Isolation of an IHF-deficient mutant of a *Pseudomonas aeruginosa* mucoid isolate and evaluation of the role of IHF in *algD* gene expression

Ina Delic-Attree, Bertrand Toussaint, Alexandra Froger, John C. Willison and Paulette M. Vignais

Author for correspondence: Bertrand Toussaint. Tel: +33 76 883483. Fax: +33 76 885185.
e-mail: BT@girose.ceng.cea.fr

CEA/Grenoble, Laboratoire de Biochimie Microbienne (CNRS URA 1130 alliée à l'INSERM), DBMS, 17 rue des Martyrs, 38054 Grenoble Cedex 9, France

The role of integration host factor (IHF) in the regulation of alginate synthesis was investigated in a mucoid strain of *Pseudomonas aeruginosa* (strain CHA) isolated from a cystic fibrosis patient. *Escherichia coli* strain BL21(DE3) was made IHF-deficient by inactivation of its chromosomal IHF genes, *himA* and *himD*, then used as host strain to overproduce *P. aeruginosa* IHF. The purified recombinant IHF protein was used to determine the affinity of IHF for the two IHF binding sites in the *algD* promoter. The K_d values were determined to be 130 nM for *algD* IHF site 2 and about 2 μ M for *algD* IHF site 1. Two IHF-deficient mutants of *P. aeruginosa* strain CHA were constructed by insertional inactivation of the *himA* gene, and the activity of the *algD* promoter was determined using transcriptional fusion with *xylE* as reporter gene. The expression of *algD*, the structural gene for GDP-mannose dehydrogenase, was decreased three- to fourfold in the *himA* mutants under conditions of high salinity and nitrogen limitation. Assays of alginate production by cultures grown on agar plates indicated that the IHF-deficient mutants synthesized 50% less polymer than the mucoid parental strain. These results demonstrate clearly that although IHF is dispensable for alginate production, *himA* expression is required for full activation of *algD* expression.

Keywords: alginate, integration host factor, cystic fibrosis, *Pseudomonas aeruginosa*

INTRODUCTION

The overproduction of alginate by strains of *Pseudomonas aeruginosa* that chronically infect cystic fibrosis (CF) patients is an important virulence factor in these infections. It has been shown that the polysaccharide capsule promotes adherence to the epithelial cells of the respiratory tract (May *et al.*, 1991), inhibits phagocytosis (Krieg *et al.*, 1988) and protects the bacteria from certain antibiotics (Slack & Nichols, 1981). Overproduction of alginate confers a mucoid phenotype upon colonies; strains that initially cause infections of the respiratory tracts of CF patients are typically non-mucoid, but with the progression of the disease, mucoid variants emerge and become predominant.

Most of the genes involved in alginate biosynthesis are clustered at 34 min on the *P. aeruginosa* chromosome and

probably form a single operon (Chitnis & Ohman, 1993; reviewed by May & Chakrabarty, 1994a). The first gene of the cluster, *algD*, codes for GDP-mannose dehydrogenase, which catalyses the unidirectional oxidation of GDP-mannose to GDP-mannuronic acid, the immediate precursor of alginate. Transcription of the alginate biosynthetic cluster, preceded by *algD*, is controlled by a complex regulatory network. Activation of *algD* has been observed only in mucoid *P. aeruginosa* variants and correlates directly with alginate production. Various environmental conditions, such as high osmolarity, nitrogen or phosphate starvation, and ethanol-induced dehydration, promote the activation of *algD* transcription (May & Chakrabarty, 1994a).

The *algD* promoter has a complex structure and DNA topology may play an important role in transcriptional regulation of the *algD* gene. At least two histone-like elements appear to be involved, namely AlgP (AlgR3) (Deretic & Konyecsni, 1990; Kato *et al.*, 1990) and

Abbreviations: CF, cystic fibrosis; IHF, integration host factor.

integration host factor (IHF). Indeed, IHF consensus sequences able to bind purified IHF from *Escherichia coli* have been identified within the *algD* promoter. Recently, Wozniak (1994) has shown, by mutational analysis of the *algD* promoter, that the IHF-binding sites are *cis*-acting elements required for high level *algD* transcription. It was therefore of interest to determine directly the effect of inactivation of the IHF genes on *algD* expression and alginate synthesis.

In a previous study, we demonstrated that *P. aeruginosa* contains IHF protein, which was purified from the mucoid strain CHA and shown to bind to the *algD* promoter *in vitro* (Toussaint *et al.*, 1993a). More recently, we have reported the isolation and sequencing of the structural *himA* and *himD* genes, which encode the two IHF subunits (Delic-Attree *et al.*, 1995). In the present study, we overproduced *P. aeruginosa* IHF in *E. coli* and determined the *in vitro* affinity of IHF for the *algD* promoter. In addition, we constructed an IHF-deficient mutant of the mucoid *P. aeruginosa* strain CHA, and obtained direct *in vivo* evidence for the involvement of IHF in alginate synthesis.

METHODS

Media and growth conditions. *E. coli* strains were grown on LB medium (Sambrook *et al.*, 1989) supplemented with tetracycline (10 µg ml⁻¹), ampicillin (100 µg ml⁻¹), kanamycin (25 µg ml⁻¹) or chloramphenicol (12.5 µg ml⁻¹). *P. aeruginosa* strain CHA, a mucoid CF isolate obtained from the Centre Hospitalier Universitaire of Grenoble was grown in LB liquid medium or on Pseudomonas Isolation Agar (PIA; Difco) plates. The medium was supplemented with the appropriate antibiotics: tetracycline, 300 µg ml⁻¹ for PIA plates and 100 µg ml⁻¹ for LB medium; carbenicillin, 1 mg ml⁻¹ for PIA and 300 µg ml⁻¹ for LB. The minimal medium used was that of Vogel & Bonner (1956). All cultures were grown aerobically at 37 °C.

Construction of an IHF-deficient *E. coli* host strain for overproduction of heterologous IHF. A derivative of *E. coli* BL21(DE3), which is used as a host strain for expression systems based on the T7 phage promoter (Studier *et al.*, 1990), was constructed as follows. Strains JRY827 (LE392, *himD*: :cam^R) and JRY826 (LE392, *himA*: :Tn10) were infected with the transducing phage P1vir, as described by Miller (1972). Strain BL21(DE3) (Novagen) was then infected first with phage from JRY827, with selection for chloramphenicol resistance, then with phage from JRY826, with selection for both chloramphenicol and tetracycline resistance. The resulting strain, JW186 [BL21(DE3), *himA*: :Tn10, *himD*: :cam^R], was resistant to 10 µg tetracycline ml⁻¹ on agar plates, but not in liquid medium. Colony formation generally required 48 h incubation, compared to < 24 h for the parental strain. Strain JW186 lost viability rapidly upon storage at -20 °C, but was stable for up to 12 months at -80 °C. This strain could not be rendered competent by the standard CaCl₂ method, and was therefore transformed by electroporation. The latter procedure was carried out in a Bio-Rad Gene Pulser Unit, using the manufacturer's recommended conditions.

Nucleic acid methodology. Restriction enzymes, DNA polymerase I (Klenow fragment) and T4 DNA ligase were purchased from Boehringer Mannheim. Treatment of DNA with enzymes, subcloning of DNA and transformations were performed as described by Sambrook *et al.* (1989). PCR reactions were done

with *Taq* DNA polymerase (Promega), as recommended by the manufacturer. Plasmid DNA was purified with Qiagen columns (Qiagen Corp.). Southern blot hybridizations and detections were performed as described in the Boehringer Mannheim manual with digoxigenin-labelled DNA probes. DNA sequencing was performed by the chain-termination method (Sanger *et al.*, 1977) using a Sequenase DNA sequencing kit (United States Biochemical) and [³⁵S]dATPαS (1000 Ci mmol⁻¹, 37 TBq mmol⁻¹; Amersham).

Overproduction and purification of *P. aeruginosa* IHF. To construct the overexpression vector pAF9, the *himA* and *himD* genes of *P. aeruginosa* were first amplified by PCR, using plasmids pIA281 and pIA3 (Delic-Attree *et al.*, 1995) as templates. The primers used for amplification of *himA* were OIP2 5'-AACATATGGGGGCTCTGACGAA and OIP1 5'-GGCTAAGGGCCGTTCGAACA. The 338 bp *Nde*I-*Hind*III fragment so generated was cloned in pGEM-T (Promega) to give pGTa. The primers OIP4 5'-GCAAGCTT-GAAAAAGAAAGGGAAA and OIP3 5'-CGGCCTCACTA-TAGCCTAGGCA were used to amplify the *himD* gene. They generated a 329 bp *Hind*III-*Bam*HI fragment which was cloned in pGEM-T to give pGTb. The integrity of the inserts in pGTa and pGTb was verified by DNA sequencing. Next, the *Nde*I-*Hind*III and *Hind*III-*Bam*HI fragments from pGTa and pGTb, respectively, were cloned together in pET-9 (Studier *et al.*, 1990) resulting in plasmid pAF9, in which the *himA* and *himD* genes are under the control of the strong bacteriophage T7 transcription and translation signals. For overexpression, *E. coli* JW186 harbouring pAF9 was grown aerobically at 37 °C in 200 ml SOC medium (Sambrook *et al.*, 1989). Induction was initiated at an OD₆₆₀ of 0.6 by the addition of 1 mM IPTG. IHF was obtained by the two-step purification procedure previously described (Toussaint *et al.*, 1993b).

DNA binding assays and affinity measurements. DNA binding assays were performed essentially as described by Toussaint *et al.* (1993b). When using crude extracts in gel-shift experiments, a DNA fragment containing the high-affinity binding site of the *algB* promoter (Wozniak & Ohman, 1993) was used, since in the case of the *algD* promoter, crude extracts contained additional proteins that gave rise to a shift similar to that produced by IHF. The DNA fragment containing the *algB* promoter was prepared by PCR, using primers 5'-AGCTAAGCTTACCGCCTCGGGGCC and 5'-TACGAATTCCACGG-ATGGCTCCGGGT and genomic DNA from strain CHA as template. A 280 bp fragment extending from -158 to +122 nt relative to the transcription start point of *algB* was generated. The *algD* fragment containing both IHF sites, generated by PCR reaction, was a 486 bp fragment extending from -290 to +196 nt relative to the *algD* start of transcription. Following electrophoresis, the gel was dried and exposed to a storage PhosphorScreen which was then introduced in a Phosphor-Imager apparatus (Molecular Dynamics) for image treatment. For the affinity measurements, total counts from both free DNA and DNA-IHF complex were determined and used to calculate the fraction of free DNA at each IHF concentration. These values were plotted on a graph showing the fraction of free DNA as a function of IHF concentration. The IHF concentration at which 50% of DNA was complexed (*K_d*) was then estimated from this graph.

Construction of a *himA* mutant of *P. aeruginosa*. Analysis of the complete nucleotide sequence of the 1.8 kb *Hind*III insert of pIA281, containing the wild-type *himA* gene, revealed no convenient unique restriction site in *himA* for its insertional inactivation. Therefore, the following strategy was adopted. The 3' and 5' flanking regions of *himA* were amplified in two separate PCR reactions. The oligonucleotides used to amplify

the 5' flanking region were M α 1 5'ACCCGGGGCAGAC-CGCGCGC, which anneals 60–80 nt downstream from the *Pst*I cloning site and contains a *Sma*I site (underlined) at its 5' end, and M α 2 5'TGGATCCGCAATTTTCAGCTTTCG, which is complementary to nt 14–32 downstream from the initiation codon of *himA* and has a *Bam*HI site introduced at its 5' end. PCR amplification with M α 1 and M α 2 generated a unique product of 490 bp that was cloned in pGEM-T (Promega), to give pIA30. Amplification of the 3' flanking region was performed with M α 3 (5'AGGATCCCGATCACGGCCCG-CCG), which anneals to nt 214–223 downstream from the ATG codon and has a *Bam*HI site introduced at the 5' end, and with M α 4 (5'CCTGCAGATGGAGGATGA), which is complementary to the sequence at and just upstream of the second *Pst*I cloning site of pIA281. PCR amplification with primers M α 3 and M α 4 generated an 845 bp fragment that was cloned in pGEM-T, giving pIA31. The two PCR fragments were ligated together in pGEM-T, generating the plasmid pIA32. In this construct a 181 bp internal part of the gene was removed and a unique *Bam*HI site was created. Next, a 1.4 kb *Ava*I–*Eco*RI fragment containing the *tet* gene from pBR322 was blunt-ended with the Klenow enzyme and was ligated into the blunt-ended *Bam*HI site of pIA32. The orientation of *tet* was determined by restriction analysis, and two plasmids, pIA33A and pIA33B, differing with respect to the orientation of the *tet* gene, were selected. To carry out gene replacement as described by Schweizer (1992), the *Sma*I–*Pst*I inserts from pIA33A and pIA33B were subcloned into pNOT19. Next, the MOB cassette from pMOB3 was cloned into the unique *Not*I site of the pNOT19 derivatives. The two resulting suicide plasmids, pIA35A and pIA35B, were then transferred to *P. aeruginosa* CHA by triparental conjugation, using pRK2013 as a helper plasmid (Konyecsni & Deretic, 1988). For complementation experiments, a 1.8 kb *Hind*III fragment containing the wild-type *himA* gene was extracted from pIA281 (Delic-Attree *et al.*, 1995) and cloned into pUCP20 (West *et al.*, 1994).

Immunoblotting experiment. *E. coli* and *P. aeruginosa* cultures were grown overnight in 30 ml LB medium. Cells were harvested by centrifugation and lysed by sonication in 50 mM Tris/HCl, 20 mM EDTA buffer, pH 8. Cell extracts were subjected to PAGE (0.1% SDS, 16%, w/v, polyacrylamide) (Laemmli, 1970) in Tris/Tricine buffer, pH 8. The proteins were then transferred to the nylon membrane by electroblotting and filters were probed with antibodies against *E. coli* IHF. The bound antibodies were detected by chemiluminescence using an ECL kit (Amersham). Bands were scanned with an Arcus II scanner (AGFA) and analysed with ImageQuant software (Molecular Dynamics).

Alginate determination and XylE activity. Alginate was obtained from cells grown on solid PIA medium. Approximately the same number of cells (10^8), grown overnight, was spread on plates and incubated for 24 h at 37 °C. Bacteria were scraped from the plates and resuspended in 0.9% NaCl. Alginate was measured in supernatant fluids after centrifugation (12000 g, 30 min) by the borate/carbazole method (Knutson & Jeanes, 1968; May & Chakrabarty, 1994b). Purified alginate (Sigma) was used as a standard. To construct plasmid pIA111 carrying the *algD*–*xylE* transcriptional fusion, the *algD* promoter from pVD2X (Deretic *et al.*, 1987) was subcloned as a *Hind*III–*Xba*I fragment in pVDX18 (Konyecsni & Deretic, 1988). The activity of the *xylE* reporter gene was determined in supernatants after centrifugation at 12000 g for 15 min as described by Konyecsni & Deretic (1988); one milliunit of catechol 2,3-dioxygenase corresponds to the formation at 25 °C of 1 nmol 2-hydroxymuconic semialdehyde min⁻¹. Protein concentrations were determined by the method of Bradford

(1976) using bovine serum albumin, fraction V (Sigma), as a standard.

RESULTS

Overproduction and purification of *P. aeruginosa* IHF

To overproduct *P. aeruginosa* IHF in *E. coli*, the *himA* and *himD* genes, previously cloned from strain CHA (Delic-Attree *et al.*, 1995), were independently amplified by PCR and subcloned in the pET-9 expression vector. The resulting plasmid, pAF9, was introduced into strain JW186, a derivative of *E. coli* BL21(DE3), in which the chromosomal *himA* and *himD* genes had been inactivated. By using a host strain unable to synthesize *E. coli* IHF, the purification of *P. aeruginosa* IHF was achieved without interference from the homologous *E. coli* protein. We checked by a gel-mobility shift assay that crude extracts prepared from JW186 did not contain active IHF (Fig. 1a, lane 2); in contrast, when crude extracts were prepared from strain JW186 harbouring pAF9, a stable complex due to IHF was obtained (Fig. 1a, lane 3). Strain JW186 has also been used to overproduce IHF from *Rhodobacter capsulatus* (unpublished work) and should have general use as host strain for the overproduction of wild-type or mutant IHF proteins from a wide range of bacteria.

The purification of IHF from strain JW186(pAF9) was achieved by the two-step procedure of Toussaint *et al.* (1993b). Extracts of cells from an IPTG-induced culture were first loaded onto a heparin-Sepharose column, and the proteins were eluted by using a linear gradient of 0–1 M NaCl. This resulted in a high degree of purification of IHF, which was contained in the 0.8 M NaCl fraction (Fig. 1b, lane 2). However, this fraction, as analysed by SDS-PAGE and staining with silver nitrate, contained a major contaminant with a molecular mass of 15 kDa. This contaminant was eliminated by passage of the fraction through a Mono-S cation-exchange column, from which pure IHF was eluted at between 0.44 and 0.48 M NaCl (Fig. 1b, lane 4). The yield of the purification procedure was estimated to be 0.5 mg IHF per litre of induced culture.

Binding of *P. aeruginosa* IHF to the *algD* promoter

Two IHF binding sites have been identified within the promoter of the *P. aeruginosa* *algD* genes, one of which (site 1) spans positions –80 to –68 relative to the *algD* transcriptional start point, and the other of which (site 2) is located downstream from the transcription start point, at positions +79 to +105. The affinity of site 1 for IHF from *E. coli* was previously found to be about two orders of magnitude lower than the affinity of site 2 (Wozniak, 1994). In the present study, we determined the affinity of purified IHF protein from *P. aeruginosa* for the two *algD* IHF binding sites. The K_d for *algD* site 1 was found to be about 2 μ M and the K_d for site 2 was 130 nM (data not shown). With the fragment containing both *algD* sites, a unique complex was observed (Fig. 2), with a K_d for IHF of 130 nM, identical to that obtained with the fragment containing site 2 alone; this suggests that only site 2 was

Fig. 1. Purification of overproduced *P. aeruginosa* IHF. (a) Gel-mobility shift assay with the 280 bp DNA fragment of the *algB* promoter containing the IHF binding site (see Methods). Lanes: 1, free DNA; 2, crude extract of *E. coli* JW186 (300 µg protein); 3, crude extract of JW186(pAF9) (50 µg protein). (b) SDS-PAGE analysis of the protein fractions obtained during purification. Lanes: 1, crude cell extract of JW186(pAF9); 2, heparin-Sepharose 0.8 M NaCl fraction; 3, Mono-S 0.40–0.44 M NaCl fraction; 4, Mono-S 0.44–0.48 M NaCl fraction. The gel was stained with silver nitrate.

Fig. 2. IHF binding affinity to a fragment of the *algD* promoter containing two IHF binding sites. The 486 bp *algD* promoter fragment (approx. 10 fmol) was incubated in the presence of various concentrations of purified *P. aeruginosa* IHF: 0, 5, 10, 20, 40, 100, 200, 300, 600, 1000 nM (lanes 1–10, respectively). Reaction mixtures (15 µl) were loaded on 6% PAGE/0.5 × TBE.

occupied and that the presence of site 2 did not enhance IHF binding to site 1.

Intracellular concentration of IHF in *P. aeruginosa* CHA

The occupancy of binding sites *in vivo* may vary with the concentration of IHF in the cell. Indeed, recent studies have demonstrated that the intracellular fluctuation of IHF in *E. coli* affects several biological functions of that protein (Ditto *et al.*, 1994). The intracellular concentration of *P. aeruginosa* IHF was determined by Western blot

analyses with *E. coli* anti-IHF antibodies. These antibodies recognize both IHF subunits of *P. aeruginosa* (the β -subunit is less well recognized), as well as the histone-like protein of 9 kDa, HU, which shares significant amino acid homology with IHF. The overproduced IHF protein, purified to homogeneity, was used for calibration (Fig. 3, lanes 1–4). The same amounts of *P. aeruginosa* cells, harvested at different points of growth, were prepared as described by Ditto *et al.* (1994) and directly loaded onto the gel. As can be seen in Fig. 3, the level of IHF did not change significantly between the early- (OD₆₀₀ 0.1) and the mid-exponential (OD₆₀₀ 0.8) phase of growth, but increased more than tenfold when the cells entered the stationary phase (OD₆₀₀ 2.0). The relative amount of IHF did not change further during late-stationary phase. The estimation of the amount of IHF was based on the facts that: (i) *P. aeruginosa* cultures in the exponential and the stationary phase of growth contain 1.5×10^9 viable cells per OD₆₀₀ ml⁻¹ unit and (ii) this quantity of cells contains approximately 280 µg protein. In the stationary phase, the intracellular concentration of *P. aeruginosa* IHF was estimated to be 0.2 ng IHF per µg total cell protein, that is about one-thirtieth of the IHF concentration in *E. coli* cells at the same phase of growth (Ditto *et al.*, 1994). The low abundance of IHF in *P. aeruginosa* cells compared to *E. coli* was confirmed by the relative binding activity of IHF in crude extracts. About 20 times more protein from *P. aeruginosa* than from *E. coli* was needed to obtain a similar level of binding (data not shown).

Inactivation of the *himA* gene in a mucoid strain of *P. aeruginosa*

In order to investigate the *in vivo* role of IHF in alginate production, a derivative of the mucoid *P. aeruginosa* strain, CHA, carrying a deletion in the *himA* gene, was

Fig. 3. Determination of the intracellular concentration of IHF in *P. aeruginosa* CHA cells. Lanes 1–4, purified IHF protein (2.7, 27, 136 and 272 ng protein, respectively). Lanes 5–8, total extract of CHA cells harvested at an OD₆₀₀ of 0.1, 0.8, 2.0 and 3.5, respectively, then adjusted to an OD₆₀₀ of 0.5. Cells were lysed in the gel-loading buffer, and separated by SDS-PAGE (0.1% SDS, 16% polyacrylamide). Proteins were blotted onto the membrane and developed by antibodies against *E. coli* IHF, as described in Methods. The protein of higher mobility present in *P. aeruginosa* crude extracts which cross-reacts with anti-IHF antibodies corresponds to *P. aeruginosa* HU.

Fig. 4. (a) Construction of *P. aeruginosa* strains CHA-A2 and CHA-B2, mutated in *himA*, by the allelic exchange technique. A partial restriction map of plasmid pIA35, which contains the inactivated *himA* gene of *P. aeruginosa* CHA, is shown. Plasmids pIA35A and pIA35B differ in the orientation of the tetracycline (*tet*) resistance gene inserted into *himA*. The unique *NotI* site used to ligate the MOB cassette from pMOB3 is underlined. Selection for the transconjugants that were Tc^R and Cb^S resulted in *P. aeruginosa* strains CHA-A2 and CHA-B2. Abbreviations: S, *SmaI*; E, *EcoRI*; B, *BamHI*; A, *AvaI*; H, *HindIII*; P, *PstI*; Tc^R, resistance to tetracycline; Cb^R, resistance to carbenicillin; Cb^S, sensitivity to carbenicillin. (b) Gel-mobility shift assay with an *algB* DNA fragment. A PCR-generated DNA fragment containing the IHF binding sequence from the *algB* promoter was labelled and incubated with cell extracts from *P. aeruginosa* CHA (lane 1), CHA-A2 (lane 2), CHA-B2 (lane 3), CHA-A2(pIA282) (lane 4) and CHA-B2(pIA282) (lane 5). The IHF-*algB* complex is indicated with an arrow. Another complex, indicated with an asterisk, is due to an unidentified protein. (c) Western blot of total cellular protein extracts of *E. coli* and *P. aeruginosa* strains using antibodies against *E. coli* IHF. Lanes: 1, *E. coli* HB101; 2, *P. aeruginosa* CHA; 3, CHA-A2; 4, CHA-B2. The IHF and HU proteins that cross-react with anti-IHF antibodies are indicated.

constructed. Gene replacement in strain CHA was carried out as described in Methods, using plasmids (pIA35A and B) in which the *tet* gene was inserted in the two opposite orientations with respect to *himA* transcription. Allelic exchange between the CHA chromosome and pIA35 at

the *himA* locus takes place by a double recombination event. Strains in which a double crossover had occurred were identified by screening tetracycline-resistant transconjugants for sensitivity to carbenicillin (Fig. 4a). Two carbenicillin-sensitive isolates, designated CHA-A2 and

CHA-B2, were then tested by Southern hybridization to confirm the presence of the correct *himA*:*tet* insertion. The absence of plasmid, and the presence of a single copy of the *tet* gene inserted in the *himA* gene, were further verified by using either the MOB cassette from pMOB3 or the *tet* gene as a probe (data not shown).

The absence of functional IHF in the mutants was confirmed by gel retardation experiments, using a DNA fragment containing the IHF-binding site from the *algB* promoter (Fig. 4b). A complex with the same mobility as that formed with purified *P. aeruginosa* IHF was observed with crude extracts from strain CHA, but not with crude extracts from strains CHA-A2 and CHA-B2. This band was restored in the mutant strains complemented with the wild-type *himA* gene cloned on a 1.8 kb *Pst*I fragment (Fig. 4b, lanes 4 and 5). An additional band, which was observed with extracts from all strains (indicated with an asterisk), is probably due to an additional, unidentified protein, which also binds to the *algB* promoter (unpublished data).

Further confirmation that the mutants lacked IHF protein was provided by Western blotting using antibodies against *E. coli* IHF (Fig. 4c). The mutant strains lacked the β subunit of IHF (encoded by *himD*), as well as the α subunit, suggesting that the α subunit is required for the synthesis of the β subunit, or to stabilize it, e.g. by heterodimer formation. A decrease in the level of β subunit following deletion of *himA* has also been observed in *E. coli* (Ditto *et al.*, 1994). The HU protein (9 kDa) was present in similar amounts in all strains, showing that IHF deficiency had no effect on the synthesis of HU.

Effect of *himA* inactivation on *algD* gene expression

Transcriptional fusions between the *algD* promoter and the *xylE* reporter gene were constructed, then introduced into the parental strain CHA and the *himA* mutants. The transcriptional activities of *palgD* were determined by quantitative catechol dioxygenase assays.

Several reports have shown that induction of *algD* transcription depends on the mucoid status of a given strain and on various environmental stimuli. We observed a tenfold increase in the level of *algD* transcription in the mucoid strain CHA when the NaCl concentration in the medium was increased from 0 to 0.3 M (Fig. 5a), in accordance with earlier results that defined the expression pattern of *algD* (DeVault *et al.*, 1989). In the *himA* mutants, expression of the *palgD*-*xylE* fusion did not differ from that in strain CHA when cells were grown in LB medium without NaCl, but in the presence of 0.3 M NaCl, when *algD* is fully induced, Xyle specific activity in the *himA* mutants was only 25–35% of that observed in the parental CHA cells. Nevertheless, the level of *palgD* transcription in the *himA* mutants remained two- to threefold higher than that observed in the parental and the two mutant strains under non-activating conditions (Fig. 5a). This result suggests that IHF is not absolutely required for basal *algD* expression, but is

Fig. 5. Analysis of *algD* expression in *himA* mutants using quantitative catechol dioxygenase (CDO) assays. *P. aeruginosa* strains harbouring the *algD*-*xylE* fusion plasmid pIA111 were grown to an identical cell density (OD_{600} 2.0) in LB medium either with or without 0.3 M NaCl (a), or in minimal medium containing either $(NH_4)_2SO_4$ or KNO_3 (each 0.2%) as nitrogen source (b). The results shown are means from at least three experiments. Results are plotted as a percentage of the fully induced parental (CHA) activity level; 100% specific activity corresponded to 19000–21000 mU (mg protein)⁻¹ (a) and 500–700 mU (mg protein)⁻¹ (b).

necessary for full activation under conditions of high salinity.

Another environmental factor reported to activate *algD* expression is nitrogen limitation (May & Chakrabarty, 1994a). We measured the activity of the *palgD*-*xylE* fusion in minimal medium supplemented either with ammonia or with nitrate as the nitrogen source. In the IHF⁺ parental strain CHA the level of *algD* transcription was increased tenfold in the medium containing nitrate compared to that in medium containing ammonia, while in the *himA* mutants the *algD* promoter was not activated under those conditions (Fig. 5b).

We have shown above that transcriptional activation of the *algD* gene is affected in the *himA* mutants CHA-A2 and CHA-B2. Since a strong activation of *algD* is a necessary step in alginate overproduction leading to a mucoid phenotype we examined the synthesis of alginate in the mutant strains. The initial screening of the mutants on agar plates containing tetracycline did not allow us to select double recombinants, such as CHA-A2 and CHA-B2, on the basis of a non-mucoid phenotype. When grown on PIA plates, a medium reported to induce alginate production, the colonies clearly synthesized less alginate than the parental strain, CHA, but were still mucoid. The difference in the mucoid aspect of the colonies was most obvious when CHA-A2 and CHA-B2 were restreaked on the same PIA plate alongside the parental CHA strain. This difference was quantified by determining the amount of alginate synthesized (Fig. 6). The amount of alginate produced by strain CHA was comparable to that of other CF isolates (e.g. Deretic *et al.*, 1987). The quantity of alginate synthesized by the mutants was approximately 50% of that synthesized by the parental strain, CHA. Complementation of the CHA-A2 and CHA-B2 mutants with plasmid pIA282, containing the intact *himA* gene, restored the level of alginate synthesis to the amounts measured in CHA. This shows

Fig. 6. Alginate production in *P. aeruginosa* strains. Alginate was isolated from cultures grown for 24 h on PIA plates and its amount determined by the carbazole method (Knutson & Jeanes, 1968). The results are means from at least three experiments.

that the decrease in alginate synthesis was indeed due to the lack of IHF and not to a secondary mutation.

DISCUSSION

The results described in this paper confirm and extend the report of Wozniak (1994) concerning the role of IHF in *algD* gene expression. The two IHF binding sites in the *algD* promoter were shown to bind purified, recombinant *P. aeruginosa* IHF with K_d values similar to those reported previously for *E. coli* IHF, suggesting that the two proteins have similar binding activity. The K_d values of 130 nM for *algD* IHF site 2 and 1.9 µM for *algD* IHF site 1 determined with *P. aeruginosa* IHF are higher than the 1–20 nM determined for the binding of *E. coli* IHF to its specific sites (reviewed by Nash, 1996), although values in the range of 100 nM have also been found in *E. coli* (Freundlich *et al.*, 1992).

In late-stationary phase, the IHF concentration in *E. coli* may be as high as 84 µM (Ditto *et al.*, 1994). In the present study, we found that the IHF content of *P. aeruginosa* increased when the cells entered stationary phase, as found in *E. coli* (Ditto *et al.*, 1994; Aviv *et al.*, 1994), but the level, in the range 200–500 nM in exponential phase and about 3 µM in stationary phase, was about 30 times lower than in *E. coli*. The IHF concentration in exponential phase is probably insufficient to saturate both sites in the *algD* promoter *in vivo*, even if all the IHF is available for binding. However, the major increase in the intracellular content of IHF occurs at the phase of growth where *algD* transcription and alginate synthesis reach their maximal levels (Leitão & Sá-Correia, 1995) and may be critical for the *in vivo* role of IHF in the *algD* activation.

Mutational analysis of the *algD* IHF binding sites has shown that both are required for full-level *algD* expression (Wozniak, 1994). In the present study, the isolation of an IHF-deficient mutant of *P. aeruginosa* allowed us to confirm the role of IHF protein in *algD* expression and to examine more clearly the role this protein plays *in vivo* in the regulation of *algD* expression and alginate synthesis. The *algD* gene is highly regulated and its expression is

modulated by environmental conditions such as nitrogen limitation, osmolarity or ethanol. It has been established that the topological state of the promoter DNA plays a role in transcriptional activation of environmentally regulated genes (Higgins *et al.*, 1990). It was initially thought that IHF acts essentially as an architectural element, promoting the bending of DNA and allowing the interaction between an activator, bound at sequences known as far upstream sites (FUS) from the transcriptional start site, and σ^{54} -containing RNA polymerase. Such is the case for *nifH*, which has a σ^{54} -dependent promoter activated by the NifA regulator, a member of the NtrC subfamily (Hoover *et al.*, 1990). Activation of *algD* depends on two regulatory proteins, AlgR (Deretic *et al.*, 1989) and AlgB (Wozniak & Ohman, 1991; Goldberg & Dahnke, 1992), which both belong to the same NtrC subfamily. However, *algD* transcription does not require σ^{54} but rather the product of *algU*. AlgU is a sigma factor related to *E. coli* stress-response σ^E factor (Deretic *et al.*, 1994; Hershberger *et al.*, 1995). The position of the upstream IHF binding site 1 located between the transcriptional start site and the FUS suggests that IHF functions as an architectural element that enhances the interactions of RNA polymerase with activating proteins, including AlgR. In *algR* mutants, the activation of *algD* by nitrogen limitation is abolished, but some response to increased osmolarity is still observed (Mohr *et al.*, 1990). Under these two activating conditions, we observed that our IHF mutants were not able to achieve maximal *algD* transcription. This is the only known example where IHF is involved in the activation of a σ^E -type promoter. The relatively high residual *algD* activity (25–35%) measured in the IHF mutants under activating conditions, an activity that correlates with alginate production (50% compared to the parental strain, CHA), can be explained either by a non-specific activation by *trans*-activators that do not require IHF or by the involvement of other histone-like elements like AlgR3. Indeed, in addition to IHF, several proteins able to bind and bend the *algD* promoter DNA have been identified. The cAMP receptor protein from *E. coli* binds the *algD* promoter between positions –399 and –288 upstream from the transcriptional start site (DeVault *et al.*, 1991). It has been shown also that the synthetic peptide derived from AlgR3 (also called AlgP), a *P. aeruginosa* H1-like histone protein, can bind to the *algD* promoter (Deretic & Konyecsni, 1990), and a general function for AlgR3 (AlgP) in the organization of the bacterial nucleoid has been proposed (Deretic *et al.*, 1992). Although a direct role of a CRP-like protein and of AlgR3 (AlgP) in the regulation of *algD* is still questionable, a participation of those proteins in the modulation of the local promoter topology cannot be excluded.

Mutational analysis of IHF site 2, located downstream from +1 of transcription, has shown that site 2 is indeed required for transcriptional activation of *algD*; furthermore, deletion of the 3' region downstream from that site resulted in a tenfold decrease in *algD* expression (Wozniak, 1994). The role of IHF may be to facilitate interactions between a factor bound to a 3' enhancer and RNA

polymerase. Preliminary results from our laboratory indicate that IHF binding to site 2 in the *palgD* 3' region induces a strong looping out of DNA, an effect consistent with the proposed DNA bending function of IHF. Furthermore, we have recently isolated a DNA-binding protein able to bind specifically to the 3' *palgD* region, downstream from IHF site 2, and are currently in the process of characterizing it (unpublished data). This unusual organization of regulatory elements is not unknown for bacterial promoters. Perhaps the best-known example is a developmentally regulated *Caulobacter* flagellar gene, *flaN*, whose activation requires both a 3' enhancer element and an IHF binding site situated just upstream of it (Gober & Shapiro, 1992).

ACKNOWLEDGEMENTS

The authors thank Drs H. A. Nash (NIH, Bethesda, USA), J. Rouvière-Yaniv (Institut de Biologie Physico-Chimique, Paris) and M.-A. Mandrand (INSA, Lyon) for generous gifts of anti-IHF antibodies, *E. coli* JRY827 and JR826 strains, and phage P1vir, respectively. This work was supported in part by grant from the Association Française de Lutte contre la Mucoviscidose (no. 94003, to B. Toussaint).

REFERENCES

- Aviv, M., Giladi, H., Schreiber, G., Oppenheim, A. B. & Glaser, G. (1994). Expression of the genes coding for the *Escherichia coli* integration host factor are controlled by growth phase, *rpoS*, ppGpp and by autoregulation. *Mol Microbiol* **14**, 1021–1031.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Chitnis, C. E. & Ohman, D. E. (1993). Genetic analysis of the alginate biosynthetic cluster of *Pseudomonas aeruginosa* shows evidence of an operonic structure. *Mol Microbiol* **8**, 583–590.
- Delic-Attree, I., Toussaint, B. & Vignais, P. M. (1995). Cloning and sequence analysis of the genes coding for the integration host factor (IHF) and HU and proteins of *Pseudomonas aeruginosa*. *Gene* **154**, 61–64.
- Deretic, V. & Konyecsni, W. M. (1990). A procaryotic regulatory factor with a histone H1-like carboxy-terminal domain: clonal variation of repeats within *algP*, a gene involved in regulation of mucoidy in *Pseudomonas aeruginosa*. *J Bacteriol* **172**, 5544–5554.
- Deretic, V., Gill, J. F. & Chakrabarty, A. M. (1987). Gene *algD* coding for GDPmannose dehydrogenase is transcriptionally activated in mucoid *Pseudomonas aeruginosa*. *J Bacteriol* **169**, 351–358.
- Deretic, V., Dikshit, R., Konyecsni, W. M., Chakrabarty, A. M. & Misra, T. K. (1989). The *algR* gene, which regulates mucoidy in *Pseudomonas aeruginosa*, belongs to a class of environmentally responsive genes. *J Bacteriol* **171**, 1278–1283.
- Deretic, V., Hibler, N. S. & Holt, S. C. (1992). Immunocytochemical analysis of AlgP (H_p1), a histonelike element participating in control of mucoidy in *Pseudomonas aeruginosa*. *J Bacteriol* **174**, 824–831.
- Deretic, V., Schurr, M. J., Boucher, J. C. & Martin, D. W. (1994). Conversion of *Pseudomonas aeruginosa* to mucoidy in cystic fibrosis: environmental stress and regulation of bacterial virulence by alternative sigma factors. *J Bacteriol* **176**, 2773–2780.
- DeVault, J. D., Berry, A., Misra, T. K., Darzins, A. & Chakrabarty, A. M. (1989). Environmental sensory signals and microbial pathogenesis: *Pseudomonas aeruginosa* infection in cystic fibrosis. *Bio/Technology* **7**, 352–357.
- DeVault, J. D., Hendrickson, W., Kato, J. & Chakrabarty, A. M. (1991). Environmentally regulated *algD* promoter is responsive to the cAMP receptor protein in *Escherichia coli*. *Mol Microbiol* **5**, 2503–2509.
- Ditton, M. D., Roberts, D. & Weisberg, R. A. (1994). Growth phase variation of integration host factor level in *Escherichia coli*. *J Bacteriol* **176**, 3738–3748.
- Freundlich, M., Ramani, N., Mathew, E., Sirko, A. & Tsui, P. (1992). The role of integration host factor in gene expression in *Escherichia coli*. *Mol Microbiol* **6**, 2557–2563.
- Gober, J. W. & Shapiro, L. (1992). A developmentally regulated *Caulobacter* flagellar promoter is activated by 3' enhancer and IHF binding elements. *Mol Biol Cell* **3**, 913–926.
- Goldberg, J. B. & Dahnke, T. (1992). *Pseudomonas aeruginosa* AlgB, which modulates the expression of alginate, is a member of the NtrC subclass of prokaryotic regulators. *Mol Microbiol* **6**, 59–66.
- Hershberger, C. D., Ye, R. W., Parsek, M. R., Xie, Z.-D. & Chakrabarty, A. M. (1995). The *algT* (*algU*) gene of *Pseudomonas aeruginosa*, a key regulator involved in alginate biosynthesis, encodes an alternative σ factor (σ^B). *Proc Natl Acad Sci USA* **92**, 7941–7945.
- Higgins, C. F., Hinton, J. C. D., Hulton, C. S. J., Owen-Hughes, T., Pavitt, G. D. & Seirafi, A. (1990). Protein H1: a role for chromatin structure in the regulation of bacterial gene expression and virulence? *Mol Microbiol* **4**, 2007–2012.
- Hoover, T. R., Santero, E., Porter, S. & Kustu, S. (1990). The integration host factor stimulates interaction of RNA polymerase with NIFA, the transcriptional activator for nitrogen fixation operons. *Cell* **63**, 11–22.
- Kato, J., Misra, T. K. & Chakrabarty, A. M. (1990). AlgR3, a protein resembling eukaryotic histone H1, regulates alginate synthesis in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **87**, 2887–2891.
- Knutson, C. A. & Jeanes, A. (1968). A new modification of the carbazole analysis: application to heteropolysaccharides. *Anal Biochem* **24**, 470–481.
- Konyecsni, W. M. & Deretic, V. (1988). Broad-host-range plasmid and M13 bacteriophage-derived vectors for promoter analysis in *Escherichia coli* and *Pseudomonas aeruginosa*. *Gene* **74**, 375–386.
- Krieg, D. P., Helmke, R. J., German, R. F. & Mangos, J. A. (1988). Resistance of mucoid *Pseudomonas aeruginosa* to nonopsonic phagocytosis by alveolar macrophages *in vitro*. *Infect Immun* **28**, 546–556.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Leitão, J. H. & Sá-Correia, I. (1995). Growth-phase-dependent alginate synthesis, activity of biosynthetic enzymes and transcription of alginate genes in *Pseudomonas aeruginosa*. *Arch Microbiol* **163**, 217–222.
- May, T. B. & Chakrabarty, A. M. (1994a). *Pseudomonas aeruginosa*: genes and enzymes of alginate synthesis. *Trends Microbiol* **2**, 151–157.
- May, T. B. & Chakrabarty, A. M. (1994b). Isolation and assay of *Pseudomonas aeruginosa* alginate. *Methods Enzymol* **235**, 295–304.
- May, T. B., Shinabarger, D., Maharaj, R., Kato, J., Chu, L., DeVault, J. D., Roychoudhury, S., Zielinski, N., Berry, A., Rothmel, R. K., Misra, T. K. & Chakrabarty, A. M. (1991). Alginate synthesis in *Pseudomonas aeruginosa*: a key pathogenic factor in chronic pulmonary infections of cystic fibrosis patients. *Clin Microbiol Rev* **4**, 191–206.
- Miller, J. H. (1972). In *Experiments in Molecular Genetics*, pp. 4549–4557. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Mohr, C. D., Martin, D. W., Konyecsni, W. M., Govan, J. R. W.,

- Lory, S. and Deretic, V. (1990).** Role of the far-upstream sites of the *algD* promoter and the *algR* and *rpoN* genes in environmental modulation of mucoidy in *Pseudomonas aeruginosa*. *J Bacteriol* **172**, 6576–6580.
- Nash, H. A. (1996).** The *E. coli* HU and IHF proteins: accessory factors for complex protein–DNA assemblies. In *Regulation of Gene Expression in Escherichia coli*. Edited by E. C. C. Lin & A. S. Lynch. Austin, TX: R. G. Landes (in press).
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977).** DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Slack, M. P. E. & Nichols, W. W. (1981).** The penetration of antibiotics through sodium alginate and through the exopolysaccharide of a mucoid strain of *Pseudomonas aeruginosa*. *Lancet* **ii**, 502–503.
- Schweizer, H. P. (1992).** Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable *oriT* and the counter-selectable *Bacillus subtilis* *sacB* marker. *Mol Microbiol* **6**, 1195–1204.
- Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990).** Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol* **185**, 60–89.
- Toussaint, B., Delic-Attree, I. & Vignais, P. M. (1993a).** *Pseudomonas aeruginosa* contains an IHF-like protein that binds to the *algD* promoter. *Biochem Biophys Res Commun* **196**, 416–421.
- Toussaint, B., Delic-Attree, I., de Sury d'Aspremont, R., David, L., Vinçon, M. & Vignais, P. M. (1993b).** Purification of the Integration Host Factor homolog of *Rhodobacter capsulatus*: cloning and sequencing of the *hip* gene, which encodes the β subunit. *J Bacteriol* **175**, 6499–6504.
- Vogel, H. J. & Bonner, D. M. (1956).** Acetylornithinase of *Escherichia coli*: partial purification and some properties. *J Biol Chem* **218**, 97–106.
- West, S. E. H., Schweizer, H. P., Dall, C., Sample, A. K. & Runyen-Janecky, L. J. (1994).** Construction of improved *Escherichia–Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. *Gene* **128**, 81–86.
- Wozniak, D. J. (1994).** Integration host factor and sequences downstream of the *Pseudomonas aeruginosa algD* transcription start site are required for expression. *J Bacteriol* **176**, 5068–5076.
- Wozniak, D. J. & Ohman, D. E. (1991).** *Pseudomonas aeruginosa* AlgB, a two-component response regulator of the NtrC family, is required for *algD* transcription. *J Bacteriol* **173**, 1406–1413.
- Wozniak, D. J. & Ohman, D. E. (1993).** Involvement of the alginate *algT* gene and integration host factor in the regulation of the *Pseudomonas aeruginosa algB* gene. *J Bacteriol* **175**, 4145–4153.

Received 15 January 1996; revised 3 April 1996; accepted 31 May 1996.