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 *fliC*, 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 (OD<sub>600</sub> 4·0) onto LB 1·5 % (w/v) agar and harvesting all bacteria from parallel agar
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-\textit{P. mirabilis} flagellin antiserum (a gift from H. L. T. Mobley, University of Maryland, USA; used at a dilution of 1:100000). Cell elongation was assessed after 4 h seedling 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 \textit{Escherichia coli} XL-1 Blue transformants by hybridization with a probe internal to TnphoA (Gygi et al., 1995a). A phage library in ADashII (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 ADII24 was subcloned into pBluescript II KS. The resulting recombinant plasmid pH1 was digested with PstI and religated to obtain plasmid pH2, carrying 4.6 kbp DNA. Plasmid pH2 was digested with Ncol/EcorV, followed by blunt-ending using T4 DNA polymerase and religation to obtain plasmid pH20, carrying 2.4 kbp insert DNA, which complemented the IC24 mutant swarming defect when supplied in trans. DNA manipulations, transformation and selection of \textit{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). Formamidine/formaldehyde-denatured RNA (10 µg 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-Spfl fragment from pDG201 was used as a \textit{ppaA} probe, an approximately 650 bp \textit{HimB} fragment from plasmid \textit{pbr} DC, \textit{C}_{4} was used as a \textit{flC} probe (Belas & Flaherty, 1994), an approximately 800 bp \textit{Avai} fragment from pRB1 was the \textit{flbDC} probe (Furness et al., 1997) and an approximately 400 bp Bgfl-BssHII fragment from p200XP4 was the \textit{lrp} probe (Hay et al., 1997). All were labelled by random priming with \textit{[α-32P]dATP} (Amersham). Autoradiographs were exposed to Fuji-RX film and transcript intensities were assessed using a Kodak Digital Science DC200 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 \textit{P. mirabilis} U6450 was previously identified due to its aberrant swarming colony on LB medium containing 1.5% (w/v) agar, 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
translocating membrane ATPase

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 XbaI–HindIII probe from pDG200. Plasmid pH1.1 is pBluescript II KS containing an approximately 6.5 kbp EcoRI insert. Plasmid pH2 is pBluescript II KS carrying an approximately 2-4 kbp EcoRI–PstI insert. Plasmid pDG201 is pBluescript II KS containing an approximately 2-4 kbp Ncol–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, Ncol; P, PstI; X, XbaI.

**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 β-Dashili library of P. mirabilis wild-type (Gygi et al., 1995a) with a 1-5 kbp XbaI–HindIII probe from pDG200. Plasmid pH1.1 is pBluescript II KS containing an approximately 6.5 kbp EcoRI insert. Plasmid pH2 is pBluescript II KS carrying an approximately 2-4 kbp EcoRI–PstI insert. Plasmid pDG201 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 β-Dashili library of P. mirabilis wild-type (Gygi et al., 1995a) with a 1-5 kbp XbaI–HindIII probe from pDG200. Plasmid pH1.1 is pBluescript II KS containing an approximately 6.5 kbp EcoRI insert. Plasmid pH2 is pBluescript II KS carrying an approximately 2-4 kbp EcoRI–PstI insert. Plasmid pDG201 is pBluescript II KS containing an approximately 2-4 kbp EcoRI–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, Ncol; 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 an 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 pH2 revealed that 16 bp 5' of this ORF was a 297 bp ORF, orfI, 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. P37836) 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 pH2 (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 Hpnp polypeptide of Helicobacter pylori, which possesses a high histidine content (47%), two cysteine pairs, and which binds, in descending order of affinity, the cations Ni2+, Zn2+, Co2+, Cu2+, Cd2+ and Mn2+ (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
gene was maximal in differentiated swarm cells (Fig. 4). This pattern is similar to the expression of the strongly induced \textit{flhDC} flagellar master operon (Fig. 4; Furness \textit{et al.}, 1997), but the \textit{ppaA} mRNA was present at an approximately 100-fold lower concentration. The approximately 2.5 kbp \textit{ppaA} transcript size of the \textit{ppaA} mRNA is compatible with transcription initiating 5' of \textit{orf1} and terminating at the predicted terminator sequence 3' of \textit{ppaA}.

Reduced expression of the \textit{flhDC} master operon and \textit{lrp} regulator in the \textit{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 \textit{flhDC}, the levels of which control flagellar biogenesis and cell division, and correlate with swarm-specific cell hyperflagellation and filamentation in \textit{Serratia liquefaciens} and \textit{P. mirabilis} (Eberl \textit{et al.}, 1996; Furness \textit{et al.}, 1997). This was investigated by Northern blot hybridization of RNA samples collected at regular intervals during a seeding differentiation cycle (Gygi \textit{et al.}, 1997). The 1.3 kbp flagellin \textit{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 \textit{flhDC} transcript (Furness \textit{et al.}, 1997) and the approximately 700 bp mRNA of the \textit{lrp} gene (Fig. 5), expression of which is a requirement for \textit{Proteus} swarming, specifically flagellar gene hierarchy expression and cell elongation (probably via \textit{flhDC}) (Hay \textit{et al.}, 1997).

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 \textit{flhDC}. The observation that the \textit{ppaA} mutant has correspondingly reduced expression of \textit{lrp}, which is essential for swarm-specific hyperexpression of \textit{flhDC} (Hay \textit{et al.}, 1997), suggests that cations might influence expression of flagella via \textit{lrp}. An effect of ion homeostasis on swarming would not be surprising as NaCl prevents swarming (Allison \& Hughes, 1991a) and represses \textit{E. coli} flagellar biogenesis at the transcriptional level (Shi \textit{et al.}, 1993), and intracellular Ca$^{2+}$ levels influence the chemotactic response in \textit{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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REFERENCES


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