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Editor's Choice

Surface swarming motility by *Pectobacterium atrosepticum* is a latent phenotype that requires O antigen and is regulated by quorum sensing

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We describe a previously cryptic phenotype associated with the opportunistic phytopathogen *Pectobacterium atrosepticum* (Pca): surface swarming. We found that when Pca was spotted onto plates containing <0.5 % (w/v) agar, the culture produced copious amounts of extracellular matrix material containing highly motile cells. Once produced, this 'slime layer' spread rapidly across the plate either as an advancing front or as tendrils. Transposon mutagenesis was used to identify mutants that were affected in swarming. Hypo-swarmer mutants mostly carried insertions in a horizontally acquired island (HAI5), which encodes a cluster of genes involved in O antigen biosynthesis. Hyper-swarmer mutants mostly carried insertions in *hexY*, a known antagonist of the class I flagellar master regulator, $FlhD_4C_2$. In addition, we found that the nucleoid protein, histone-like nuclear structuring protein 2 (H-NS2), also regulated swarming behaviour. A mutant in which *hns2* was overexpressed displayed a hyper-swarming phenotype, whereas a mutant in which the *hns2* ORF was inactivated had a hypo-swarming phenotype. Swarming was also regulated by quorum sensing (QS) and by the carbon source being utilized. We show, using a range of epistasis experiments, that optimal swarming requires both motility and O antigen biosynthesis, and that H-NS2 and QS both promote swarming through their effects on motility.

INTRODUCTION

Pectobacterium atrosepticum (Pca) is a member of the *Enterobacteriaceae*. Pca is an opportunistic phytopathogen which causes damage to a number of crops, most importantly potato in temperate climates (Charkowski *et al.*, 2012; Mole *et al.*, 2007). Indeed, Pca was recently voted to be among the top 10 plant-pathogenic bacteria worldwide by experts in the field (Mansfield *et al.*, 2012). Pca can cause potato blackleg, rotting of the stems in the field, and also leads to loss of tubers in storage. The generic mechanisms by which Pca effects these outcomes are now reasonably well established. The organism secretes a welter of exceptionally active plant cell wall degrading exoenzymes (PCWDEs) including pectate lyases, pectinases, cellulases and proteases (Hugouvieux-Cotte-Pattat *et al.*, 1996). Collectively, these agents digest plant cell walls, leading to osmotic lysis of the host cells and the release of nutrients (cell wall carbohydrates and amino acids) which support microbial growth. The synthesis and secretion of PCWDEs is under the control of quorum sensing (QS) (Jones *et al.*, 1993; Pirhonen *et al.*, 1993; Smith *et al.*, 2006; Barnard *et al.*, 2007). Pca encodes an *N*-acylhomoserine lactone synthase called ExpI, which synthesizes 3-oxo-hexanoyl-L-homoserine lactone (OHHL) (Bainton *et al.*, 1992a, b). As the population cell density of the culture increases, so too does the concentration of OHHL, until it reaches a critical threshold value. At this point PCWDE production is strongly activated [or more strictly, de-repressed (Charkowski *et al.*, 2012; Smith *et al.*, 2006; Barnard *et al.*, 2007)].

In addition to PCWDEs, Pca also encodes a number of other virulence determinants and factors important for survival *in planta*. These include pathways for the synthesis of phytotoxic secondary metabolites such as coronofacic acid (Bell *et al.*, 2004), siderophores (Bell *et al.*, 2004; Monson *et al.*, 2013), and genes involved in attachment/agglutination (Bell *et al.*, 2004; Pérez-Mendoza *et al.*, 2011a, b). Some of these virulence and adaptation functions are encoded within the 'core' genome of Pca, while others are encoded on the horizontally acquired

Abbreviations: ECA, enterobacterial common antigen; HAA, hydroxyalkanoic acid; HAI, horizontally acquired island; H-NS, histone-like nuclear structuring protein; OHHL, 3-oxo-hexanoyl-L-homoserine lactone; Pca, *Pectobacterium atrosepticum*; Pcc, *Pectobacterium carotovorum*; PCWDE, plant cell wall degrading exoenzyme; qRT-PCR, real-time reverse-transcription PCR; QS, quorum sensing; Tn, transposon.

One supplementary table is available with the online version of this paper.

islands (HAIs). In addition, Pca displays vigorous swimming motility. As in many other motile Gram-negative organisms, flagella synthesis is regulated by proteins encoded by the class I flagellar genes, *flhD* and *flhC*. Based on the *E. coli* paradigm, FlhD and FlhC form a hexameric complex ($FlhD_4C_2$) which stimulates expression of the class II flagellar genes (encoding the hook and basal body). The class II regulon also includes *fliA*, an alternative sigma factor which is required for expression of all class III genes (encoding the flagellar filament, chemotaxis machinery and motor switch complex proteins). In addition, in *Pectobacterium carotovorum* (Pcc, closely related to Pca), $FlhD_4C_2$ is also required for optimal production of many PCWDEs (Cui *et al.*, 2008; Chatterjee *et al.*, 2009). $FlhD_4C_2$ accomplishes this by negatively regulating the expression of a global repressor of PCWDE production, *hexA*, (Cui *et al.*, 2008) and by activating the expression of several positive regulators of virulence, including *gacA*, *rsmB* [which encodes an RNA antagonist of RsmA (Cui *et al.*, 2008)] and *hexY* [also known as *rsmC* (Chatterjee *et al.*, 2009)]. The *hexY* gene encodes a small (14 kDa) protein that is thought to bind and sequester $FlhD_4C_2$, thereby antagonizing the function of the latter. Consequently, *hexY* mutants are hypermotile and overexpress PCWDEs (Shih *et al.*, 1999; Cui *et al.*, 1999).

We describe a new QS-regulated behavioural phenotype of Pca: surface-associated swarming. This swarming phenotype is associated with the exuberant production of surfactant on agar surfaces. To the best of our knowledge, this form of social motility has not been reported – and has certainly not been characterized in detail – in the *Pectobacteria* before. Surface swarming was found to be dependent upon a cluster of O antigen-encoding genes located on a horizontally acquired island in the Pca genome, HAI5. In addition, transposon mutagenesis was used to identify novel regulators of the phenotype. These regulators seem to work primarily by modulating factors that affect motility.

METHODS

Preparation of agar plates and observation of swarming. As reported by many other groups working on the swarming phenotype, in order to obtain consistent results it was important to prepare the plates following a strict standard procedure. Unless otherwise stated, we routinely used 0.4× MacConkey agar base supplemented with 0.5–1 % carbon source and any indicated supplements (e.g. OHHL). Once poured, the plates were left with the lids off to solidify in a laminar flow cabinet for 15 min. As soon as the medium had solidified, aliquots (2–5 µl) of overnight culture were spotted onto the surface of the plate and allowed to adsorb for 10 min with the lid of the plate off. The plates were then incubated at 25 °C in a humidified container to develop. Typically, the wild-type [Pca strain SCRI1043 (Bell *et al.*, 2004)] displayed robust swarming after 48 h.

Transposon mutagenesis. Transposon (Tn) mutagenesis was carried out as described by Williamson *et al.* (2008), except that we used TnDS1028*lacZ*::Cm. The sites of transposon insertion were determined by random-primed PCR as described by Williamson *et al.* (2008).

RNA extraction and qRT-PCR. RNA was extracted from cells grown on the surface of plates containing 0.35× MacConkey agar base supplemented with 0.5 % (v/v) glycerol. The cells were scraped off, transferred into RNAlater solution (Ambion) and incubated on ice for 1 h before sedimentation by centrifugation at 3200 ***g*** for 15 min, 4 °C. Cell pellets were incubated in 1 mg ml^{-1} lysozyme for 15 min at room temperature and total RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The resulting RNA (200 ng) was utilized as a template for reverse transcription and conversion into cDNA, as described by Chung *et al.* (2013). Real-time reverse-transcription PCR (qRT-PCR) samples were prepared with diluted cDNA (1 : 5 in water) as a template and primers specific to the specified genes (primer sequences are given in Table S1, available in *Microbiology* Online). All qRT-PCR analyses were carried out on samples from at least three independent cultures. Representative results are shown.

RESULTS

Wild-type Pca displays swarming motility

Pca has been shown to be highly motile in liquid media and in semi-solid agar (Shih *et al.*, 1999), but has never been reported to display surface swarming motility. During the course of experiments aimed at examining carbon source utilization by Pca, we noticed that when cultures of wild-type Pca were spotted onto half-strength MacConkey base agar supplemented with 0.5 % (v/v) glycerol, the resulting colonies developed a slimy, 'mucoid' appearance, and spread rapidly from the point of inoculation across the surface of the plate (Fig. 1a, left). This was accompanied by a pronounced colour change in the plates. Initially, the growing Pca colonies appeared pink/red and were surrounded by a zone of red precipitated bile, indicative of ongoing glycerol fermentation. However, once the colony began to exhibit signs of mucoidy it spread away from the point of

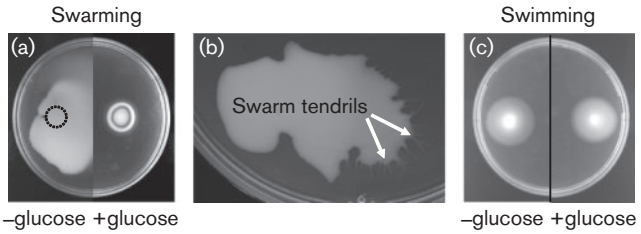

Fig. 1. Surface swarming and swimming phenotypes in the absence and presence of glucose. (a) Surface swarming was evaluated on 0.35× MacConkey agar base supplemented with 0.5 % (v/v) glycerol with or without 1 % (w/v) glucose as a carbon source, as indicated. (b) Close-up image of the edge of a wild-type swarm on glycerol-containing plates. (c) Swimming was evaluated in LB plates containing 0.35 % (w/v) agar with or without 1 % (w/v) glucose, as indicated. Swarm plates were allowed to develop for 48 h at 25 °C. Swim plates were photographed after 20 h.

inoculation, and the agar in the vicinity of the advancing slime layer turned yellow (indicative of peptone utilization and subsequent alkalinization of the agar).

Surface spreading was optimal when the MacConkey agar base medium was diluted to 0.35–0.5 ×; no mucoidy was seen on full-strength MacConkey/glycerol medium (containing 1.5 %, w/v, agar). We also observed surface spreading on some other agar formulations (not containing bile salts) such as Eiken agar (Eiken Chemical Company, Japan), as long as the agar concentration was low. This observation was reminiscent of the conditions required for optimal surface swarming motility reported in many previous studies for other organisms (Partridge & Harshey, 2013; Kearns 2010). Consistent with this, close inspection of the swarm edge revealed distinct tendrils (Fig. 1b) and microscopic examination of an aliquot taken from the edge of an advancing slime layer revealed that the sample was teeming with motile cells (data not shown).

Swarming by Pca is carbon-source-dependent

In addition to glycerol, the plant cell wall components galacturonic acid (1 %, w/v) and polygalacturonic acid (1 %, w/v) also supported vigorous swarming on 0.5 × MacConkey agar plates. However, other sugars such as arabinose, rhamnose and glucose (each at 1 %, w/v) did not support swarming. Indeed, glucose strongly suppressed swarming when added to glycerol-containing MacConkey agar (Fig. 1a). By contrast, swimming motility was unaffected by the addition of glucose (Fig. 1c).

A horizontally acquired genomic island, HAI5, encodes genes required for swarming by Pca

The obvious mucoid colony morphotype and yellow colouration associated with swarming on MacConkey agar plates provided a simple visual screen for mutants affected in this phenotype. Wild-type Pca was mutagenized with TnDS1028*lacZ*::Cm [derived from TnDS1028 (Williamson *et al.*, 2008) but with a *lacZ* cassette cloned into the *Kpn*I site (N.R. Williamson and G.P.C. Salmond, unpublished data)] and the resulting Cm^R mutants were screened visually on 0.5 × MacConkey/glycerol agar. Of approximately 12 200 mutants screened, 11 displayed strongly diminished swarming. Random-primed PCR/sequencing was used to identify the Tn insertion site in each mutant. Notably, nine of the 11 insertions were located within a single horizontally acquired island, HAI5, encoding a cluster of genes (ECA1416–ECA1442) involved in O antigen biosynthesis. The two other hypo-swarmers were disrupted in *waaL2* (ECA0161; unlinked to the HAI5 cluster, and encoding an O antigen ligase responsible for linking O antigen and enterobacterial

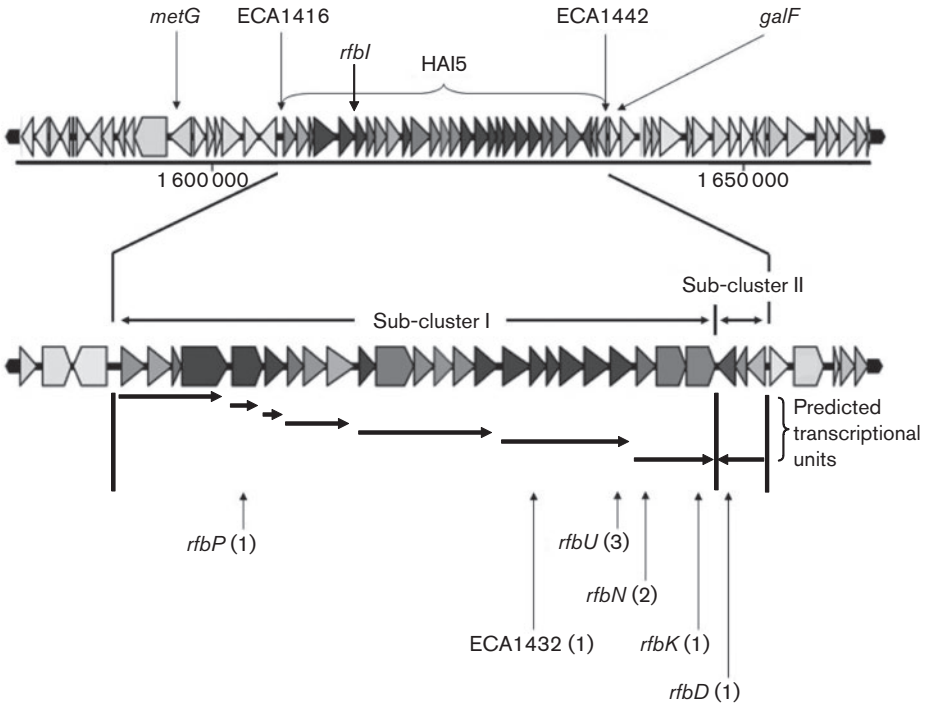

Fig. 2. Genomic context of HAI5 and the location of Tn insertions in this locus. The figure shows the region of the chromosome containing HAI5 (ECA1416–ECA1442), flanked by two representative 'marker genes' (*metG* and *galF*). Note the darker shading of the HAI5 ORFs (indicating a higher AT content). Numbers in parentheses indicate the number of insertions obtained in the indicated ORF. Transcriptional units were predicted using BioCyc.

common antigen to the LPS core) and *hns2* (see below). The genetic organization of HAI5 is shown in Fig. 2. This AT-rich cluster of genes comprises two convergent subclusters. Subcluster I encodes 24 predicted ORFs (*wecA* (ECA1416)→*rfbK* (ECA1439)) organized into multiple predicted transcriptional units (Fig. 2). Subcluster II encodes 3 ORFs (*rfbA* (ECA1442)→*rfbD* (ECA1440)) in one predicted transcriptional unit.

The motility behaviour of the *waaL2* and *rfbP* mutants (compared with the wild-type) is shown in Fig. 3a (swimming) and Fig. 3b (swarming). Although the HAI5 and *waaL2* mutants showed varying degrees of swimming motility in soft agar, they failed to swarm; it seems that motility alone is not sufficient to promote swarming. None of the genes encoding the enterobacterial common antigen [encoded by *wecA*→*wecG* (ECA4210–ECA4200)] were hit in our screen, indicating that O antigen biosynthesis is likely to be the major contributor towards the observed swarming phenotype. In a previous study, Evans *et al.* (2010) showed that a mutant in the *rfbI* gene (encoding CDP-6-deoxy-delta-3,4-glucoseen reductase) of HAI5 was less susceptible to infection by an LPS-dependent bacteriophage and also lacked O antigen (although it retained the enterobacterial common antigen). It therefore appears that HAI5 encodes the main O antigen biosynthetic cluster in Pca. Furthermore, Evans *et al.* (2010) also pointed out that the *rfbI* mutant displayed impaired virulence *in tuber*, suggesting that surface swarming may play a direct role in pathogenesis.

O antigen biosynthetic clusters generally contain three classes of gene; those involved in nucleotide sugar biosynthesis, glycosyltransferases and genes involved in O antigen processing. Six of the nine insertions in HAI5 disrupted glycosyltransferase-encoding genes; *rfbU*, encoding a probable mannosyltransferase, contained three independent Tn insertions, *rfbN* (which encodes a likely rhamnosyltransferase) was hit by two Tn insertions and ECA1432 (encoding a putative glycosyltransferase) was hit by one insertion (Fig. 2). The remaining insertions were in *rfbK* (encoding a phosphomannomutase), *rfbP* (encoding an undecaprenyl-phosphate galactose phosphotransferase) and *rfbD* (encoding a putative dTDP-4-dehydrorhamnose reductase). The *rfbD* gene is the last of a tricistronic operon (subcluster II of HAI5) also encoding *rfbA* (alternatively known as *rmlA*, a glucose-1-phosphate thymidylyltransferase) and *rfbC* (*rmlC*; a dTDP-6-deoxy-D-glucose-3,5 epimerase). Together, *rfbA*, *rfbC* and *rfbD* encode a pathway that is partially responsible for the synthesis of dTDP-L-rhamnose, a biosynthetic precursor of O antigen. In order to synthesize dTDP-L-rhamnose fully, the pathway also requires dTDP-D-glucose 4,6-dehydratase (RmlB), which is absent from subcluster II. In Pca, the nearest homologue of *rmlB* (E-value 10^{-117}) is encoded by gene ECA4206 (also known as *rffG*), part of the unlinked enterobacterial common antigen cluster. In *E. coli*, *rffG* has been shown functionally to complement an *rmlB* mutant (Marolda & Valvano, 1995). The terminal position of the *rfbD* gene in subcluster II meant that the *rfbD* mutant lent itself to facile complementation. Therefore, and to confirm the importance of *rfbD* in swarming, we complemented the *rfbD* mutant with a low-copy vector (pWKS30) in which *rfbD* was expressed from the plasmid-encoded *lac* promoter. As shown in Fig. 3c, this restored swarming to wild-type levels. We conclude that the O antigen biosynthetic cluster located on HAI5 encodes functions essential for optimal swarming by Pca.

With the exception of the mutant in *rfbK* (the terminal ORF in subcluster I) the HAI5 mutants all displayed pronounced aggregation when liquid cultures were allowed to sit static on the bench for a few minutes (Fig. 4). Presumably, this phenotype is related to the altered cell surface properties of these mutants.

Swarming is regulated by HexY

In addition to the hypo-swarming LPS mutants described above, we also identified a large number of mutants that had a hyper-swarmer phenotype compared with the wild-type. Five of these mutants (denoted NH3C, SB1I, SB4B, SB5R and SB3K) immediately drew our attention because they spread so effusively that the surface of the plate rapidly became flooded; i.e. they were superswarmers. The swarming behaviour of NH3C is shown in Fig. 5(a).

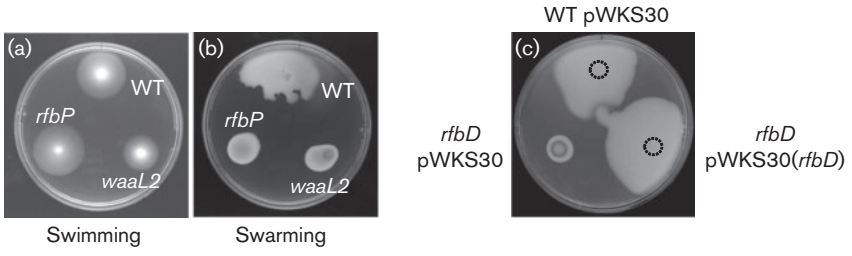

Fig. 3. Motility phenotypes of selected mutants defective in swarming. The figure shows the swimming (a) and swarming (b) phenotypes of the *rfbP* and *waaL2* mutants compared with the wild-type (WT). (c) Swarming behaviour of the wild-type containing the low-copy vector pWKS30, the *rfbD* mutant (NH3E) containing pWKS30, and NH3E containing pWKS30(*rfbD*). Motility phenotypes were assayed as described in the legend to Fig. 1, except that in (c) the plate also contained 50 μg ml^{-1} ampicillin.

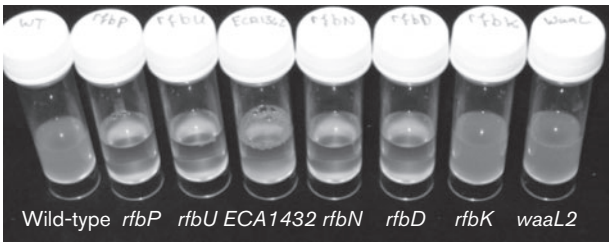

Fig. 4. Auto-aggregation of HAI5 mutants. LB-grown aerated cultures of the indicated mutants and the wild-type progenitor were allowed to sit without agitation for a few minutes before photographing. Note how many of the mutants auto-aggregate and settle.

These mutants also displayed hyper-motility in swim agar plates (e.g. Fig. 5b for NH3C). This hypermotile phenotype was reminiscent of that reported previously for *hexY* mutants of Pca (Shih *et al.*, 1999). Consistent with this, PCR-based mapping and sequencing of the transposon insertions revealed that four of the five superswarmer mutants contained insertions that disrupt the *hexY* transcript. Mutants SB1I and SB5R contained insertions within the *hexY* ORF. The *hexY* ORF itself is relatively short (390 bp) but is preceded by a long (525 bp) 5′ untranslated region. Insertions in this 5′ region have been shown previously to lead to loss of gene function (Shih *et al.*, 1999). Mutants SB4B and NH3C contained insertions in this upstream region. With the exception of SB4B, all of the *hexY* mutants also overproduced secreted protease on bioassay plates (data not shown).

HexY is thought to act by binding $FlhD_4C_2$, thereby preventing the latter from activating the transcription of *gacA* and *fliA*. If this mechanism is also involved in regulating surface swarming, we would predict that overexpression of *flhD*/*flhC* might out-titrate the endogenous HexY and thereby phenocopy the *hexY* mutant. This was indeed the case; arabinose-induced expression of *flhD*/*flhC* from the tightly controlled P_{ara} promoter on pBAD30 led to precocious and effusive surface swarming (Fig. 5c).

Surface swarming is influenced by the Rcs phosphorelay system

In many organisms, perturbations affecting the cell surface – including alterations in O antigen structure – can be

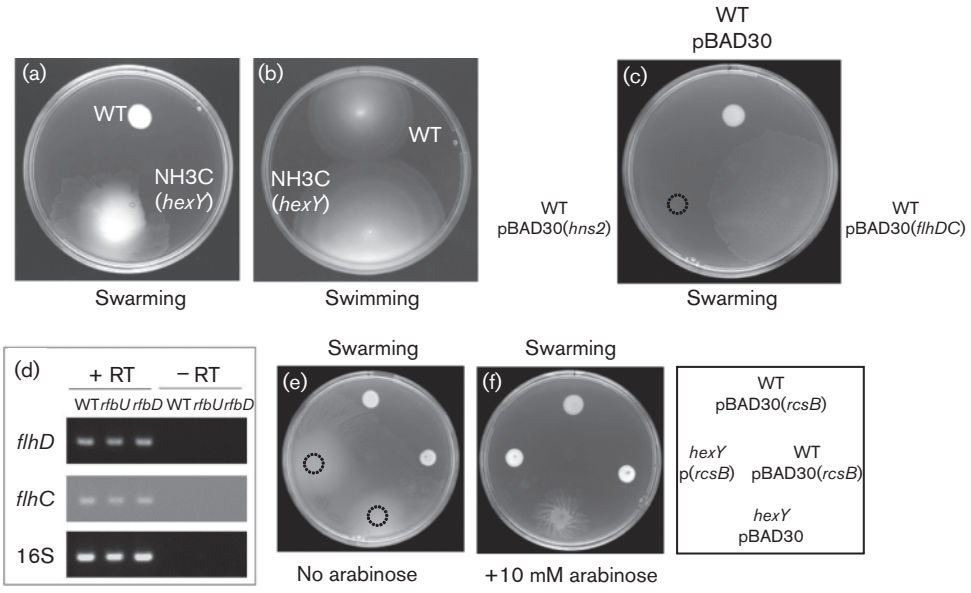

Fig. 5. Characterization of a superswarmer *hexY* mutant. Swarming (a) and swimming (b) phenotypes of the *hexY* mutant NH3C. Note that due to the precocious swarming behaviour of the *hexY* mutant, the swarm plates were photographed after overnight incubation and not 48 h incubation. The wild-type (WT) colony had not yet begun to swarm at this stage. (c) Expression of *flhDC* from pBAD30 in wild-type Pca promotes swarming. The plate contained 10 mM arabinose to induce expression of the cloned gene. The plate also contains a spot of wild-type Pca containing pBAD30(*hns2*). Note that induction of *hns2* expression from pBAD30 prevented cell growth. The plate was photographed after overnight incubation (at which point the wild-type colony had not yet begun to swarm). (d) qRT-PCR analysis of *flhD* and *flhC* transcript levels and (as a control) 16S rRNA levels in the wild-type, *rfbU* (NH1B) and *rfbD* (NH3E) mutant. RNA was extracted from cells grown on the surface of plates containing 0.35× MacConkey agar base supplemented with 0.5 % (v/v) glycerol. The cells were scraped off and harvested after 48 h at 25 °C. '±RT' indicates whether the extracted RNA was reverse transcribed (RT) or not. (e,f) Suppression of swarming by Rcs signalling. The wild-type and *hexY* mutant (NH3C) contained either pBAD30 alone or pBAD30(*rcsB*), as indicated. In (f) expression of *rcsB* from pBAD30 was induced with 10 mM arabinose.

detected by a sensor kinase (RcsC) which, in turn, phosphorylates a response regulator (RcsB). In combination with RcsA, phospho-RcsB directly represses *flhD*/*flhC* expression and decreases motility (Francez-Charlot *et al.*, 2003; Andresen *et al.*, 2010). We therefore wondered whether the mutants in HAI5 might be swarm-defective because their altered cell surface (O antigen) properties were activating the Rcs signalling pathway. If this were so, we would predict that *flhD* and *flhC* transcript levels might be lower in the HAI5 mutants than in the wild-type. However, this was not the case (Fig. 5d). This may suggest that (i) Rcs signalling is not involved, or (ii) Rcs signalling is involved but *flhDC* is not the principle target of RcsB~P action in these conditions. Consistent with the notion that Rcs signalling does play a role in regulating Pca swarming, we note that overexpression of *rcsB* (from pBAD30) could suppress the exuberant swarming phenotype associated with the *hexY* mutant (Fig. 5e, f). Moreover, one of the many hyper-swarmer mutants that we isolated contained a Tn insertion in *rcsD*. RcsD acts as a phosphotransfer intermediary between RcsC and RcsB (Majdalani & Gottesman, 2005). Consequently, Rcs signalling should be blocked in the *rcsD* mutant. Therefore, the hyper-swarming phenotype of the *rcsD* mutant (NH1A4E) strongly suggests that the cell envelope integrity (Rcs) signalling pathway is 'on' in our assay conditions and is important for optimal swarming.

Swarming motility requires functional flagella

Since $FlhD_4C_2$ activates flagella gene expression, we next examined the role of motility in Pca swarming. FliC encodes the main flagella filament protein and consequently, a mutant in *fliC* is non-motile (Fig. 6a). Notably, the *fliC* mutant also failed to swarm (Fig. 6b) and we were unable to bypass this swarming defect by overexpressing *flhD*/*flhC* from pBAD30 (Fig. 6c). Coupled with our earlier observation that *rfb* mutants are motile but swarm-defective (Fig. 3), this result indicates that motility is a necessary but not sufficient prerequisite for optimal swarming. To investigate this further, we examined whether overexpression of *flhD*/*flhC* could bypass the swarming defect in the *rfbK* and *rfbP* mutants. It did (Fig. 6d, e), however, the resulting swarms were 'dry' and non-mucoid, indicating that although *flhD*/*flhC* overexpression can compensate for the swarm defect, it does not strictly complement it.

The nucleoid protein, H-NS2, regulates swarming behaviour

Unlike the *hexY* mutants, the fifth superswarming mutant (SB3K) did not display enhanced production of secreted protease (data not shown). Sequence analysis of the DNA flanking the transposon insertion in SB3K revealed that it was located 297 bp upstream of the translational start of *hns2*. This was unexpected since we had also isolated a mutant (NH3B) carrying an insertion located 204 bp within the *hns2* ORF and this had the opposite phenotype, abolishing swarming (Fig. 7a). H-NS is a histone-like nucleoid structuring protein (Dorman, 2004; Fang & Rimsky, 2008) and is known to repress the transcription of around 5 % of genes in *E. coli* (Hommais *et al.*, 2001). The Pca genome encodes three *hns* homologues: *hns1* (ECA1665), *hns2* (ECA2328) and *hns3* (ECA2893). The *hns1* and *hns3* genes are located on the AT-rich genomic islands HAI7 and HAI13, respectively. By contrast, *hns2* is located adjacent to *galU* within the core genome. It has been proposed that H-NS may act generically as a 'genome sentinel' by silencing the expression of genes on horizontally acquired AT-rich genomic islands (Ali *et al.*, 2012). In this regard, we note that the O antigen-encoding genes are located on one such AT-rich island, HAI5. However, since the *hns2* knockout mutant, NH3B, had a hypo-swarmer phenotype, it seems unlikely that H-NS2 acts by repressing the expression of O antigen biosynthetic genes on HAI5. Consistent with this, levels of two representative HAI5 transcripts (*rfbA* and *rfbP*) were similar in surface-grown cells of SB3K, NH3B and the wild-type (Fig. 7e). Instead, and noting that there was a strong correspondence between the swarming and swimming phenotypes of SB3K and NH3B (Fig. 7a, b) it seems more likely that H-NS2 elicits

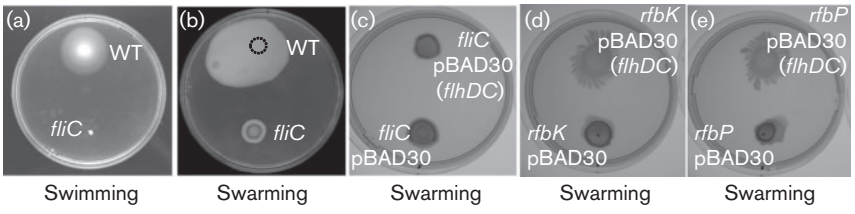

Fig. 6. Motility is required for optimal swarming. The swimming (a) and swarming (b) phenotype of the wild-type (WT) and *fliC* mutant. (c) Expression of *flhDC in trans* does not bypass the swarming defect in the *fliC* mutant. The plate contained 10 mM arabinose to induce expression of *flhDC* from pBAD30. Expression of *flhDC in trans* can partially bypass the swarming defect in the *rfbK* (NH3J) and *rfbP* (NH2D) mutants (d) and (e). The plates in (c)–(e) each contained 10 mM arabinose to induce expression of *flhDC* from pBAD30.

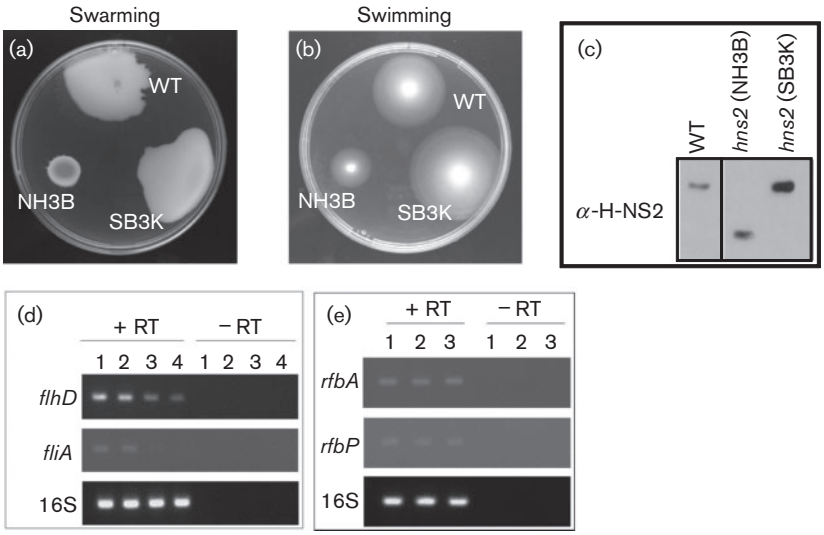

Fig. 7. Divergent phenotypes of *hns2* mutants. The swarming (a) and swimming (b) phenotypes of the NH3B and SB3K mutants, compared with the wild-type (WT). NH3B contains a transposon within the *hns2* ORF. SB3K carries a transposon insertion upstream of the *hns2* ORF. Swarming and swimming were determined as described in the legend to Fig. 1. (c) Western blot analysis of H-NS2 expression in surface-grown cells of the wild-type, NH3B and SB3K, as indicated. Cells were grown on 0.35× MacConkey agar base supplemented with 0.5 % (v/v) glycerol and scraped off after 48 h at 25 °C (at which point the SB3K was swarming effusively). The cells were washed in PBS and lysed by sonication. Identical amounts of protein extract were resolved by SDS-PAGE and transferred to PVDF membrane. After blocking, the membrane was probed with a primary polyclonal antibody raised against the first 64 residues of the conserved *S. enterica* H-NS protein. (d) qRT-PCR analysis of *flhD* and *fliA* transcript levels and (as a control) 16S rRNA levels in the wild-type (lane 1), SB3K (lane 2), NH3B (lane 3) and *expI* mutant (lane 4). (e) qRT-PCR analysis of *rfbA* and *rfbP* transcript levels in the wild-type (lane 1), SB3K (lane 2) and NH3B (lane 3). The data shown in (d) and (e) were obtained using RNA prepared as described in the legend to Fig. 5.

its effect(s) by modulating flagella-dependent motility. Indeed, *flhD* and *fliA* transcript levels were lower in the *hns2* knockout strain (NH3B) than in the wild-type or strain SB3K (Fig. 7d, lane 3). This effect of H-NS on motility is not likely to be restricted only to Pca and its relatives; previous workers have noted that in *Salmonella enterica* serovar Typhimurium, loss of *hns* function is also accompanied by a reduction in motility (Hinton *et al.*, 1992).

The location of the insertion in SB3K made us wonder whether the transposon might have disrupted the binding site of the repressor of *hns2* expression, thereby leading to overexpression of the gene. This would account for the inverse phenotypes associated with SB3K and the corresponding knockout mutant, NH3B. Western blot analysis revealed that H-NS2 was indeed more highly expressed in SB3K than it was in the wild-type (Fig. 7c). Unfortunately, when we tried overexpressing *hns2* in the wild-type from pBAD30, cell growth was arrested (Fig. 5c), so it was not possible to examine the effect(s) of H-NS2 overexpression on swarming. Previous workers have also noted that inappropriate overexpression of H-NS is cytotoxic in *S. enterica* (Hinton *et al.*, 1992). In contrast to SB3K, the insertion in NH3B was located at a position (residue 68) just downstream of a predicted protein domain (PFAM DUF_4164, residues 22–65) and resulted in the production of an apparently stable (but presumably non-active) truncated protein (Fig. 7c). It should be noted that the antibody used to probe the blots was raised against a peptide corresponding to the first 64 residues of *S. enterica* H-NS protein (Hinton *et al.*, 1992).

Surface swarming is regulated by QS

In the closely related organism, Pcc, motility is regulated by QS. We therefore investigated whether QS might regulate swarming in Pca. A Pca *expI* mutant MC3 (Burr *et al.*, 2006) that was unable to synthesize the QS molecule OHHL failed to swarm (Fig. 8a) unless the swarm plate was supplemented with chemically synthesized OHHL (Fig. 8b). In *Pseudomonas aeruginosa*, the genes encoded by HAI5 subcluster II (*rfbA*, *rfbC* and *rfbD*) are under the control of the rhl component of the QS system (Aguirre-Ramírez *et al.*, 2012). Consequently, one formal possibility was that QS might impinge on swarming in Pca by affecting the expression of genes within HAI5. However, transcript levels of *rfbA* (and also transcript levels of the subcluster I gene, *rfbP*) were unaffected in the *expI* mutant relative to the wild-type (Fig. 8d). It therefore seems likely that QS influences swarming through its effects on motility

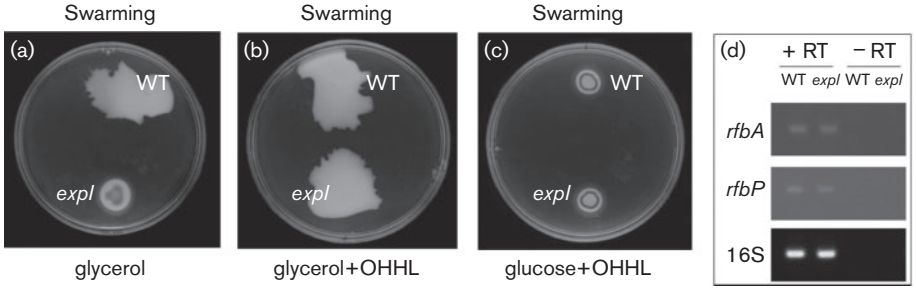

Fig. 8. Quorum sensing controls swarming motility. Swarming motility of the wild-type and an *expI* mutant (TB6) when grown on 0.35× MacConkey agar base supplemented with 0.5 % (v/v) glycerol and (a) no additives, (b) 5 μM OHHL, and (c) 5 μM OHHL and 1 % glucose. (d) qRT-PCR analysis of *rfbA* and *rfbP* transcript levels and (as a control) 16S rRNA levels in the wild-type and *expI* mutant (TB6). The data were obtained using RNA prepared as described in the legend to Fig. 5.

rather than O antigen biosynthesis. Indeed, and consistent with reports that QS regulates motility in Pcc (Chatterjee *et al.*, 2010) *flhD* and *fliA* transcript levels were decreased (relative to the wild-type) in the *expI* mutant (Fig. 7d, lane 4). Interestingly, the ability of OHHL to restore swarming to the *expI* mutant could be suppressed in the presence of glucose (Fig. 8c) indicating that the effect of QS is subordinate to catabolite repression.

DISCUSSION

We describe a previously cryptic QS-regulated phenotype – surface swarming – associated with the opportunistic phytopathogen, *Pectobacterium atrosepticum*. We show, using Tn mutagenesis, that O antigen production plays a key role in facilitating swarming. Interestingly, and although we also show that motility is a necessary prerequisite for Pca swarming, we did not identify any Tn mutants in the genes encoding the motility apparatus in our screen. Presumably, and given the large target size presented by the flagella gene clusters, this may be due to a latent bias in the insertion probability of the Tn used. Indeed, and consistent with the notion of possible Tn 'hotspotting', we obtained five independent Tn insertions in a relatively small regulator gene (*hexY*) and two independent hits in *hns2*. However, analysis of an independently isolated *fliC* mutant proved that loss of motility does affect swarming. Moreover, we note that (i) the loss of *hexY* would be expected to influence motility indirectly by affecting 'free' levels of the master regulator of motility $FlhD_4C_2$ and (ii) that the *hns2* mutant was found to display diminished *flhDC* gene expression. Similarly, QS was found to affect swarming, most likely by modulating *flhDC* expression. None of the swarm-defective regulatory mutants we identified apparently affected expression of the O antigen-encoding genes on HAI5.

Like other Gram-negative bacteria, the external leaflet of the outer membrane of Pca is enriched in LPS. The LPS molecule comprises three distinct units: a hydrophobic lipid A moiety (which anchors the LPS to the cell envelope), an oligosaccharide core, and an extended glycan-containing polysaccharide (the O antigen) (Reyes *et al.*, 2012). In some enterobacteria, the O antigen is replaced by a different polysaccharide, the enterobacterial common antigen (ECA). Both O antigen and ECA are attached to the lipidA::core by the same enzyme, O antigen ligase (encoded by *waaL*). Our data and those of earlier workers strongly suggest that O antigen has a dual function; it not only serves as a structural component of the LPS layer but can also be released in quantity under certain conditions to act as a surfactant and/or hydrating agent.

This is not the first study to implicate O antigen in swarming. For example, Toguchi *et al.* (2000) were the first to report that Tn mutants of *S. enterica* serovar Typhimurium defective in LPS synthesis were also defective in swarming. More recently, Girgis *et al.* (2007) found that in *Escherichia coli*, loss of *rfa* gene (LPS biosynthesis) function led to impaired swimming and swarming motility. This was not simply due to diminished cell envelope structural integrity (a trivial, yet plausible explanation) since motility could be largely restored in the *rfa* mutants by introducing secondary mutations in components of the Rcs phosphorelay system. The same team also found that in *E. coli*, an *hns* mutant displayed defective swimming and swarming motility, and that this defect could be largely overcome in the presence of a secondary mutation in *rcsB* (Girgis *et al.*, 2007). They suggested that H-NS down-regulates *rcsAB* expression, which, in turn, should lead to an increase in *flhDC* expression. Inactivation of *hns* should therefore lead to greater Rcs signalling and concomitantly lower *flhDC* expression. This model would be consistent with our observations that *fliA* and *flhD* transcript levels are low in the *hns2* knockout mutant, NH3B. Alternatively, it is also possible that H-NS2 may directly regulate *flhDC* expression, and this is not without precedence (Soutourina *et al.*, 1999). These workers showed that H-NS binds to the regulatory region upstream of *flhD* in *E. coli* and activates transcription of the gene in a manner dependent on

sequence elements located between the transcriptional start site and translational start site (Soutourina *et al.*, 1999). An *hns2* knockout such as NH3B would therefore be expected to display reduced *flhD* expression (and therefore, reduced swarming) which is what we observed.

Morgenstein *et al.* (2010) investigated swarming motility in *Proteus mirabilis* and found that it was abolished in a *waaL* mutant (which was defective in both ECA and O antigen biosynthesis) and in a *wzz* mutant (which was defective in O antigen biosynthesis only) but not in a *wzyE* mutant (defective in ECA synthesis only). This suggests that like Pca, *P. mirabilis* specifically requires O antigen for optimal swarming. In this regard, Pca encodes two *waaL*-like genes, which are just 37 % identical at the amino acid level: *waaL1* (ECA0162) and *waaL2* (ECA0161). Since the loss of *waaL2* function could not be compensated by the presence of *waaL1* (which is located upstream of *waaL2*) it seems likely that WaaL2 is specifically involved in linking O antigen to the lipid A core. Presumably, WaaL1 has a subtly different function to WaaL2 (e.g. it may be involved in linking ECA to the lipid A core). However, altered O antigen biosynthesis in the *P. mirabilis waaL* mutant led to a marked reduction in *flhDC* transcription; no such change in *flhDC* transcript levels was observed in the Pca O antigen mutants. Unlike both Pca and *P. mirabilis*, swarming by *Serratia marcescens* is dependent on ECA rather than O antigen, and the swarm-defective *wec* mutants (unable to make ECA) are also defective in motility (Castelli & Véscovi, 2011; Castelli *et al.*, 2008). However, and in common with *P. mirabilis*, the swarming defect in the *wec* mutants could be traced to reduced *flhD*/*flhC* expression levels. It therefore seems that different bacteria employ subtly different mechanisms to fine-tune their swarming behaviour. Presumably, Pca has bypassed the need to regulate *flhDC* expression directly because it utilizes a post-transcriptional (HexY-dependent) mechanism to regulate motility. This regulator of $FlhD_4C_2$ has so far only been found in the *Pectobacteria*, *Dickeya* and *Brenneria*, and is not present in any of the other species discussed above.

Several previous workers have postulated a cell density dependence for swarming (Partridge & Harshey, 2013; Daniels *et al.*, 2004) and in some cases, a well-characterized mechanism (QS) has been directly implicated. For example, in *Serratia liquefaciens*, QS regulates swarming by controlling production of a cyclic lipodepsipentapeptide surfactant, serrawettin (Lindum *et al.*, 1998), while in *Serratia* sp. ATCC39006, QS regulates production of a hydroxyalkanoic acid (HAA) surfactant by regulating transcription of the *rhlA* gene (Williamson *et al.*, 2008). Although it is not yet known whether it is QS-controlled, HAAs also act as surfactants in other *Serratia* species; *S. rubidaea*-derived rubiwettin is known to be a mixture of HAA-like compounds (Matsuyama *et al.*, 1990). It may be that a similar surfactant is QS-regulated in Pca, although it should be noted that the genome sequence of Pca reveals no *rhlA* homologues, and we have no experimental evidence for the involvement of surfactants other than O antigen. Indeed, our qRT-PCR data suggest that the main route by which QS impacts on swarming is through activation of *flhDC* transcription.

To conclude, we have shown that swarming behaviour in Pca requires O antigen and motility. Many of the genes involved are common to other bacterial species where swarming has been well characterized, yet the detailed interconnections between these components are clearly subtly different in Pca. The O antigen biosynthetic locus has been identified through mutagenesis, as have a number of regulatory inputs, including QS, a global regulatory protein (H-NS2) and HexY. All of these regulatory factors affect the expression or activity of the motility regulator, $FlhD_4C_2$. Having now characterized the Pca swarming phenotype itself, and the key genes involved, our current work is aimed at understanding better the complex interplay between HexY, QS, $FlhD_4C_2$, H-NS2 and the Rcs phospho-relay system in regulating the social motility of Pca.

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