The *Pseudomonas fluorescens* transcription activator AdnA is required for adhesion and motility

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The locations of two mutations that prevent adhesion of *Pseudomonas fluorescens* Pf0-1 to sand columns and seeds (*adn*, adhesion) were identified. Both lie in a single gene showing homology to the NtrC/NifA family of transcription activators. The predicted 55 kDa protein encoded by *adnA* is most closely related to activators involved in expression of flagellar proteins, consistent with the lack of flagella in *adnA* strains. Constitutive *adnA* expression restored motility and adhesion to an *adnA* strain, demonstrating that the observed phenotypes are due to lack of AdnA and not a consequence of other mutations or polar effects of mutations in *adnA* on other genes.

**Keywords:** adhesion, flagella, transcription regulation

INTRODUCTION

Fluorescent pseudomonads are common soil bacteria. They have been investigated for their potential use in bioremediation and for biocontrol of agricultural pathogens (de Lorenzo *et al*., 1993; Lajoie *et al*., 1993; Simons *et al*., 1996). However, performance of strains introduced for these purposes into soil in field trials has been inconsistent (van Veen *et al*., 1993; Simons *et al*., 1996). One likely source of this inconsistency is the failure of the introduced cells to become established and colonize the soil to a sufficient degree when faced with an established community of micro-organisms in the soil.

Numerous traits contribute to the survival of a particular bacterial strain in the soil. Cells must be able to adapt to changing temperature, nutrient availability and osmolarity. Biotic factors, such as avoiding predation by other organisms and resistance to antibacterial compounds produced by other species, also play a role. One general survival strategy is attachment to a surface and growth of a biofilm (Costerton *et al*., 1995, 1999). By this mechanism, cells have access to the nutrients adsorbed by the surface and may be protected from exogenous antibiotics and competitive colonization by other species.

Motility is often recognized as a factor contributing to adhesion and colonization of both biologic (Piette & Idziak, 1992; Ramphal *et al*., 1991; Scharfman *et al*., 1996) and abiotic surfaces (Korber *et al*., 1994; O’Toole & Kolter, 1998a, b; Williams & Fletcher, 1996), but the exact relationship between the two is somewhat obscure. It may be that motility is required to overcome the repulsion between the negatively charged bacterial cell and a negatively charged surface. In the case of *Pseudomonas aeruginosa* however, the most recent data indicate that it is the FlID protein, located at the tip of the flagella, that mediates a specific interaction with mucins (Arora *et al*., 1998).

In a previous study, 3500 Tn5 insertion mutants of *Pseudomonas fluorescens* strain Pf0-1 were screened for reduced binding to quartz sand columns (DeFlaun *et al*., 1990). Three defective mutants were identified. These mutants also displayed defects in adhesion to a variety of seeds and soil (DeFlaun *et al*., 1994). Examination of these strains indicated that two mutants, Pf0-5 and Pf0-10, lacked flagella and were non-motile. The insertions Pf0-5 and Pf0-10 have now been localized and the disrupted genes identified as the same ORF, specifying a...
putative transcriptional regulator named AdnA belonging to the NtrC/NifA family of activators.

**METHODS**

**Bacterial strains and media.** The bacterial strains and plasmids used in this study are listed in Table 1. P. fluorescens strain Pf0-1, which was isolated from agricultural soils in Sherborn, MA, USA (Compeau et al., 1988). Pseudomonas strains were grown on nutrient agar, Luria-Bertani agar (LB) or minimal medium (Stanier et al., 1966) at 30 °C. Escherichia coli strain DH5α was used for cosmid and plasmid constructions and grown at 37 °C. The following antibiotics were used for selection: tetracycline, 15 μg ml−1; kanamycin, 50 μg ml−1; ampicillin, 100 μg ml−1.

**Cloning of P. fluorescens DNA flanking Tn5 insertion mutations.** A Tn5-specific probe was prepared by labelling a 1.55 kb BamHI–BglII fragment of pKan1 (Hächler et al., 1991) with [32P]CTP by extension of random hexamer primers using the Klenow fragment of E. coli DNA polymerase. Chromosomal DNA from P. fluorescens Pf0-5 and Pf0-10 was isolated by the method of Beji et al. (1987). DNA from each mutant was cleaved with restriction endonucleases and Southern blot analysis was performed using the Tn5-specific probe (Sambrook et al., 1989). Tn5 contains a single BamHI site 3′ to the kanamycin resistance gene and does not contain an EcoRI site. BamHI–EcoRI fragments were isolated from Pf0-5 and Pf0-10 and ligated into pUC18. Selection for kanamycin-resistant transformatants yielded plasmids pPF3A4 and pPF3A2.

**Isolation of the wild-type adnA gene.** P. fluorescens gene-specific DNA probes were isolated by digesting pPF3A4 and pPF3A2 with EcoRI and HpaI to produce 4.5 kb and 3.95 kb fragments, respectively. The fragments were labelled with [32P]CTP and random hexamer primers. Genomic DNA from strain Pf0-1 was digested to completion with BamHI. DNA fragments (9–13 kb) were electroeluted from agarose gel slices and ligated to pLAFR5 cosmid arms. Cosmid arms were produced by first linearizing pLAFR5 with ScaI and then digesting with BamHI to produce two DNA fragments of 1975 and 1.75 kb (Keen et al., 1988). The ligation buffer contained 10 mM ATP to suppress blunt end ligation at the ScaI site. The ligation mix was packaged in vitro (Gigapack II gold packaging extract; Stratagene) and transduced into E. coli DH5α.

**DNA sequence analysis.** To sequence the adnA gene, a 6.2 kb BamHI–EcoRI fragment of pBP2 was subcloned into pBluescript II to form plasmid pPF1B. Both strands of DNA were sequenced manually using a Sequenase kit (Amersham). Similarity searches were performed using the National Center for Biotechnology Information blast server (Altschul et al., 1990). The sequence was deposited in GenBank with the accession number AF312695.

**Inducible adnA expression.** Plasmid pPC100 was constructed by inserting a PCR fragment consisting of the adnA ORF flanked by AfIII and EcoRI sites into the broad-host-range plasmid pJB866 (Blatny et al., 1997). This manipulation places adnA under control of the Pm promoter and XylS activator, both derived from the TOL plasmid pWWO of P. putida (Mermod et al., 1986). pPC100 was constructed by deleting a 923 bp AfIII fragment from pPC100, thus removing residues.

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**Table 1. Strains and plasmids used**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td>recA1 endA1 bsdR17(1351m) supE44 thi-1 relA1 λ− φ80d/lacZΔM15</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>DH5α</td>
<td>Δ(lacZYA-argF)U169</td>
<td></td>
</tr>
<tr>
<td>S17.1</td>
<td>recA thi pro bsdR` M− RP4 2-Tc::Mu-Km::Tn7</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><strong>P. fluorescens strains</strong></td>
<td>Cloning vector, amp</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>Pf0-1</td>
<td>Wild-type</td>
<td>Compeau et al. (1988)</td>
</tr>
<tr>
<td>Pf0-5</td>
<td>Pf0-1 adnA::mini-Tn5 kan</td>
<td>DeFlaun et al. (1990)</td>
</tr>
<tr>
<td>Pf0-10</td>
<td>Pf0-1 adnA::mini-Tn5 kan</td>
<td>DeFlaun et al. (1990)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td>INC−tra kan</td>
<td>Hächler et al. (1991)</td>
</tr>
<tr>
<td>pUC18 containing marA::Tn5, amp kan</td>
<td></td>
<td>Ditta et al. (1980)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>orfRK2, mobRP4, expression vector carrying XylS and Pm, tet</td>
<td>Blatny et al. (1997)</td>
</tr>
<tr>
<td>pJB866</td>
<td>6.5 kb EcoRI–BamHI fragment of Pf0-1 DNA containing adnA cloned into pBluescript, amp</td>
<td>This paper</td>
</tr>
<tr>
<td>pPF3A2</td>
<td>7.25 kb EcoRI–BamHI fragment of Pf0-10 DNA cloned in pUC18, amp kan</td>
<td>This paper</td>
</tr>
<tr>
<td>pPF3A34</td>
<td>6.5 kb EcoRI–BamHI fragment of Pf0-5 DNA in pUC18, amp kan</td>
<td>This paper</td>
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<tr>
<td>pLAFR5</td>
<td>Cosmid cloning vector, tet</td>
<td>Keen et al. (1988)</td>
</tr>
<tr>
<td>pPF3B2</td>
<td>11 kb BamHI fragment of Pf0-1 DNA containing adnA cloned into pLAFR5, tet</td>
<td>This paper</td>
</tr>
<tr>
<td>pPC100</td>
<td>16 kb adnA gene cloned into AfIII–EcoRI site of pJB866, tet</td>
<td>This paper</td>
</tr>
<tr>
<td>pPC101</td>
<td>Deletion of 923 bp AfIII fragment from adnA on pPC100, tet</td>
<td>This paper</td>
</tr>
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</table>
encoding amino acids 1–309 from adnA and shifting the translation reading frame of the residual adnA sequence. To verify that adnA expression from pPC100 was inducible, E. coli cells were grown with and without 3-methylbenzoate and examined for differences in whole-cell protein profiles. LB was inoculated with an overnight culture of DH5α carrying pPC100 or pPC101 and each was grown at 30°C in 95% ethanol was added to one, and an equal volume of 95% ethanol was added to the control. Incubation was continued for 16 h at 30°C. Cells were collected by centrifugation and lysed by resuspension in 100 µl 10 mM Tris/HCl, 0.1 mM EDTA, pH 7.5 and 100 µl 2 x SDS loading buffer. 10 µl lysate was resolved by SDS-PAGE in 10% gels. Proteins were stained with 0.01% Coomassie blue for 2 h and photographed.

**Complementation.** Cosmids pPBF2 and pPBF3 were mobilized from E. coli strains into P. fluorescens in triparental matings using pRK2013 to supply transfer functions in trans (Ditta et al., 1980). pPC100 and pPC101 (see above) were initially transformed into E. coli strain S17.1, then transferred to Po-1 and mutant strains in biparental matings. Relative to *Pseudomonas* strains carrying control plasmid pPC101, strains carrying pPC100 exhibited reduced growth rates in Luria broth. However pPC101 had little effect on the exponential growth phase before resuming exponential growth. Therefore care was taken to start complementation experiments with cultures at the same growth state and optical density.

**Motility.** Motility was assayed in MMO, 0.2% glutamate, 0.3% agar at 30°C. Strains were inoculated into the agar with a sterile needle and growth away from the inoculation point was recorded.

**Adhesion.** The adhesion assay was based on the assay described by O’Toole & Kolter (1998b). An exponential phase culture (20 µl) growing in MMO + 0.2% glutamate was added to 1 ml MMO + glutamate in borosilicate glass tubes. The tubes were incubated for 6 h at 30°C without agitation, then rinsed with ddH₂O and stained with 1% crystal violet. Excess stain was removed by rinsing in ddH₂O. Adhesion was detected as a ring of stain on the tube wall at the air/media interface.

**RESULTS AND DISCUSSION**

**Co-localization of Tn5 insertions in adhesion mutants**

The Tn5 insertion sites of the adhesion-defective mutants Po-5 and Po-10 were compared by Southern blot analysis of chromosomal DNA using a Tn5-specific probe (Fig. 1a). Digestion of Po-5 and Po-10 DNA with KpnI and ClaI yielded identically sized Tn5-containing fragments, while digestion of these DNAs with BamHI and EcoRI yielded fragments of distinct sizes. This hybridization pattern suggested that the Tn5 insertion sites in these two mutants were physically close.

Chromosomal DNA from the wild-type strain Po-1 was digested with a number of restriction enzymes and analysed by Southern analysis using probes generated from DNA flanking the Tn5 insertion sites in Po-10 and Po-5 (Fig. 1b). Identical bands were observed in all cases, clearly showing that the Tn5 insertions in Po-5 and Po-10 were located in the same region of the DNA sequence. The difference in size between Po-5 and Po-10 BamHI fragments (Fig. 1a) are due to the different insertion sites of Tn5 in the chromosome. The difference in size of EcoRI fragments hybridizing with the Tn5 probe may be due to sequence rearrangements that occurred following the Tn5 insertion into one of these strains.

The Tn5 insertion junction sites were cloned by digesting chromosomal DNA from Po-5 and Po-10 with EcoRI and BamHI, ligating the fragments into pUC18 and selecting for kanamycin-resistant transformants. The insertion junctions in the resulting plasmid DNAs, pPfA34 and pPfA32, were sequenced using a primer complementary to the end of the transposon. Distinct DNA sequences were obtained for Po-5 and Po-10 (data not shown). Thus, although the Tn5 insertions in
Pf0-5 and Pf0-10 are physically close, they are independent mutations.

Isolation of cosmid clones complementing the motility defect of Pf0-5 and Pf0-10

A library containing BamHI fragments from strain Pf0-1 was constructed in the cosmid pLAFR5, packaged in vitro and transduced into E. coli. The transductants were screened by colony hybridization using a probe containing DNA flanking the Tn5 insertion in Pf0-5. Two clones, containing cosmids pPBF2 and pPBF3, hybridized to the Pf0-5 DNA probe. Both clones also hybridized to a probe containing DNA flanking the Tn5 insertion in Pf0-10.

Digestion of these cosmids with several restriction enzymes showed that both clones contained an identical 11 kb BamHI fragment. Further subcloning demonstrated that adnA resided within this fragment. One gene, called adnA, contains the sites of Tn5 insertion in Pf0-5 and Pf0-10 (Fig. 2). The other genes encode homologues of flagella structural (fliD, fliE, fliF) and regulatory (fliS, fleS, fleR). The function of orf99 is unknown, but it shares similarity with its positional homologue in P. aeruginosa, orf97. Regions with a similar or identical genetic organization are found in P. aeruginosa, V. cholerae and V. parahaemolyticus (Arora et al., 1997; Klose & Mekalanos, 1998a; Kim & McCarter, 2000).

The predicted adnA ORF encodes a 491 residue protein with a predicted molecular mass of 55-5 kDa. A potential ribosome-binding site is located 11 nt upstream of the initiation codon. There is a 172 nt intergenic region upstream of the adnA initiation codon, but no σ74, σ28 or σ54 promoters were identified. A potential σ70-dependent promoter was identified beginning 135 nt upstream of the adnA coding region (TTGACTgtgcacggttttttgacT-TAACT: upper case letters, conserved nucleotides; lower case letters, less well conserved ’spacer’ region).

**Fig. 3.** Alignment of AdnA with homologues. Alignments were created with BLAST. Amino acid residues identical to AdnA are boxed. FleQ, P. aeruginosa; FlaK, V. parahaemolyticus; FlrA, V. cholerae; NtrC, Salmonella typhimurium. Conserved residues involved in Mg2+ binding (aa 11–12) and potential phosphorylation sites (aa 59) are underlined. The double underline indicates the predicted position of a helix-turn-helix motif based on alignment with S. typhimurium NtrC.
AdnA is a transcription regulator

The predicted adnA-encoded protein sequence was compared to known proteins using the blast alignment tool (Altschul et al., 1990). AdnA showed strong homology to the NtrC/NifA family of transcriptional activators of $\sigma^+$-dependent promoters (Fig. 3) (Kustu et al., 1991; Morett & Segovia, 1993). These activators respond to environmental stimuli by activating transcription of adaptive genes, often as the response to environmental stimuli by activating transcription of adaptive genes (Kustu et al., 1991; Morett & Segovia, 1993). In the case of NtrC, the sensor kinase NtrB phosphorylates the amino-terminal regulatory domain of NtrC in response to the nitrogen status of the cell. A second mechanism, illustrated by the regulation of the activator NifA by NifL in response to oxygen and fixed-nitrogen levels, requires an interaction between the proteins, but not phosphorylation of the activator. A third mechanism is illustrated by P. putida XylR. Here, binding of a small aromatic ligand (toluene) directly to the amino terminus of the activator regulates XylR activity. AdnA, FleQ, FlrA and FlaK all contain two conserved acidic residues common to phosphorylation-regulated activators (Fig. 3), but a third highly conserved aspartate phosphorylation site is changed to threonine, serine or asparagine (Stock et al., 1990). Although the conserved aspartate residue is missing, serine and threonine can be phosphorylated, leaving open the possibility that an unrecognized kinase exists for AdnA. The regions immediately downstream of fleQ, flaK and flrA contain $\sigma^4$-dependent, two-component systems named fleSR, flaLM and flrBC (Ritchings et al., 1995; Klose & Mekalanos, 1998a; Kim & McCarter, 2000). Transcription of fleSR and flrBC is activated by FleQ and FlrA, respectively, so it seems likely that the P. fluorescens fleSR homologues will be regulated by AdnA.

Complementation of Pf0-5 with a cloned adnA gene

A broad-host-range plasmid, pPC100, was constructed in which adnA expression is under control of the activator XylIS and the inducible XylIS-dependent promoter Pm, both from the TOL plasmid from P. putida (Blatny et al., 1997; Mermod et al., 1986). When this

with AdnA is greatest between residues 142–375 (66% identical in all four proteins), which encompass amino acids involved in ATP binding and hydrolysis, and transcription activation. The amino-terminal 141 residues are expected to regulate AdnA activity and are less well conserved with the other homologues except for FleQ. The carboxyl terminal 116 residues are moderately conserved and contain the helix–turn–helix DNA binding domain. The AdnA transcription regulator presumably affects adhesion by altering expression of structural genes required for adhesion. The demonstrated role of AdnA in flagella synthesis suggests that, as in P. aeruginosa, flagella are critical for adhesion.

It is striking that in P. fluorescens, P. aeruginosa, V. cholerae and V. parahaemolyticus there appears to be a conserved regulatory system controlling polar flagella synthesis. The regulation of flagella synthesis in these species is distinct from the synthesis of peritrichous flagella in enteric bacteria in that $\sigma^+$ plays an important role in early stages of flagella synthesis, before the flagella-specific $\sigma^+$ is required (Helmann, 1991). In all four cases, there is an activator homologous to AdnA that does not have an adjacent sensor protein (Klose & Mekalanos, 1998a; Stewart & McCarter, 1996; Arora et al., 1997). Signal transduction through $\sigma^+$-dependent activators occurs by one of three mechanisms (Morett & Segovia, 1993). In the case of NtrC, the sensor kinase NtrB phosphorylates the amino-terminal regulatory domain of NtrC in response to the nitrogen status of the cell. A second mechanism, illustrated by the regulation of the activator NifA by NifL in response to oxygen and fixed-nitrogen levels, requires an interaction between the proteins, but not phosphorylation of the activator. A third mechanism is illustrated by P. putida XylR. Here, binding of a small aromatic ligand (toluene) directly to the amino terminus of the activator regulates XylR activity. AdnA, FleQ, FlrA and FlaK all contain two conserved acidic residues common to phosphorylation-regulated activators (Fig. 3), but a third highly conserved aspartate phosphorylation site is changed to threonine, serine or asparagine (Stock et al., 1990). Although the conserved aspartate residue is missing, serine and threonine can be phosphorylated, leaving open the possibility that an unrecognized kinase exists for AdnA. The regions immediately downstream of fleQ, flaK and flrA contain $\sigma^4$-dependent, two-component systems named fleSR, flaLM and flrBC (Ritchings et al., 1995; Klose & Mekalanos, 1998a; Kim & McCarter, 2000). Transcription of fleSR and flrBC is activated by FleQ and FlrA, respectively, so it seems likely that the P. fluorescens fleSR homologues will be regulated by AdnA.

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plasmid was introduced into *E. coli* DH5α, a high level of a 55 kDa protein was synthesized in the presence of the inducer 3-methylbenzoate, indicating that the expected ORF is being transcribed and translated in *E. coli* (Fig. 4).

Plasmids pPC100 or pPC101 (a control plasmid with a deletion in *adnA*), were transferred to Pf0-1, Pf0-5 or Pf0-10, and motility and adhesion assays were performed. Strains carrying pPC100 re-establish exponential growth more slowly than strains carrying pPC101 when an overnight culture is used as an inoculum. Therefore, all complementation assays were initiated with actively growing cultures adjusted to equal cell density just prior to inoculation. The defective motility and adhesion of Pf0-5 and Pf0-10 were complemented by pPC100 but not by pPC101 (Fig. 5). The constitutive low-level transcription from Pm was sufficient for complementation in these experiments. These results demonstrate that the phenotypic effects of the Tn5 insertion in Pf0-5 and Pf0-10 are due to a lack of AdnA and not mutations elsewhere in the chromosome or polar effects of the insertion on expression of other genes.

As is the case with other bacteria, it is likely that synthesis of flagella proteins in *P. fluorescens* will proceed through a cascade mechanism in which one activator regulates expression or activity of more activators (Arora et al., 1997; Helmann, 1991; Klose & Mekalanos, 1998a) causing the flagella to be assembled in a defined order. Future experiments directed at identifying genes regulated by AdnA and the proteins that control *adnA* expression will provide clues to the position of AdnA in this hierarchy.

**Fig. 5.** Complementation of adhesion and motility defects by cloned *adnA*. (a) Adhesion of Pfo-1, Pfo-5 and Pfo-10 to borosilicate tubes, without plasmid, or carrying pPC100 (*'adnA'*) or pPC101 (*'ΔadnA'*). (b) Motility assay of Pfo-1, Pfo-5 or Pfo-10 alone or carrying plasmids pPC100 or pPC101. The presence of *adnA* restored adhesion and motility to Pfo-5 and Pfo-10. Pfo-1 is the wild-type control.

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