined the effect of different mutations on swarming of strain 168 swrA sfp+ on LB and, in particular, on synthetic B-medium.

**Effect on swarming of signalling mutations in the ComPA signalling pathway: comA and phrC are essential for dendritic swarming**

In liquid cultures, transcriptional activity of the srfA promoter, which determines surfactin production and competence in *B. subtilis*, is subject to multiple levels of positive and negative regulatory mechanisms (Fig. S1, available with the online version of this paper). In particular, the regulation of the srf promoter for co-expression of the genes srfA, srfC, and comA, which are required for the non-ribosomal synthesis of the cyclic lipopeptide surfactin, includes a two-component signal-transduction pathway. This is composed of ComP (histidine kinase) and ComA (response regulator), responding to at least two peptide pheromones, ComX and CSF (formed from the precursor PhrC), which accumulate in stationary phase. These presumed quorum-sensing mechanisms also positively control competence in liquid cultures, through the expression of comS, also from the srf promoter. ComS is known to displace MecA from a complex with ComK, targeting the latter for proteolysis by ClpC/ClpP (Turgay et al., 1998). The liberated transcription factor ComK then regulates a large number of genes, including flgM (flagella biogenesis) and late competence genes (Berka et al., 2002).

We first constructed isogenic strains in the sfp+ (and the sfp background, as appropriate) with mutations of genes in, or directly related to, the ComPA pathway: comQ, comX, oppD, rapC, comA, phrC, comS, mecA and comK. In swim tests, on both B-medium and LB, we found that the mecA strain, particularly on LB, showed a reduction in motility. The srfB mutant also showed some reduction in swimming, while all the other mutants showed swimming motility similar to the parental strain. However, in staining tests for flagella in cells grown in liquid B-medium, most mutants appeared to produce normal levels of flagella; these mutants included the mecA strain. The exception was srfB, which produced very few flagella in liquid culture, and this was confirmed in cells taken from an abortive srfB swarm on B-medium (data not shown).

The mutants in the sfp+ background were then examined for effects on swarming on both B-medium and LB. A representative series of the most relevant results is shown in Fig. 2. Somewhat surprisingly, when compared with the reported low level of srf expression by several of the mutants in liquid culture, with the exception of comA, all mutants, including phrC, produced a large zone of...
surfactin. This is defined as the transparent region extending from the central mother colony to the visible perimeter of the zone, and is normally detected as spreading just ahead of the swarm front (position indicated by black arrows in the relevant panels in Figs 2a and 3). Notably, comXP (and comQ, not shown), although having substantially reduced levels of expression of srf (lacZ fusion data) in liquid cultures (Hahn & Dubnau, 1991; Cosby et al., 1998; Schneider et al., 2002; Lanigan-Gerdes et al., 2007), produced large surfactin zones. Overall, these results highlighted apparently important differences between liquid cultures and cells on swarm plates, with respect to the regulation of srf expression.

Concerning the swarming profiles shown in Fig. 2, and compared with the WT, comXP, rapC and comK, together with comQ and oppD (not shown), displayed subtle alterations in the pattern of swarming on B-medium, while the comS mutant showed little change. However, all these mutants swarmed robustly with little effect on the migration speed. Presumably, therefore, ComXP, ComK, RapC, ComS, ComQ and OppD are relatively unimportant for production of surfactin in these surface communities on the synthetic medium. On LB (Fig. 2b), the comXP, comQ (not shown) and rapC mutants gave major pattern changes, while comK, comS and oppD (not shown) displayed, at most, relatively subtle pattern changes.

In contrast to the relatively minimal effects of comXP, comQ and rapC mutations, as shown in Fig. 2, the phrC mutation, and to a lesser extent mecA, blocked surfactin-dependent swarming at an early stage on both B-medium and LB, although with distinctive phenotypes. The comA mutant was also blocked in swarming at an early stage on B-medium, but swarmed relatively robustly on LB, although with a major pattern difference, compared with the WT. As indicated above, in particular on LB, the reduced swimming of the mecA mutant, consistent with a reduction in sigD expression, and therefore flagella production (Rashid et al., 1996; Liu & Zuber, 1998), may explain the limited swarming on rich medium. However, the mecA mutant displayed relatively normal swimming on B-medium, and staining indicated levels of flagella similar to those of the WT 168 strain (data not shown). The premature arrest of swarming of this mutant on B-medium is therefore likely to be due to another function of mecA that does not involve sigD-dependent transcription of hag.

Regarding the comA mutant, this was apparently, as expected, deficient in surfactin production, and this could explain the poor swarming on B-medium. However, in the case of the phrC strain, despite early cessation of migration, an extended surfactin-like zone continued to expand well beyond the swarm front (see Fig. 2a). We concluded that, although the phrC mutant might have some reduction in surfactin synthesis, an additional role for phrC in swarming, independent of surfactin production, appeared to be a possibility. To test this, we analysed the effect on swarming on LB of both phrC and comA mutations in the strain 168 sfp background, in which the surfactin synthases are not activated because of the absence of a functional sfp gene (Nakano et al., 1992). The results showed that, in this surfactin-defective background, swarming was arrested early, not only in the phrC strain, but also in the comA mutant (Fig. S2, available with the online version of this paper). Therefore, both comA and phrC appear to control additional functions required for swarming.

Fig. 2. Composite photographs of the swarming profile of different mutants in the comPA signalling pathway, and the parental strain 168 swrA sfp+ on B-medium and LB. Swarms were set up as described in Methods on 0.7% agar B-medium (a) and 0.7% agar LB (b), and incubated for 36 and 16 h, respectively. (a) Black arrows indicate the boundary of the surfactin zone for comA and phrC; other swarms reached the edge of the plates. (b) White arrows mark the limit of the bacterial swarm front for mecA and phrC. Other details are described in the text. Bar, 1 cm.
One possibility for an additional swarming function under ComA control was that comS (co-expressed with srfABCD), and therefore the late competence genes, were required for swarming. However, as shown in Fig. 2, neither comS nor comK mutants in the sfp<sup>+</sup> background were defective in swarming on either B-medium or LB. In fact, other members of the large comA regulon provide several candidates that might be required for swarming, in addition to surfactin synthases (see Discussion). The nature of a possible additional role for phrC in swarming is addressed below.

**Identification of other genes required for swarming on B-medium: yvzB, swrB, codY and abrB**

The *B. subtilis* genome contains the gene yvzB that encodes a 17 kDa protein (compared with the 32 kDa flagellin, Hag) with 41% identity in the region corresponding to the C-terminal domain of Hag. Such a protein might contribute to flagella function in some way, and therefore we constructed a strain (sfp<sup>+</sup>) with yvzB deleted. While the deletion strain swarmed apparently normally on LB (not shown), it swarmed more slowly and arrested prematurely on 0.7% B-medium agar, compared with the WT (Fig. 3). Moreover, on B-medium 0.9% agar, swarming was even more severely curtailed (data not shown).

Kearns & Losick (2003) identified a mutant swrB (ylxL) that was deficient in swarming on LB; Werhane et al. (2004) also showed that swrB had some role in motility and that it was linked to control of sigD expression. Interestingly, we found that staining for flagella indicated that very few flagella were produced by the swrB mutant on B-medium agar. When we investigated the swarming of this mutant on the B-medium, as shown in Fig. 3, it showed a novel phenotype: prematurely arrested, with characteristic curved, but stunted, dendrites.

CodY, a GTPase sensor, has been reported to be a negative regulator of the *srfA* operon (Seror & Sonenshein, 1996) and flagella synthesis (Mirel et al., 2000; Bergara et al., 2003). Therefore, we examined swarming of the sfp<sup>+</sup> strain with codY disrupted, on B-medium and LB. Swarming on LB was slower and showed a modest pattern change (data not shown). In contrast, on B-medium, as shown in Fig. 3, while swarming was accompanied by an extensive zone of surfactin, migration was halted quite early. We also tested a point mutation in codY (S215A), in which the serine residue, recently reported to be phosphorylated, was replaced by alanine (Joseph et al., 2005; Macek et al., 2007). As shown in Fig. 3, this mutant also prematurely halted swarming. In fact, with both codY mutants, the extent of swarming gave variable results in replicate experiments: swarming was either completely blocked, or gave small dendrites that prematurely arrested, or, alternatively, swarms were restricted to a single dendrite that then arrested early. This suggested that CodY and, in particular, its phosphorylation are required to sustain normal outward elongation or migration of dendrites on B-medium. Similarly to codY, abrB encodes a global regulator (Ogura et al., 2001) implicated in the negative regulation of *srfA* transcription in liquid cultures, while also repressing production of sigma H and therefore phrC expression (see Supplementary Fig. S2; Hamoen et al., 2003). As also shown in Fig. 3, an abrB mutant, although producing an extensive surfactin zone, was blocked in swarming at an early stage, failing to form dendrites.

**Correlating the function of some swarming genes with specific early steps in swarming on B-medium: high-magnification microscope analysis**

Fig. 4(a) illustrates some distinctive early stages of the normal swarming process. We are able to confirm that these stages are very reproducible and lead to the highly branched patterns obtained on the synthetic B-medium, with both the laboratory strain 168 sfp<sup>+</sup> and the non-domesticated 3610 (Julkowska et al., 2004, 2005). Thus, 7–8 h after inoculation with 10<sup>4</sup> cells, cells in the mother...
The colony (inoculum spot) began to form long septated cells that clustered or aggregated as microcolonies (see Fig. 4a1). After 11–12 h (up to six generations), secretion of surfactin was first detected as a spreading zone just ahead of the edge of the mother colony (see Fig. 4a2 and 4a3). When the surfactin zone reached about 2.5 mm from the edge of the mother colony, large groups of cells emerged from the edge of the mother colony in the form of ‘buds’ (Fig. 4a1), constituting a monolayer of quite closely packed normal-sized cells. At 12–13 h, the bud-like structures migrated outwards into the surfactin zone, but they remained tethered to the mother colony (Fig. 4a3). These structures formed the nascent dendrites that then continued to migrate as monolayers of cells up to 17–18 h post-inoculation. The dendrites were initially largely unbranched, but then at around 1.25–1.5 cm in length they began to form initial branches leading to highly branched patterns (Fig. 4a4). Note that the number of long septated chains of cells progressively increased in the mother colony, becoming the dominant form after 13–14 h. Electron microscopy and staining analyses have shown that these cells are not flagellated (unpublished data). Similar long chains of cells have been described in the early stages of B. subtilis pellicles (Kobayashi, 2007), while Kearns & Losick (2005) have identified subpopulations of similar long chains of cells in liquid cultures, apparently due to a stochastic switch inactivating sigma D, and therefore reducing transcription of an operon encoding genes for flagellin and autolysins. In swarming dendrites on B-medium, long septated cells, together with aggregations of cells, progressively appeared from 17–18 h, and gradually spread outwards from the base. This was shortly followed by multilayering of dendrites seeded by microcolony formation, and normally proceeded from the base of dendrites.
In order to assign, if possible, mutations to the different specific stages of development of dendrites on B-medium shown in Fig. 4(a), the mutants were examined at the microscopic level as described below. Concerning the surfactin non-producing sfp mutant, we previously reported that the characteristic early stage buds are formed, but the subsequent stage leading to dendrite formation on B-medium, as shown in Fig. 4(a3), did not develop (Julkowska et al., 2004). Subsequently, we discovered that this mutant was incorrectly classified, and mutants of this type (strain 168 sfp) have now been reinvestigated. In this mutant, swarming was blocked completely at a stage preced ing bud formation (Fig. 4b1). In the comA mutant, surfactin production was greatly reduced (as indicated by the relatively small surfactin zone) and, similar to the sfp mutant, no buds or dendrites developed. In fact, as shown in Fig. 2(a), in the comA mutant, non-dendritic migration bypassed bud formation and continued for some time along a uniform front, but then stopped. The hag mutant, lacking flagella, as previously shown (Julkowska et al., 2005), made a large visible surfactin-like zone, extending well beyond the mother colony, but the mutant was nevertheless blocked early in swarming on B-medium (Fig. 3). Microscope analysis (Fig. 4b2) revealed that this mutant failed to produce buds. However, in this case, a novel phenotype was observed, with large numbers of stationary small clusters of cells, present just beyond the periphery of the mother colony. These clusters may represent abortive attempts to form pre-dendrite buds. The codY mutants also formed buds, which, with a low probability, can develop into dendrites that then, nevertheless, arrest early, as shown above in Fig. 3. The process in codY mutants sometimes arrested at the (very small) bud stage, while the abrB mutant formed full-sized buds, which did not develop into dendrites. In both mutants, all the cells in the bud were progressively converted to the long septated form (Fig. 4b4 and 4b5). In the mecA and the yveB mutant (lacking a Hag-like protein), buds formed that were apparently normal (not shown), but subsequent migration of dendrites was prematurely arrested (Figs 2 and 3). The swrB (yxL) gene has been implicated in motility (Werhane et al., 2004), and, indeed, we found a substantial reduction in stainable flagella in this mutant. Nevertheless, unlike the hag mutant, bud formation appeared to be normal in the swrB mutant, but the dendrites subsequently formed were unusually curved, and arrested early. These properties of swrB, compared with the hag mutant completely lacking flagella, indicate that changes in the quantity of flagella can also have profound effects on the swarming process. Unfortunately, at present, the actual role(s) of flagella in swarming in B. subtilis is unclear.

In contrast to the abrB, codY, mecA, swrB and yveB mutants, which could all apparently form pre-dendrite buds, the phrC mutant bypassed bud formation, with dendrites commencing deep inside the mother colony (Fig. 4b3), giving a unique phenotype that is described in more detail below. Finally, we also note that, without exception, at least at some stage all the mutants displayed the switch to make long septated cells.

Evidence that phrC plays a novel role in swarming

The 40 aa PhrC peptide is translocated across the cytoplasmic membrane, apparently with no cleavage of the N-terminal signal sequence (Stephenson et al., 2003). Subsequent processing by different cell-wall-associated proteases results in the release of the CSF pentapeptide from the C terminus of PhrC (Lanigan-Gerdes et al., 2007). Studies in liquid cultures have clearly demonstrated the presence of CSF in culture supernatants (around 10 nM), and that CSF can be imported by the Opp permease into the cytosol, where it promotes activation of srf expression via competition with RapC (Core & Perego, 2003).

The microscopy analysis of the phrC mutant on B-medium, described above, indicated an apparent bypass that replaced early bud formation with a novel mechanism of dendrite initiation. Nevertheless, subsequent migration of the mutant was severely restricted. This, together with other indications that phrC may have a role in swarming that is independent of any effect on srf expression, prompted us to examine in more detail the function of phrC in swarming. First, we tested whether the level of surfactin production was a major limiting factor for swarming. For this purpose, we utilized a derivative of the phrC strain ectopically expressing the srfABCD operon constitutively from a xyl promoter, in addition to expression from the normal srf promoter. This strain (see Methods) was used to inoculate B-medium swarm plates in the presence of 1 % xylose in order to induce maximal expression from the P_{xyl–srf} promoter. The photographs in Fig. 5 show that, while additional surfactin production enhanced swarming, the migration of the phrC P_{xyl–srf} strain, compared with the isogenic phrC P_{xyl–srf} mutant, was significantly slower, branching was frequently abortive, and swarming always arrested prematurely. Overall, these results provide further evidence that the major swarming defect in the phrC mutant is largely independent of any reduction in surfactin production. We next tested whether the role of phrC in swarming depended on import of CSF via the Opp permease. In fact, the oppD mutant, like rapC that lacks the known CSF target, swarmed essentially normally, indicating that re-import of CSF is not an important factor in swarming.

A mixed swarm (synergy) experiment reveals a migration defect in the phrC mutant

In view of the above results indicating that phrC may have a novel role in swarming, possibly not involving CSF, we attempted to determine more directly whether the swarming defect of the phrC mutant could be restored or not by the extracellular supply of CSF. To do this, we carried out a ‘synergy’ experiment (Fig. 6). Thus, two swarming-defective strains (otherwise growing normally in liquid
The phrC mutant. In fact, the results proved to be more complex, as revealed when, in order to visualize individual cells, dendrites in the successful swarming community (90 % phrC) were examined at high magnification. First, dendrite initiation was clearly exclusively of the V-shaped type (data not shown), which is characteristic of the phrC mutant (see Fig. 4b3). However, unexpectedly, whereas both strains (non-fluorescent phrC and fluorescent sfp) were found in approximately the expected ratio (10:1 sfp⁺:sfp) in the mother colony and at the base of dendrites, the GFP-labelled cells were clearly in the great majority towards the extremity of the dendrite (Fig. 6c). This indicated that, in the mixed swarm, while the phrC mutant cells were able to initiate formation of at least novel (V-shaped) dendrites, these dendrites were then also populated by the sfp phrC⁺ bacteria, which subsequently outpaced the phrC cells during migration.

The above analysis provides evidence that phrC cells have a migration defect apparently independent of any change in surfactin production, the level of which, in the mixed swarm, is sufficient for normal extension and branching of dendrites by the sfp cells, once dendrite formation is initiated. Finally, since there was no apparent complementation of the phrC strain in the swarm with 90 % phrC⁺ cells, the results again suggested that CSF was not required for swarming. Two further control experiments were then carried out in order to establish more directly whether either an sfp or a phrC mutant could be successfully complemented by the isogenic WT in a mixed swarm. In these experiments, the mutant strain, together with strain 168 sfp⁺ phrC⁺ carrying a constitutively expressed gfp reporter, were mixed in different proportions and used to inoculate swarm plates. As shown in Fig. 7, in the mixed sfp⁺+sfp swarm, full complementation of the sfp defect was observed. Thus, both cell types were found in the tips of dendrites in proportions virtually identical to those in the input inoculum. Similar proportions were obtained when the middle and base of the dendrites were examined (data not shown). Thus, surfactin produced by the sfp⁺ partner in the community was available, and sufficient for swarming of the surfactin non-producing strain. This was also the case when only 10 % of inoculated cells were sfp⁺, although the overall swarming rate in this mixed swarm was slower than normal (data not shown). In the second mixed community control experiment, the phrC strain was co-inoculated with GFP-labelled WT phrC⁺ cells. An apparently normal swarm was obtained, and the ratio of GFP⁺/GFP⁻ cells in dendrites of different lengths was examined, and typical results are presented in Table 2. Strikingly, in contrast to the observed complementation of the sfp mutant described above, there was always a substantial deficit (at least 10-fold) of phrC mutant cells in all regions of dendrites, while in the mother colony the phrC cells were detected in the expected ratio (data not shown), reflecting the number of input phrC cells present in the inoculum. The results therefore confirmed those in Fig. 6 showing that any CSF generated by the phrC⁺ strain was not able to complement the swarming deficiency of the phrC mutant.

**Fig. 5.** Additional synthesis of surfactin from an xyl promoter is not sufficient to rescue swarming of the phrC mutant. (a) The phrC⁺ strain, also expressing the srfABCD operon from an xyl promoter, was allowed to swarm on a B-medium swarm plate containing 1 % xylose (which provides constitutive surfactin synthesis) for 24 h at 30 °C. (b) The isogenic phrC mutant. (c) and (d) Enlargements of sections of plates (a) and (b), respectively. The phrC mutant swarms more slowly and arrests at 2.5–3 cm, with branching of dendrites significantly reduced. In contrast, consolidation/multilayering (indicated by dark colouring close to the central regions) is accelerated in the phrC mutant. The arrows indicate the limit of the surfactin zone. Bars, (b) 1 cm, (d) 0.5 cm.
We suggest, as discussed below, that the simplest interpretation for these results is that swarming is facilitated by a cell-associated form of PhrC, rather than by free CSF.

**DISCUSSION**

Our previous studies (Julkowska et al., 2004, 2005) have demonstrated that dendritic swarming in *B. subtilis* follows a developmental-like process, which when carried out under optimal conditions, in particular with humidity between 55 and 70%, as confirmed in this study, occurs with reproducible, clearly defined stages. A major objective in this study was to identify genes involved in particular stages of this dendritic swarming process.

The control of *srfA* expression in liquid cultures is associated with a complex network of regulators (see Fig. 6). A synergy experiment with a mixed swarm, *sfp* (gfp) + *phrC*: apparent complementation of *sfp* but not of *phrC*. Swarm plates were set up as described in Methods, and incubated for 36 h on B-medium. (a) Plates were inoculated with the mutants *sfp* *phrC* (gfp) and *sfp* + *phrC*, which were both derived from 168 *swrA*. (b) Plates were inoculated with a mixture of the two strains in the indicated proportions, with the *sfp* mutant additionally expressing gfp from the ribosomal promoter rpmGB inserted into the amyE locus. The arrows indicate the surfactin ring. (c) From the swarm inoculated with 90% *phrC* (no GFP) and 10% *phrC* (gfp), fluorescent images were taken in situ at the tip, the middle and the base of the dendrite when dendrites reached about 1.5 cm (17 h). All these regions are therefore still in a monolayer. Fluorescence and visible light images are merged. The results show that, while the input is only 10% *sfp* (gfp) cells, these constitute the vast majority of cells in the tips, as they dramatically outpace the *phrC* cells. Bar, 150 μm.

**Fig. 6.** A synergy experiment with a mixed swarm, *sfp* (gfp) + *phrC*: apparent complementation of *sfp* but not of *phrC*. Swarm plates were set up as described in Methods, and incubated for 36 h on B-medium. (a) Plates were inoculated with the mutants *sfp* *phrC* (gfp) and *sfp* + *phrC*, which were both derived from 168 *swrA*. (b) Plates were inoculated with a mixture of the two strains in the indicated proportions, with the *sfp* mutant additionally expressing gfp from the ribosomal promoter rpmGB inserted into the amyE locus. The arrows indicate the surfactin ring. (c) From the swarm inoculated with 90% *phrC* (no GFP) and 10% *phrC* (gfp), fluorescent images were taken in situ at the tip, the middle and the base of the dendrite when dendrites reached about 1.5 cm (17 h). All these regions are therefore still in a monolayer. Fluorescence and visible light images are merged. The results show that, while the input is only 10% *sfp* (gfp) cells, these constitute the vast majority of cells in the tips, as they dramatically outpace the *phrC* cells. Bar, 150 μm.

**Fig. 7.** Complementation of *sfp* in a mixed swarm with WT (GFP labelled). The mixed swarm was set up in the indicated proportions (input). Swarms were incubated for 17 h until dendrites reached 1.5 cm. Images of dendrite tips were taken in situ with a fluorescence microscope, and proportions of fluorescent and non-fluorescent cells were determined (see Methods). Results are presented as the percentage of *sfp* cells reaching the dendrite tip (output). Constitutive GFP is expressed from the λ phage *P*φ inserted into amyE, in the *sfp* + strain. The many rafts of five to six aligned cells seen here typically develop in dendrite tips when swarming at this early stage is arrested some minutes following removal of the Petri dish lid. The normally dispersed, but closely packed, monolayer of cells is then rapidly converted to these pseudo-crystalline formations. Bar, 10 μm.
Table 2. A mixed swarm, \textit{phrC}×\textit{phrC}+ \textit{sfp} still shows no complementation of the \textit{phrC} (CSF) function

Strain \textit{phrC}+, with \textit{sfp} expressed constitutively from the \textit{\textit{iP}_R} promoter in \textit{amyE}, together with strain \textit{\textit{\alpha}pC}, were co-inoculated in the indicated proportions on B-medium swarm plates and incubated at 30 °C for 14 h (0.5 cm dendrites) or 17 h (1.5 cm dendrites), before subsequent counting of fluorescent and non-fluorescent cells (see Methods). Input indicates the percentage of strain \textit{\textit{phrC} in the inoculum and output is the percentage of \textit{\textit{\alpha}pC} detected in dendrite tips. The results are the means ± SD from two different experiments, in which at least two dendrites were analysed in each experiment.

<table>
<thead>
<tr>
<th>Input</th>
<th>Output (% \textit{\textit{\alpha}pC} cells)</th>
<th>Dendrite 0.5 cm</th>
<th>Dendrite 1.5 cm</th>
</tr>
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<tbody>
<tr>
<td>\textit{\textit{\alpha}pC}×\textit{phrC}+</td>
<td>Base Tip</td>
<td>Base Tip</td>
<td></td>
</tr>
<tr>
<td>10% + 90%</td>
<td>0</td>
<td>0</td>
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<tr>
<td>50% + 50%</td>
<td>1.3 ± 0.2</td>
<td>0.4 ± 0.2</td>
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<tr>
<td>90% + 10%</td>
<td>9 ± 7</td>
<td>7 ± 2</td>
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S1); however, the physiological importance of this complex network is not clear. In fact, the results of the mutational analysis revealed that genes, such as \textit{comXPQ}, \textit{phrC}, \textit{oppD} and \textit{rapC}, were apparently dispensable for surfactin production on the agar plates, and were of relatively little importance for swarming. Thus, with the exception of \textit{sfp} and \textit{comA}, all mutants, including \textit{comXP}, produced a large surfactin-like zone ahead of the swarm front on either B-medium or LB. This was surprising, since in liquid cultures, as cells enter stationary phase, the histidine kinase \textit{ComP}, activated by the endogenous signalling pheromone \textit{ComX} and, to a lesser extent, CSF (imported via the \textit{OppD} permease to inhibit \textit{RapC}) are essential for activation of \textit{ComA} to drive transcription of \textit{srfABCD} (van Sinderen \textit{et al.}, 1990; Nakano \textit{et al.}, 1991; Solomon \textit{et al.}, 2003).

Evidently, our results indicate that regulation of \textit{srf} expression during swarming appears to be significantly different from that demonstrated in liquid cultures, indicating that the physiology of the cells is quite different on swarm plates. One might also now question whether the activation of \textit{srf} expression in stationary-phase liquid cultures has any physiological relevance for a process that appears more designed for controlling motility on a surface. Since, in particular, \textit{comXPQ} were essentially dispensable for surfactin production on B-medium swarm plates, this suggests that phosphorylation activation of \textit{ComA} under these conditions might rather, for example, depend predominantly upon acetyl phosphate. This phosphate-group donor appears to be involved in activating many response regulators, at least in \textit{E. coli} (Wolfe \textit{et al.}, 2003), and one report has implicated acetyl phosphate in \textit{ComA} activation in \textit{B. subtilis} (Kim \textit{et al.}, 2001).

Interestingly, control by acetyl phosphate would link swarming directly to central metabolism. However, we cannot exclude a role for other histidine kinases in \textit{ComA} activation, although evidence for such cross-talk is rare. Alternatively, other global regulators acting directly on the \textit{srfABCD} promoter might be important regulators of \textit{srfA} transcription on swarm plates.

Importantly, in this study, from careful microscopy analysis, we were able, for what we believe is the first time, to clearly identify specific morphologically distinct stages in the development of the dendritic swarming process. This greatly facilitates genetic analysis and, consequently, we were able, as shown in Fig. 4, to assign roles for several genes to distinct early stages in the initiation of swarming. Thus, \textit{comA}, \textit{sfp} and \textit{hag} mutants failed to form the characteristic pre-dendrite buds. In the \textit{abrB} mutant, and frequently the \textit{codY} strain, dendrites arrested at the bud stage. The \textit{phrC} mutant bypassed normal bud formation, while \textit{codY}, \textit{mecA}, \textit{swrB} and \textit{yvzB} mutants formed buds that only developed short or stunted dendrites. Briefly, to recapitulate results of other mutational analyses on B-medium, \textit{comS}, \textit{comK}, \textit{comXP} and \textit{comQ} mutants showed only subtle changes in pattern formation, while mutations in \textit{oppD}, \textit{degU} (data not shown) or \textit{rapC} had relatively little effect on any aspect of swarming. On LB, mutations in \textit{hag}, \textit{phrC} and \textit{mecA} also arrested swarming early, and \textit{comXP}, \textit{comQ}, \textit{rapC} and \textit{comA} mutants all showed major pattern changes compared with the WT, while \textit{comK}, \textit{oppD} and \textit{comS} mutants displayed more subtle, if any, pattern changes. Surprisingly, we also found that swarming on B-medium, in contrast to some previous studies with swarming on LB (Kearns \textit{et al.}, 2004; Calvio \textit{et al.}, 2005), does not require \textit{swrA}. Finally, robust swarming on both LB and B-medium required \textit{hag}, \textit{mecA} and \textit{phrC}, but the requirement for swarming in the two media differed with respect to mutations in \textit{codY}, \textit{comA} and \textit{yvzB}.

Previous studies have shown that, in liquid cultures, MecA acts to regulate the action of ComK and is also implicated in control of sigma D and hence flagellin synthesis (Liu & Zuber, 1998; Rashid \textit{et al.}, 1996). However, at least on B-medium, the \textit{mecA} mutant appeared to produce numbers of flagella similar to those of the WT, as determined by staining (data not shown). This suggests other possible roles for MecA in swarming under these conditions that might include inappropriate expression of the ComK regulon (Hahn \textit{et al.}, 1995). Interestingly, we also identified a putative novel flagellin (encoded by \textit{yvzB}), which we showed is essential for normal swarming on B-medium. However, for the moment, we have no indication how or whether the \textit{yvzB} product might contribute to flagella formation or function.

In liquid cultures, CodY is a negative regulator of \textit{srfA} expression (Serror & Sonenshein, 1996). Nevertheless, the \textit{codY} mutants did not show a detectable increase in surfactin production on B-medium swarm agar, as indicated by the normal speed of migration and size of the spreading surfactin zone, therefore suggesting another role in swarming for this GTP sensor CodY. Interestingly, since the \textit{codY} S215A mutant displayed a similar swarming
defect to the knockout strain, we suggest that, in particular, the phosphorylated form of CodY has a specific role in swarming. Like codY, abrB negatively regulates srfA transcription in liquid cultures, while DegU is a positive regulator (Amati et al., 2004; Mäder et al., 2002). CodY, AbrB and DegU play a role in B. subtilis pellicle formation, and all are required for robust swarming of ATCC 6051 on a rich medium (Kobayashi., 2007; Verhamme et al., 2007). However, while we found that codY and abrB were essential for dendritic swarming, degU was not required (data not shown).

We have demonstrated that comA blocks swarming early on B-medium in the sfp+ strain, and surfactin production is limited to a small zone beyond the mother colony. However, surfactin-independent swarming on LB was also blocked in an sfp comA mutant. This indicated a possible dual role in swarming for this response regulator, which controls the synthesis of many proteins (Comella & Grossman, 2005), including some involved in exopolysaccharide synthesis; these proteins are likely to be candidates for a role in swarming. Intriguingly, in this study, we also obtained evidence for a novel role for the phrC gene that encodes the pheromone CSF. In liquid cultures, at least three different functions have so far been ascribed to this pentapeptide. At high concentrations, CSF, which is released from the PhrC propeptide after translocating the cytoplasmic membrane, stimulates sporulation, while also inhibiting ComA action in some way (Perego, 1997; Jiang et al., 2000; Solomon et al., 1996; Lazazzera et al., 1997, 1999). However, the only well-established function of CSF, acting at low concentration, involves its re-importation through the Opp permease and subsequent inhibition of RapC and therefore increased srf expression. In fact, under swarming conditions, we could find no clear evidence that surfactin production in the phrC mutant was limiting for swarming. Thus, we propose that phrC has a novel role in swarming, supported by evidence that (i) surfactin-independent swarming on LB by the sfp mutant required phrC, (ii) the phrC mutant was able to supply sufficient surfactin for normal swarming of the sfp strain in the mixed swam synergy experiment, but a migration defect in the phrC cells was revealed; (iii) a phrC strain expressing low amounts of surfactin exclusively from P_{srf}-srf swarmed slowly, but had none of the characteristic features of the phrC mutant (unpublished data). If phrC has a novel role in swarming, our evidence appears to show that this role was not fulfilled by CSF. Thus, in addition to the fact that the Opp permease plays no significant role in swarming, the phrC mutant in a mixed swam could not be complemented by the WT, even though, in liquid cultures, exogenous CSF can be readily taken up by phrC cells to promote ComA activation (Lazazzera et al., 1997). We cannot rule out the possibility that extracellular CSF generated under swarming conditions is, in some way, unable to diffuse freely to the surface of phrC cells. However, we prefer the alternative idea that the active factor required for swarming is cell-associated PhrC.

Another study has indicated that the PhrC signal cleavage site may be non-functional, and, indeed, PhrC was not cleaved by five known signal peptidases, either in vivo or in vitro (Stephenson et al., 2003). In contrast, Lanigan-Gerdes et al. (2007) showed that the PhrC propeptide could be cleaved to release CSF from the extreme C terminus by any one of three secreted proteases. Therefore, our results are consistent with a role in swarming for the residual PhrC 35 aa residue peptide (including the signal sequence), which should be exposed on the exterior of the cytoplasmic membrane. This anchored form of processed PhrC would clearly not be diffusible. For the moment, we have no indication what might be the function of a membrane-anchored PhrC peptide, but it is possible to speculate that interaction with other surface proteins implicated in migration could be involved.

This study has demonstrated that B. subtilis swarming as a monolayer provides a system amenable to genetic analysis and following cell fate in situ during the developmental-like process. Moreover, this system is ideal for quantitative analysis of gene expression at the single-cell level, and the spatiotemporal analysis of the expression of swarming genes is now in progress. Our initial studies indicate, interestingly, that phrC expression is clearly maximal in the mother colony and the base of dendrites, consistent with the requirement for phrC early in dendrite establishment.

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