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

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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 *Kd* values were determined to be 130 nM for *algD* IHF site 2 and about 2 pM 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* transcription 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
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 (125 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 & Bonnet (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*: cam⁰) and JRY826 (LE392, *himA*: Tn10) were infected with the transducing phage Plvir, as described by Miller (1972). Strain BL21(DE3) (Novagen) was then infected 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*: cam⁰), 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 Roche and New England BioLabs. Treatment of DNA with enzymes, subcloning of DNA and transformations were performed as described by Sambrook et al. (1989). PCR reactions were done with *Tag* DNA polymerase (Promega), as recommended by the manufacturer. Plasmid DNA was purified with Qagen 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 [³²P]dATP (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’ACATATGGGGGCTCTGACGAA and OIP1 5’GGCTAAAGGGGCGTTCGAACA. The 338 bp NdeI-HindIII fragment so generated was cloned into pGEM-T (Promega) to give pGTb. The integrity of the inserts in pGTa and pGTb was verified by DNA sequencing. Next, the NdeI-HindIII and HindIII-BamHI fragments from pGTb were cloned into pGEM-T to give pGTc. The primers OIP4 5’GAAGCTTGAGAAAGAGGGGAGA and OIP3 5’CCGCTCTACTAGCTGAGGCA were used to amplify the himD gene. They generated a 329 bp HindIII-BamHI fragment which was cloned into pGEM-T to give pG7b. The integrity of the inserts in pGTa and pG7b was verified by DNA sequencing. Overexpression, *E. coli* JW186 harbouing pG7b 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’AGCTAAGCGCTTACGCTC and 5’TACGAATTCCACGGTCCTCCTC. The 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 PhosphorImager 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 (K₅) 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 HindIII insert of pLA281, 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 Ma1 5′ACCCGGGCGACCGGCGG, which anneals 60–80 nt downstream from the PstI cloning site and contains a Smal site (underlined) at its 5′ end, and Ma2 5′TGATCCGGAATTCTAGGTTTCG, which is complementary to nt 14–32 downstream from the initiation codon of himA and has a BamHI site introduced at its 5′ end. PCR amplification with Ma1 and Ma2 generated a unique product of 490 bp that was cloned in pGEM-T (Promega), to give plA30. Amplification of the 3′ flanking region was performed with Ma3 (5′AGATCCGCGTACGCCGCGGCGG), which anneals to nt 214–223 downstream from the ATG codon and has a BamHI site introduced at the 5′ end, and with Ma4 (5′CGCGCGATGAGGATGA), which is complementary to the sequence at and just upstream of the second PstI cloning site of plA281. PCR amplification with primers Ma3 and Ma4 generated an 845 bp fragment that was cloned in pGEM-T, giving plA31. The two PCR fragments were ligated together in pGEM-T, generating the plasmid plA32. In this construct a 181 bp internal part of the gene was removed and a unique BamHI site was created. Next, a 1.4 kb Avai-EcoRI fragment containing the tet gene from pBR322 was blunt-ended with the Klenow enzyme and was ligated into the blunt-ended BamHI site of plA32. The orientation of tet was determined by restriction analysis, and two plasmids, plA33A and plA33B, differing with respect to the orientation of the tet gene, were selected. To carry out gene replacement as described by Schweizer (1992), the Smal-PstI inserts from plA33A and plA33B were subcloned into pNOT1. Next, the MOB cassette from pMOB3 was cloned into the unique NsiI site of the pNOT1 derivatives. The two resulting suicide plasmids, plA35A and plA35B, were then transferred to P. aeruginosa CHA by triparental conjugation, using pRK192 as a helper plasmid (Konyecsni & Deretic, 1988). For complementation experiments, a 1.8 kb HindIII fragment containing the wild-type himA gene was extracted from plA281 (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 lyzed by sonication in 50 mM Tris/HCl, 20 mM EDTA buffer, pH 8. Cell extracts were subjected to PAGE (10% 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 XyIE activity. Alginate was obtained from cells grown on solid PLA medium. Approximately the same number of cells (108), 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 algin (Sigma) was used as a standard. To construct plasmid plA111 carrying the algD-xyIE transcriptional fusion, the algD promoter from pVD2X (Deretic et al., 1987) was subcloned as a HindIII–Xhol fragment in pVDX18 (Konyecsni & Deretic, 1988). The activity of the xyIE 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 overproduce 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 Kd for algD site 1 was found to be about 2 μM and the Kd 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 Kd for IHF of 130 nM, identical to that obtained with the fragment containing site 2 alone; this suggests that only site 2 was
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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.

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

Intracellular concentration of IHF in 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

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 x 10⁹ viable cells per OD₆₀₀ 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

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
Fig. 3. Determination of the intracellular concentration of IHF in P. aeruginosa CHA cells. Lanes 1–4, purified IHF protein (27, 27, 136 and 272 ng protein, respectively). Lanes 5–8, total extract of CHA cells harvested at an OD_{600} of 0.1, 0.8, 2.0 and 3.5, respectively, then adjusted to an OD_{600} 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, Smal; E, EcoRI; B, BamHI; A, Aval; H, HindIII; P, PstI; Tc^r, resistance to tetracycline; Cb^s, resistance to carbenicillin; Cb^i, sensitivity to carbenicillin. (b) Gel-mobility shift assay with an aigB DNA fragment. A PCR-generated DNA fragment containing the IHF binding sequence from the aigB 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–aigB complex is indicated with an arrow. Another complex, indicated with an asterisked arrow, 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
CHAB2, 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 PstI 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 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
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 $K_a$ values similar to those reported previously for E. coli IHF, suggesting that the two proteins have similar binding activity. The $K_a$ 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 $σ^B$-containing RNA polymerase. Such is the case for nifH, which has a $σ^B$-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 & Dahne, 1992), which both belong to the same NtrC subfamily. However, algD transcription does not require $σ^B$ but rather the product of algU. AlgU is a sigma factor related to E. coli stress-response $σ^B$ 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 $σ^B$-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 indicates 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).

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