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 (OD600 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 OD_{600} 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 bloting using anti-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 seeding differentiation by harvesting cells (OD_{600} 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 λDasyll (Gygi et al., 1995a) was screened for the wild-type locus using a 1.6 kbp Xbal/HindIII hybridization probe. Several overlapping phage were restriction-mapped. A 6.5 kbp EcoRI fragment from phage λDII24 was subcloned into pBluescript II KS. The resulting recombinant plasmid pHLI was digested with PstI and religated to obtain plasmid pHL2, carrying 4.6 kbp DNA. Plasmid pHL2 was digested with Ncol/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 swimming 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 (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 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 Xbal-SpeI fragment from pDG201 was used as a pppA probe, an approximately 650 bp HincII fragment from plasmid plf8DC; C_6 was used as a flfC probe (Belas & Flaherty, 1994), an approximately 800 bp AvaI fragment from pRBFl was the flBD probe (Furness et al., 1997) and an approximately 400 bp BgflI-BssHII fragment from p200XP4 was the Lr 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, 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 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
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 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).

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 Ca²⁺ 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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**REFERENCES**


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