A swarming-defective mutant of *Proteus mirabilis* lacking a putative cation-transporting membrane P-type ATPase

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The motile *TnphoA* mutant IC24 of *Proteus mirabilis* U6450 generates an aberrant swarming colony, and was shown to be impaired in swarm cell differentiation, i.e. cell elongation and hyperflagellation, causing delayed and slower population migration across a solid growth medium. Levels of transcript from the flagellin filament gene *flhC*, the flagellar master operon *flhDC*, and the leucine-responsive regulatory protein gene *lrp*, a regulator of swarming differentiation, were reduced in IC24 mutant swarm cells. The transposon had inserted into a gene encoding a putative P-type ATPase closely related to those transporting cations across bacterial membranes. This *ppa* gene (*Proteus* P-type ATPase) was maximally expressed in differentiated swarm cells. The data suggest an effect of ion homeostasis on swarm cell differentiation, possibly mediated via the *lrp*–*flhDC* pathway.

Keywords: P-type ATPase, *Proteus mirabilis*, swarming

INTRODUCTION

Proteus mirabilis differentiates and undergoes multicellular swarming migration. When inoculated onto rich solid media, the typical short vegetative motile rods with few peritrichous flagella differentiate into swarm cells, multinucleate, aseptate filaments of up to 40-fold vegetative cell length with >50-fold greater density of surface flagella. These form multicellular rafts which migrate rapidly away from the colony via cycles of differentiation and consolidation (pauses) (Allison & Hughes, 1991a). Swarming is a factor in urinary tract pathogenicity of *Proteus* (Allison *et al.*, 1994), and hyperexpression of flagellar genes in swarm cells is accompanied by the up-regulation of virulence genes, e.g. *hpmA* encoding haemolysin (Allison *et al.*, 1992; Gygi *et al.*, 1995a, 1997). *Proteus* transposon mutants with defects in swarming have indicated the involvement of a substantial number of genes in differentiation and subsequent population migration (Allison & Hughes, 1991b; Belas *et al.*, 1991). Hyperflagellation and a

capsular polysaccharide contribute to surface translocation (Gygi *et al.*, 1995a, b, 1997; Belas, 1994), and cell elongation and the up-regulation of flagellar gene expression are closely coupled (Gygi *et al.*, 1995a) via *flhDC* master operon expression (Furness *et al.*, 1997; Hay *et al.*, 1997). The stimuli that induce swarming are not clear, although surface contact, cell density, amino acids and other products have been discussed (Allison & Hughes, 1991a; Allison *et al.*, 1993; Eberl *et al.*, 1996; Gaisser & Hughes, 1997). This report characterizes a transposon mutant, IC24, which was isolated due to its altered pattern of swarming, producing less pronounced or infrequent consolidation terraces (Allison & Hughes, 1991a). Examination of the IC24 mutant revealed that swarm cell differentiation is dampened at both the phenotypic and transcriptional level and that it consequently migrates less vigorously than wild-type. The IC24 mutation is in a gene encoding a putative membrane cation-transporting P-type ATPase.

METHODS

Cell differentiation and motility assays. Swarming migration was monitored (Gygi *et al.*, 1995b) on LB agar (1.5%, w/v, agar), and swimming motility was monitored in soft LB agar (0.3%, w/v, agar). Differentiation was assayed by seeding 200 µl stationary phase vegetative cells (OD₆₀₀ 4.0) onto LB 1.5% (w/v) agar and harvesting all bacteria from parallel agar

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plates at intervals (Gygi *et al.*, 1995a). Cell surface flagellin from OD₆₀₀ 5.0 cells isolated from seeding differentiation plates was assayed by SDS-PAGE (12.5% gel) and Coomassie brilliant blue staining of TCA-precipitated supernatant following vortexing of cells and centrifugation. The identity of flagellin was verified by Western blotting using anti-*P. mirabilis* flagellin antiserum (a gift from H. L. T. Mobley, University of Maryland, USA; used at a dilution of 1:10000). Cell elongation was assessed after 4 h seeding differentiation by harvesting cells (OD₆₀₀ 1.0) in formalin and photographing >50 cells under phase-contrast microscopy.

Cloning, sequencing and complementation. The mutated locus was isolated in plasmid pDG200 by digesting IC24 chromosomal DNA with *EcoRI/HindIII*, ligation of an approximately 2.3 kbp fraction (previously determined by Southern blot hybridization) into pBluescript II KS (Stratagene) and selecting *Escherichia coli* XL-1 Blue transformants by hybridization with a probe internal to *TnphoA* (Gygi *et al.*, 1995a). A phage library in λ DashII (Gygi *et al.*, 1995a) was screened for the wild-type locus using a 1.6 kbp *XbaI/HindIII* hybridization probe. Several overlapping phage were restriction-mapped. A 6.5 kbp *EcoRI* fragment from phage λ DII2₄ was subcloned into pBluescript II KS. The resulting recombinant plasmid pHL1 was digested with *PstI* and religated to obtain plasmid pHL2, carrying 4.6 kbp DNA. Plasmid pHL2 was digested with *NcoI/EcoRV*, followed by blunt-ending using T4 DNA polymerase and religation to obtain plasmid pDG201, carrying 2.4 kbp insert DNA, which complemented the IC24 mutant swarming defect when supplied *in trans*. DNA manipulations, transformation and selection of *P. mirabilis* were carried out as described previously (Sambrook *et al.*, 1989; Gygi *et al.*, 1997). Both strands of plasmid DNA were sequenced by primer walking using a T7 kit (Pharmacia Biotech). The nucleotide sequence was analysed using GCG software (Devereux *et al.*, 1984) and submitted to EMBL (accession no. AJ001437).

Messenger RNA (Northern) hybridization and transcript quantification. RNA was extracted by the hot-phenol method (Melton *et al.*, 1984). Formamide/formaldehyde-denatured RNA (10 μ g per track) was separated by electrophoresis through 1.2% agarose formaldehyde gels, transferred onto nitrocellulose filters (Hybond-C; Amersham) and hybridized as described by Gygi *et al.* (1995a). Equal loading of tracks was confirmed by staining rRNA bands on nitrocellulose filters with methylene blue (Sambrook *et al.*, 1989). An approximately 820 bp *XbaI-SpeI* fragment from pDG201 was used as a *ppaA* probe, an approximately 650 bp *HincII* fragment from plasmid *pflj*DC₁C₂ was used as a *fliC* probe (Belas & Flaherty, 1994), an approximately 800 bp *AvaI* fragment from pRBF1 was the *flhDC* probe (Furness *et al.*, 1997) and an approximately 400 bp *BglII-BssHII* fragment from p200XP4 was the *lrp* probe (Hay *et al.*, 1997). All were labelled by random priming with [α -³²P]dATP (Amersham). Autoradiographs were exposed to Fuji-RX film and transcript intensities were assessed using a Kodak Digital Science DC40 camera and Digital Science 1D software. Experimental error between individual determinations was approximately 10%.

RESULTS AND DISCUSSION

A mutant with a defect in swarming migration and swarm cell differentiation

The *TnphoA* mutant IC24 of *P. mirabilis* U6450 was previously identified due to its aberrant swarming colony on LB medium containing 1.5% (w/v) agar,

Fig. 1. Phenotype of the IC24 mutant. (a) Translocation velocities of wild-type (○) and IC24 mutant (△) swarm fronts. Error bars indicate mean SD from determinations on duplicate plates. (b) Left-hand side, surface flagellin of wild-type (wt) and mutant (IC24) during seeding differentiation at 2, 4, 6 and 8 h after inoculation, Coomassie-blue-stained after SDS-PAGE (12.5% gel). Protein marker sizes are in kDa. Right-hand side, cells of wild-type and IC24 mutant at 4 h differentiation under phase-contrast microscopy.

forming less pronounced ('infrequent') consolidation terraces than wild-type (Allison *et al.*, 1991b). Inoculation of this mutant into semi-solid LB medium (0.3% w/v, agar) revealed no reduction in the swimming motility of individual cells compared to wild-type, nor was there a change in growth rate in LB broth or on LB agar, both mutant and wild-type having mid-exponential generation times of 31 min (± 1.2 min). Migration of the swarm edges of both wild-type and IC24 cell populations was monitored on LB 1.5% (w/v) agar plates (Fig. 1a), revealing that the mutant was slower to initiate migration and that it achieved at most two-thirds maximum velocity. It migrated for longer than wild-type (the time until the first consolidation pause of the IC24 mutant was approximately 90 min later).

Wild-type and IC24 mutant cell differentiation was assessed by high cell density seeding of stationary phase broth cultures onto LB 1.5% (w/v) agar, which induces vegetative cells to undergo a cycle of differentiation (Gygi *et al.*, 1995a, b). Surface flagellin expression by the IC24 mutant was still induced but was significantly

Fig. 2. Isolation and characterization of the IC24 genetic locus. The recombinant plasmid pDG200 is pBluescript II KS containing an approximately 2.3 kbp *EcoRI*–*HindIII* fragment from mutant IC24 (*TnphoA* portion not drawn to scale), identified as described by Gygi *et al.* (1995a, b). Isolation of the intact locus was achieved by hybridization of a λ DashII library of *P. mirabilis* wild-type (Gygi *et al.*, 1995a) with a 1.6 kbp *XbaI*–*HindIII* probe from pDG200. Plasmid pHL1 is pBluescript II KS containing an approximately 6.5 kbp *EcoRI* insert. Plasmid pHL2 is pBluescript II KS carrying an approximately 4.6 kbp *EcoRI*–*PstI* insert. Plasmid pDG201 is pBluescript II KS containing an approximately 2.4 kbp *NcoI*–*PstI* insert. The orientation of the pBluescript II KS *lac* promoter is indicated by an arrow. The scale indicates kbp. E, *EcoRI*; H, *HindIII*; N, *NcoI*; P, *PstI*; X, *XbaI*.

reduced compared to wild-type (Fig. 1b), being between four- and eightfold less in differentiating cells at the 4 h point as determined by comparison of twofold serial dilutions of isolated flagellin (not shown). Western blotting of total cellular flagellin with anti-FliC (flagellin) antibody showed a comparable reduction and did not indicate intracellular accumulation of flagellin by the mutant (not shown). Differentiation-specific cell elongation was also reduced in the IC24 mutant (Fig. 1b) by on average threefold at 4 h, and unlike wild-type the mutant failed to generate a significant population of highly elongated cells (Fig. 1b).

A lesion in a gene encoding a P-type putative cation-translocating membrane ATPase

Initial sequencing of the DNA flanking the transposon cloned in pDG200 (Fig. 2) revealed an insertion in an ORF closely related to bacterial P-type ATPases. The smallest recombinant plasmid carrying DNA of the wild-type locus that was able to restore *in trans* wild-type swarming by the IC24 mutant was pDG201, in which a 2.4 kbp fragment contained a 2076 bp ORF, disrupted in the mutant, transcribed by the vector *lac* promoter (Fig. 2). Further sequencing of pDG201 and plasmid pHL2 revealed that 16 bp 5' of this ORF was a 297 bp ORF, *orf1*, which was N-terminally truncated on pDG201. Both ORFs were preceded by putative ribosome-binding sites but no potential promoter consensus sequences were obvious. A potential transcription terminator sequence 19 bp 3' of the 2076 bp ORF was

identified using the TERMINATOR program (Devereux *et al.*, 1984).

The disrupted ORF, which we call *ppaA* (*Proteus* P-type ATPase), encodes a predicted 73.95 kDa polypeptide which is related to bacterial P-type cation-transporting membrane ATPases. The PpaA sequence is 58% identical to that of the zinc-transporting ATPase ZntA of *E. coli* (Sofia *et al.*, 1994; Beard *et al.*, 1997; Rensing *et al.*, 1997), 35% identical to CadA (Nucifora *et al.*, 1989) or CadD of *Staphylococcus aureus* (SWISS-PROT accession no. P37386) and 34% identical to the potassium/copper-transporting ATPase CopA of *Enterococcus hirae* (Odermatt *et al.*, 1993). PpaA shares protein motifs with other putative bacterial P-type cation-transporting ATPases (Silver & Walderhaug, 1992), notably the N-terminal heavy metal binding site 3-Cys-X-X-Cys-6. Other features are shared with the P-type ATPase family such as the conserved tetrapeptide 233-Thr-Gly-Glu-Ser-236 found in the phosphatase domain and the phosphorylation site constituted by the 7-amino-acid motif 381-Asp-Lys-Thr-Gly-Thr-Leu-Thr-387. PpaA is hydrophobic, which would be consistent with it being a membrane protein (Fig. 3). The putative ion translocation region of PpaA, spanning residues 335–367, contains proline (amino acid 338) located within a hydrophobic domain. While this proline is conserved in all P-type ATPases, it appears to be flanked by cysteines in metal-ion-translocating enzymes like the copper transporters CopA or CopB of *Ent. hirae* (Odermatt *et al.*, 1993; Silver & Walderhaug, 1992). A possible function of PpaA in cation transport was investigated by comparing the growth rates of wild-type and mutant in the presence of monovalent and bivalent cations in minimal medium, but no difference was observed, the mid-exponential generation times being 51 min (± 2 min) for both strains under all conditions tested (not shown). However, this does not exclude a role for PpaA in cation transport as experimental conditions might not have been optimal and there may be other transporters in *P. mirabilis* with less specific specificity. This is supported by the finding that plasmid pHL1 (Fig. 2) can complement the cation-transport defect of an *E. coli zntA* null mutant (C. Rensing & B. Rosen, personal communication). Furthermore, the 99-amino-acid (11.1 kDa) Orf1, which does not show significant primary sequence identity to any database entry, is unusually rich in histidines (21%), contains three cysteine pairs, and is predicted to be highly hydrophilic and negatively charged (Fig. 3). These features are reminiscent of the 60-amino-acid Hpn polypeptide of *Helicobacter pylori*, which possesses a high histidine content (47%), two cysteine pairs, and which binds, in descending order of affinity, the cations Ni^{2+} , Zn^{2+} , Co^{2+} , Cu^{2+} , Cd^{2+} and Mn^{2+} (Gilbert *et al.*, 1995).

ppaA expression is maximal in swarm cells

Northern blot hybridization of wild-type *P. mirabilis* RNA collected during a swarming differentiation cycle (Gygi *et al.*, 1997) revealed that expression of the *ppaA*

Fig. 3. Hydrophobicity profiles of Orf1 and PpaA with amino acid scale (Kyte & Doolittle, 1982).

Fig. 4. Wild-type *ppaA* and *flhDC* mRNA during differentiation into swarm cells. RNA was extracted 1.5–6 h after seeding onto LB agar. Northern blots were exposed at -80°C with intensifying screens for 72 and 6 h, respectively, to visualize the *ppaA* and *flhDC* transcripts. Transcript sizes (kbp) were estimated by comparison to *in vitro* transcript RNA markers (Promega).

gene was maximal in differentiated swarm cells (Fig. 4). This pattern is similar to the expression of the strongly induced *flhDC* flagellar master operon (Fig. 4; Furness *et al.*, 1997), but the *ppaA* mRNA was present at an approximately 100-fold lower concentration. The approximately 2.5 kbp *ppaA* transcript size of the *ppaA* mRNA is compatible with transcription initiating 5' of *orf1* and terminating at the predicted terminator sequence 3' of *ppaA*.

Reduced expression of the *flhDC* master operon and *lrp* regulator in the *ppaA* mutant

The reduction in cell elongation and surface flagella during differentiation of the mutant suggested a defect in the expression of the flagellar master operon *flhDC*, the levels of which control flagellar biogenesis and cell division, and correlate with swarm-specific cell hyperflagellation and filamentation in *Serratia liquefaciens* and *P. mirabilis* (Eberl *et al.*, 1996; Furness *et al.*, 1997). This was investigated by Northern blot hybridization of RNA samples collected at regular intervals during a seeding differentiation cycle (Gygi *et al.*, 1997). The 1.3 kbp flagellin *fliC* transcript was reduced throughout this assay by approximately twofold in the mutant (Fig. 5), less pronounced than the reduction in surface and total cellular flagellin, as were levels of the approximately 1.1 kbp *flhDC* transcript (Furness *et al.*, 1997) and the approximately 700 bp mRNA of the *lrp* gene (Fig. 5), expression of which is a requirement for *Proteus* swarming, specifically flagellar gene hierarchy expression and cell elongation (probably via *flhDC*) (Hay *et al.*, 1997).

Fig. 5. mRNA levels in wild-type (wt) and *ppaA* mutant during seeding differentiation. RNA was extracted 2–6 h after seeding. Northern blots were exposed for 2 h at room temperature without intensifying screens for *fliC* transcript, and for 6 h and 18 h at -80°C with intensifying screens for the *flhDC* and *lrp* transcripts, respectively.

In summary, the results show that the IC24 mutant has lost a P-type ATPase, most probably a membrane cation transporter, that reduces swarming differentiation and dampens down the initiation and velocity of swarming. A reduction in differentiation to the hyperflagellated elongated form appears to be due to a decrease in transcription of flagellar genes, in particular reduced expression of the flagellar master operon *flhDC*. The observation that the *ppaA* mutant has correspondingly reduced expression of *lrp*, which is essential for swarm-specific hyperexpression of *flhDC* (Hay *et al.*, 1997), suggests that cations might influence expression of flagella via *lrp*. An effect of ion homeostasis on swarming would not be surprising as NaCl prevents swarming (Allison & Hughes, 1991a) and represses *E. coli* flagellar biogenesis at the transcriptional level (Shi *et al.*, 1993), and intracellular Ca^{2+} levels influence the chemotactic response in *E. coli* (Tisa & Adler, 1992). Our findings appear to lend weight to the idea that ion concentration is one of a number of factors influencing bacterial swarming differentiation.

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