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PSEUDOMONAS AERUGINOSA CONTAINS AN IHF-LIKE PROTEIN THAT BINDS TO THE *algD* PROMOTER

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SUMMARY: An IHF-like protein has been purified from a *Pseudomonas aeruginosa* strain isolated from a cystic fibrosis patient, by the rapid purification method described for the isolation of IHF from *Rhodobacter capsulatus*. The IHF of *P. aeruginosa* is an $\alpha\beta$ heterodimer (subunits of 10 and 11 kDa) similar to IHF from *Escherichia coli* and from *R. capsulatus*; the N-terminal amino acid sequences of the isolated subunits share a high degree of identity with their homologs from *E. coli*. *P. aeruginosa* IHF is able to bind to the promoter of the hydrogenase structural genes (*hupSL*) of *R. capsulatus* as do the other two IHF proteins. It is also demonstrated by gel retardation assays that *P. aeruginosa* IHF forms a stable complex with the *algD* promoter *in vitro*, an indication that the protein is involved in the regulation of *algD* gene expression in *P. aeruginosa*.

The typical phenotype of *Pseudomonas aeruginosa* strains that chronically infect cystic fibrosis patients is the mucoid phenotype that results from overproduction of the exopolysaccharide alginate (1). Alginate biosynthesis, in *P. aeruginosa*, involves a large number of genes, called *alg*, which have been cloned and sequenced (reviewed in 2 and 3). Enhancement of alginate synthesis in the respiratory tracts of cystic fibrosis patients results from the activation of the *algD* gene, which codes for GDP-mannose dehydrogenase, an enzyme that catalyzes the unidirectional oxidation of GDP-mannose to the alginate precursor, GDP-mannuronic acid. The transcription of the *algD* gene is enhanced in response to environmental stimuli, in particular in response to hyperosmolarity. Activation of the *algD* promoter is brought about by regulatory genes, in particular by $algR_1$ and $algR_2$, the products of which belong to the superfamily of the bacterial two-component signal transducing systems. The $AlgR_2$ polypeptide is a kinase which can autophosphorylate and then transfer its phosphate to $AlgR_1$, the response regulator (4). $AlgR_1$ is a regulatory protein able to bind to three sites in the *algD* promoter region (5). It is thought that $AlgR_1$, bound to upstream activating sequences, interacts with RNA polymerase holoenzyme bound at the *algD* transcription start site, through a nucleoid structure. The *algD* promoter has also been shown by DNA sequencing and transcriptional mapping (6) to contain σ^{54} (RpoN)-like recognition sequence for RNA polymerase binding, i.e. to be of the -24/-12 promoter type (7). Two other regulatory genes, $algR_3$ and *algB*, are needed for alginate synthesis (8, 2, 3) but their exact

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role is not yet known. Very recently, the *algU* gene which encodes a protein having sequence similarities with a sigma factor of *Bacillus subtilis* (Spo0H) has been shown to be essential in determining the mucoid status (9).

It has recently been shown by gel shift experiments that the histone-like element, integration host factor (IHF) from *Escherichia coli*, forms complexes with the *algD* promoter and induces DNA bending (10). In this report, we demonstrate that *P. aeruginosa* contains an IHF-like protein that binds to the *algD* promoter.

MATERIAL AND METHODS

Bacterial strains and cultures - The *Pseudomonas aeruginosa* strain CHA used in this study is a mucoid strain isolated from the lungs of a cystic fibrosis patient at the Centre Hospitalier Universitaire of Grenoble. *P. aeruginosa* CHA cells were grown aerobically in a medium containing 0.5% yeast extract, 1% tryptone and 0.2% glucose at 37°C. *Escherichia coli* DH5α cells were grown aerobically at 37°C in Luria-Bertani medium (11).

DNA manipulations - Standard recombinant DNA techniques were performed as described (11). The 270 bp DNA fragment from the *algD* promoter was obtained from total *P. aeruginosa* genomic DNA after PCR amplification using 2 oligonucleotides, the sense (5'agctaagcttgcaaagtgcatgggtcg3') PAOD1 and the antisense (5'tacgaattcgataggcatcgcggtg3') PAOD2. The fragment obtained corresponded to the -251/+14 positions relative to the mRNA start site and included *Hin*dIII and *Eco*RI restriction sites at position -251 and +14, respectively, introduced by the synthetic oligonucleotides. Plasmid pGMDp1 was obtained by inserting directly the PCR product, purified by electrophoresis on an acrylamide gel, in plasmid pGEM-T, according to the instructions of the manufacturer (PROMEGA).

Purification of *P. aeruginosa* IHF - *P. aeruginosa* cells were grown overnight aerobically in 5 l cultures yielding typically 30 g of wet cells. The cells were harvested by centrifugation, resuspended in 100 mM Tris HCl, 20 mM EDTA and lysosyme 0.2 mg/ml was added. The cell extract (50 ml), obtained after sonication and ultracentrifugation (150 000 g, 2 hours) and containing 1 g of protein, was applied to a 5 ml Econo-Pac heparin cartridge (Bio-Rad), equilibrated with buffer A (20 mM Tris HCl, 0.5 mM EDTA, 10% glycerol, pH 8) and the column was developed with a step gradient of 0 to 1 M NaCl. DNA was eluted from the heparin column at low salt concentration (between 0 and 0.25 M NaCl). The elution of proteins was monitored at 280 nm and protein content determined according to Bradford (12). The fraction that eluted from 0.65 to 0.8 M NaCl in buffer A (20 μg of protein in a volume of 10 ml) contained the IHF protein in an amount sufficient to be detectable by gel shift assays with the 274-bp DNA fragment from the *Rhodobacter capsulatus hupS* promoter. The IHF-containing fraction was dialysed for 2 hours against buffer P (10 mM phosphate, 0.5 mM EDTA, pH 7) then loaded onto a 5 ml Econo-Pac-S cartridge (Bio-Rad) equilibrated in buffer P. The column was developed with a step gradient of 0 to 1 M NaCl in buffer P. The fraction that eluted at between 0.5 M and 0.6 M NaCl (3 ml) contained the IHF protein.

DNA binding assays - End probe labeling and gel mobility shift DNA binding assays were carried out as in (13). The DNA fragments were obtained as described above from the *algD* promoter and as in (13) from the *hupS* promoter. Pure IHF proteins from *R. capsulatus* and from *E. coli* were obtained by the procedure described in (14).

RESULTS AND DISCUSSION

Purification of the IHF-like protein from *P. aeruginosa* CHA

The two-step purification procedure set up and used for the isolation of IHF from *R. capsulatus* (14) was applied as described in Methods. The soluble crude extract (50 ml, 1 g of protein) obtained after sonication and ultracentrifugation of the cells from a 5 l culture was

directly loaded onto a 5 ml Econo-Pac heparin column (Bio-Rad) which was developed by a step NaCl gradient (0 to 1 M). The protein fraction collected at between 0.5 to 0.65 M NaCl contained mainly a small protein of Mr 9000 (Fig. 1) which was identified as HU from its N-terminal amino acid sequence (MNKSELIDAIAASAD) (15, 16) and from the deduced product of the cloned *hupA* gene of *P. aeruginosa* (I. Delic-Attree and B. Toussaint, unpubl. results). (The *hupA* nomenclature has been used to name the gene encoding the α subunit of *E. coli* HU (17). This is why it is adopted here. It should not be confused with the hydrogenase genes of *R. capsulatus*, also termed *hup*; the hydrogenase structural genes in *R. capsulatus* are the *hupSL* genes.)

The protein fraction collected at between 0.65 to 0.8 M NaCl, analyzed by SDS-PAGE, contained mainly two polypeptides of 11 and 10 kDa apparent molecular masses (Fig. 1). This fraction was tested for its capacity to bind to DNA using the 274-bp DNA fragment isolated from the *R. capsulatus hupSL* promoter and shown earlier to bind *E. coli* IHF (13) and *R. capsulatus* IHF (13, 14). In the mobility shift assays, this fraction yielded a single complex of low mobility (Fig. 2, lane 3) while the HU-containing fraction gave multiple complexes of higher mobility (Fig. 2, lane 5). The presence of two polypeptides of molecular masses similar to the IHF subunits of *E. coli* (18), of *Serratia marcescens* (19) and of *R. capsulatus* (14), and the capacity of the fraction to give a single complex (Fig. 2, lane 3) was a good indication that the fraction contained the IHF protein of *P. aeruginosa*. The fraction could be further purified by cation exchange chromatography on Econo-Pac-S column (Bio-Rad) yielding a pure IHF protein (data not shown).

Identification of *P. aeruginosa* IHF by its N-terminal amino acid sequence

The N-terminal amino acid sequence of each of the two polypeptides was determined. The first sequence, GALTKAEIAERLYEE, was alignable to the corresponding amino acid

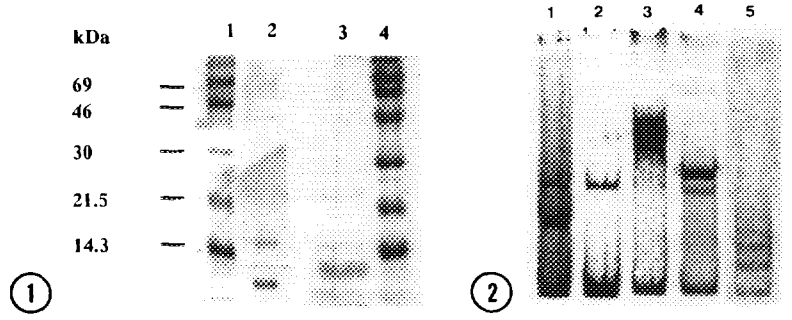

Figure 1. SDS-PAGE analysis of the protein fractions obtained from the Econo-Pac heparin column. Two SDS-15% polyacrylamide gels stained with Coomassie Blue are shown. Lanes 1 and 4, molecular weight markers; lane 2, fraction eluted at between 0.5 to 0.65 M NaCl (2 μg protein); lane 3, fraction eluted at between 0.65 to 0.8 M NaCl (1 μg protein).

Figure 2. DNA-binding activity of IHF-containing fractions studied by gel retardation assays. [^{35}S] end-labeled DNA fragments (1 ng) were used. To the 270-bp *algD* promoter fragment from *P. aeruginosa* (lanes 1, 2) or the 274-bp *R. capsulatus hupS* promoter fragment (lanes 3, 4 and 5), the following proteins were added. Lane 1, crude extract of *P. aeruginosa* (400 μg); lanes 2 and 3, the 0.65 to 0.8 M NaCl eluate (1 μg); lane 4, *R. capsulatus* IHF protein (0.5 μg); and, as a control in lane 5, the 0.5 to 0.65 M NaCl eluate containing *P. aeruginosa* HU (5 μg).

sequence of the α subunit of *E. coli* IHF, with 75 % identity between the 20 first amino acids. The second sequence, MTKSELIERTVTXQG, was alignable to the 15 N-terminal amino acids of the β subunit of *E. coli* IHF with 73 % identical amino acids (Fig. 3). The presence of a factor analogous to IHF had been suggested from the experiments of Mohr and Deretic (10) where antibodies against *E. coli* IHF were shown to crossreact with a *P. aeruginosa* protein of the size of IHF. We bring here the first direct evidence of the existence of IHF in *P. aeruginosa*; it is an αβ heterodimer similar to IHF of *E. coli* and of *R. capsulatus*.

Promoter recognition by *P. aeruginosa* IHF

The DNA binding activity of *P. aeruginosa* IHF was first demonstrated with the 274-bp DNA fragment isolated from the *R. capsulatus hupS* promoter region. This fragment contains two IHF-consensus sequences (14). By splitting the *hupS* promoter into two overlapping smaller fragments, each of them containing one of the two IHF consensus sequences, we could observe that *P. aeruginosa* IHF binds *in vitro* to the upstream IHF consensus sequence 1 (B. Toussaint, unpubl. results) whereas the IHF proteins of *R. capsulatus* and of *E. coli* bind *in vitro* to the downstream IHF consensus sequence 2 (14). The upstream "ATCAANNNNTTCTGA" consensus sequence 1 resembles more the *E. coli* IHF consensus sequence than the "CACACNNNNTTGAA" consensus sequence 2. Some differences between the bases surrounding these two sites exist, namely the dowstream site is surrounded by a series of adenines or thymines (14) whereas this is not the case for the upstream site to which *P. aeruginosa* IHF binds. The "ATCAANNNNATATT" IHF consensus sequence located in the *P. aeruginosa algD* promoter (10), which matches the *hupS* promoter upstream IHF consensus sequence 1 in 11 out of 14 residues, is also not surrounded by long poly-A or poly-T tracts (Fig. 4). Mohr and Deretic (10) have already shown that *E. coli* IHF binds to the IHF consensus sequence present in the *algD* promoter. In figure 2, *P. aeruginosa* IHF is shown to bind also to the *algD* promoter but not exactly as to the *hupS* promoter (compare lanes 2 and 3). The *in vitro* binding of *P. aeruginosa* IHF to the *algD* promoter suggests that this protein is involved in *algD* transcription. Furthermore, it has already been demonstrated (24) that deletion of a part of the *algD* promoter containing a section of the *E. coli* IHF/DNA contact region leads to a striking reduction of the *algD*

E.c IHFα				M	A	L	T	K	A	E	M	S	E	Y	L	F	D	K
P.a IHFα				G	A	L	T	K	A	E	I	A	E	R	L	Y	E	E
R.c IHFα	S	E	K	T	L	T	R	M	D	L	S	E	A	V	F	R	E	
E.c IHFβ				M	T	K	S	E	L	I	E	R	L	A	T	Q	Q	S
P.a IHFβ				M	T	K	S	E	L	I	E	R	T	V	T	X	Q	G
R.c IHFβ				M	I	R	S	E	L	I	A	K	I	A	E	E	N	P

Figure 3. Alignment of the N-terminal amino acid sequences of the α and β subunits of *P. aeruginosa* and of *R. capsulatus* (13,14) IHF proteins, determined by amino acid microsequencing with the deduced products of the *himA* gene encoding IHFα (20) and of the *hip* gene encoding IHFβ (21) in *E. coli*. (Note that the amino-terminal fMet was cleaved in P.a. IHFα but not in P.a. IHFβ as was also observed in the case of *R. capsulatus* IHF (14)).

P.a. *algD* promoter	A T C A A N N N N A T A T T
R.c. *hupS* consensus 1	A T C A A N N N N T T C T G
R.c. *hupS* consensus 2	C A C A C N N N N T T G A A
E.c. IHF consensus	A T C A A N N N N T T G A T

Figure 4. Nucleotide sequences from the *R. capsulatus* (R.c.) *hupS* promoter (22) and from the *P. aeruginosa* (P.a.) *algD* promoter (10) sharing identity with the consensus sequence for the binding of *E. coli* IHF protein (23).

promoter activity. By inducing a strong DNA bending, IHF is supposed to alter the regulation of transcription from a distant site. Recent studies (25, 26) have shown that IHF binds just upstream from the promoter of some genes whose expression requires an alternative form of RNA-polymerase which uses the *rpoN* gene product, sigma 54 (σ^{54}) factor. Although *algD* transcription does not seem to depend on σ^{54} (27), but rather on σ^{H}, encoded by *algU* (9), transcription with that other form of RNA-polymerase appears to also involve IHF.

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