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Identification of a fatty acyl responsive regulator (FarR) in *Escherichia coli*

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Abstract FarR (formerly P30) has been identified as a fatty acid and fatty acyl-CoA responsive DNA-binding protein. It is encoded by the *farR* gene (*g30*) in the citric acid cycle gene cluster of *E. coli* (*gltA-sdhCDAB-sucABCD-farR*). The amplified FarR protein specifically bound to the *farR* promoter (P_{farR}) and exhibited weak binding to the citrate synthase and lipoamide dehydrogenase promoters. Binding at P_{farR} was abolished by long-chain fatty acids and their CoA thioesters. In DNaseI footprints, FarR binding at P_{farR} protected two sites, each characterised by two related 10-bp direct repeats. It is suggested that FarR autoregulates *farR* expression and may modulate citric acid cycle expression in response to long-chain fatty acids.

Key words: FarR; P30; GntR family; Fatty acid; Fatty acyl-CoA; Transcription regulation; *Escherichia coli*

1. Introduction

FarR is an uncharacterised member of the GntR family of transcriptional regulators. They share similar N-terminal amino acid sequences that are predicted to contain analogous DNA-binding helix-turn-helix motifs [1]. The family is named after GntR, the repressor of the gluconate operon (*gntRKPZ*) in *Bacillus subtilis* [2]. The family also includes the HutC regulators of histidine utilisation in *Pseudomonas putida* and *Klebsiella aerogenes*, the KorA regulator of conjugal plasmid transfer in *Streptomyces*, and five regulatory proteins of *Escherichia coli*: PdhR (formerly GenA), the repressor of the pyruvate dehydrogenase operon (*pdhR-aceEF-lpd*); FadR, the repressor of fatty acid degradation; LctR, a potential regulator of L-lactate utilisation encoded by the *lctPRD* operon; PhnF, a potential regulator of alkylphosphonate uptake and metabolism; and FarR, an uncharacterised regulator that was formerly designated P30 [1,3,4].

The gene encoding FarR (*farR*, formerly *g30*) is situated adjacent to the major cluster of citric acid cycle genes at 16.5 min on the *E. coli* linkage map (Fig. 1) and it was previously suggested that FarR may be involved in their regulation [1,5]. Support for this suggestion was strengthened when the related *pdhR* gene was shown to encode a transcriptional regulator for the downstream pyruvate dehydrogenase (PDH) complex genes, *aceEF* and *lpd* (Fig. 1) [3]. The *farR* gene converges on the *sucABCD* operon, in which the *sucA* and *sucB* genes encode the specific E1o and E2o components of the 2-oxoglutarate dehydrogenase (ODH) complex [5]. The E3 component is encoded by the *lpd* gene which supplies lipoamide dehydrogenase subunits for assembly into the independently-regulated ODH and PDH complexes [3,6] as well as for the glycine cleavage system [7]. The single *lpd* gene is expressed from the *pdh* promoter (P_{pdh}) as part of a polycistronic *pdhR-aceEF-lpd* transcript, to satisfy the E3 requirements of the PDH complex, and separately from the *lpd* promoter (P_{lpd}), to meet the needs of the ODH complex and glycine cleavage system. It was thus envisaged that other potential functions of FarR might be to coregulate expression from P_{lpd} with *sucAB* transcription, and like many transcriptional regulators, to regulate its own expression.

During studies aimed at identifying the role of FarR, the protein was found to bind to DNA fragments containing the *farR* promoter but could be released by long-chain fatty acids and their CoA thioesters. A potential operator sequence was detected and the protein was redesignated FarR to denote its fatty acyl response.

2. Experimental

2.1. Bacterial strains and plasmids

DH5α [8] was the routine *E. coli* strain and JRG2904, a pGS701 transformant of *E. coli* BL21/λDE3 (Novagen Inc), was used for IPTG-induced amplification of the FarR protein. The source of *farR* DNA was pGS130 [9]. The *farR* coding region was enriched by PCR amplification using two primers: S243, ^{98}CGGATCCC ATGGGACACAAGCC-C^{120}; and S244, ^{885}GGAATTGTCGAC TCGGTTTCGAC^{862} (numbered according to [5] with superscripted mismatches), which simultaneously introduce an *Nco*I site at the initiation codon and a downstream *Sal*I site. The *Nco*I- and *Sal*I-digested product was subcloned downstream of the IPTG-inducible T7 promoter in *Nco*I-*Xho*I digested pET16b (Novagen Inc) to generate pGS701 (Fig. 1), and the presence of the desired nucleotide sequence was confirmed by dideoxy sequencing with representative plasmids. The *farR* promoter clones were constructed by subcloning the 2.2 kb *Eco*RV fragment of pGS130 into pBluescript SK- to give pGS702 (Fig. 1), and the 0.35 kb *BclI–Hin*cII fragment of pGS702 was subcloned into pUC119 to create pGS709 (Fig. 1). The other promoter clones illustrated in Fig. 1 have been described previously [10].

2.2. Microbiological methods, protein purification and FarR assay

Strains were cultured aerobically in L broth with ampicillin (100 μg/ml) when required. DNA was isolated and manipulated by standard methods [11]. Protein was assayed by the Bradford method and the Laemmli method was used for SDS-PAGE. Gels were stained with Coomassie brilliant blue and the molecular weight markers (BDH) were (kDa): ovotransferrin (77), bovine serum albumin (66), ovalbumin (43) and carbonic anhydrase (30). A Bio-Profil image analyser (Vilber-Lourmat) was used for densitometry. The FarR protein was purified from cell-free ultrasonic extracts of JRG2904 grown at 37°C, induced with IPTG (40 μg/ml) in late-logarithmic phase, and incubated for a further 16 h at 25°C. The purification procedure was similar to that devised for PdhR [10]. This involved heparin-agarose chromatography using buffer A (20 mM Tris-HCl, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM sodium azide, 1 mM DTT, 0.1 mM PMSF, 10% glycerol; pH 7.3) and a salt gradient with buffer B (A plus 0.5 M ammonium sulphate). Pooled fractions containing FarR were diluted 5-fold to minimise precipitation during dialysis and subjected to cation exchange chromatography using a Protein-Pak SP 15HR 1,000 Å column (Millipore) with the same buffer gradient, and dialysis. The purification was monitored by

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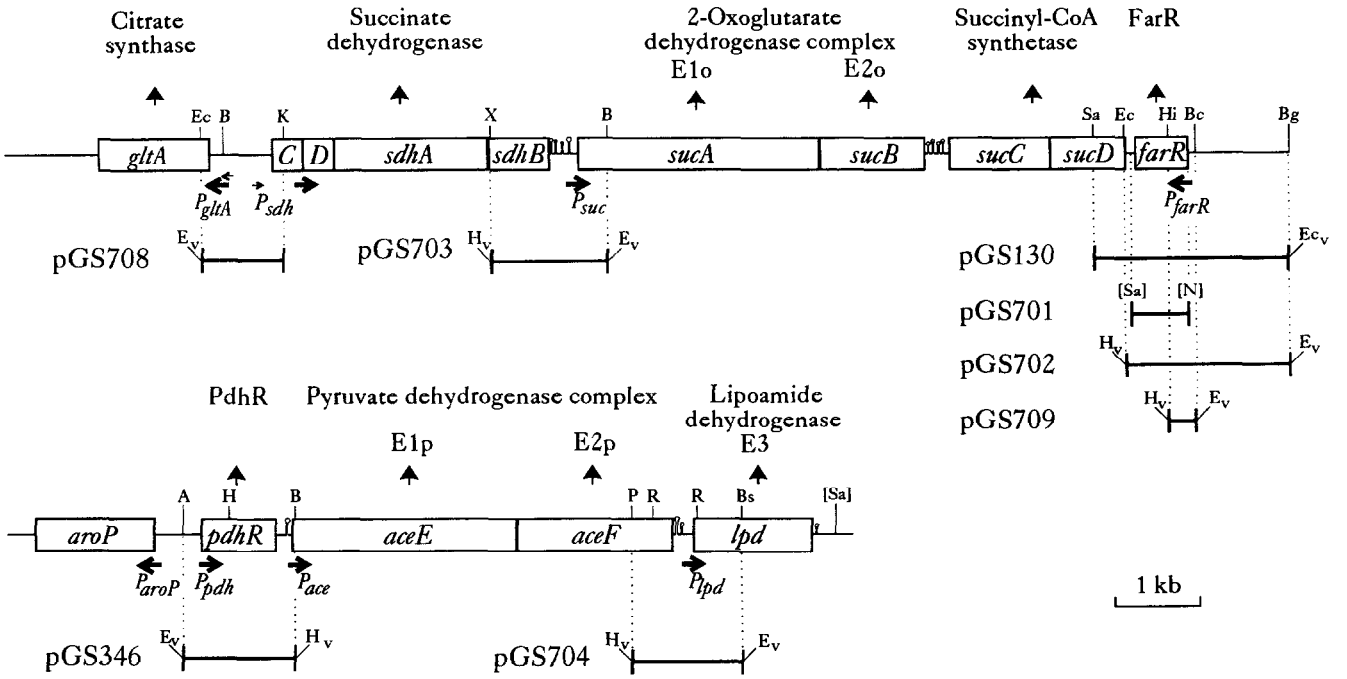

Fig. 1. Transcriptional organisation of genes encoding citric acid cycle enzymes and the PDH complex at 16.5 and 2.5 min in the *E. coli* linkage map. The genes (boxed), promoters (arrowed), regions of potential secondary structure, and DNA fragments cloned in specific plasmids, are indicated. Relevant restriction sites are: A, *Aha*III; B, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*I; Bs, *Bsp*AI; E, *Eco*RI; Ec, *Eco*RV; H, *Hin*dIII; Hi, *Hin*cII; K, *Kpn*I; [N], engineered *Nco*I; P, *Pst*I; R, *Rsa*I; [Sa], engineered *Sal*I; and X, *Xho*I; the subscript ($_v$) refers to flanking vector sites used in subcloning.

SDS-PAGE and by estimating the specific gel-retardation activity of fractions (units/μg protein) where one unit completely retards 1 pmol of *farR* promoter DNA under standard conditions.

2.3. Gel retardation and DNaseI footprinting

Gel retardation assays [12] were performed by incubating reaction mixtures (15 μl) containing, 0.1 pmol of DNA (end-labelled with [α-^{35}S]dATP), 5–200 ng of FarR protein, 3 μl of 5× bandshift buffer, and 1 μg poly(dI-dC)·poly(dI-dC), for 11 min at 37°C prior to fractionation by native PAGE and autoradiography. Potential coeffectors (5 mM) were added one minute before fractionation. Fatty acids and their CoA thioesters were from Sigma. DNaseI footprinting [12] involved incubating 10–500 ng FarR protein with 2 pmol of DNA (end-labelled with [α-^{35}S]dATP), 4 μl of 5× binding buffer and 1 μg poly(dI-dC)·poly(dI-dC) in a total volume of 20 μl, for 10 min at 37°C prior to digestion with 1 μl of 1 U/ml DNaseI (Boehringer Mannheim) for 2 min at 37°C. After phenol/chloroform extraction and ethanol precipitation, the entire sample was fractionated in a 5% acrylamide 7 M urea sequencing gel and analysed by autoradiography.

3. Results and discussion

3.1. Overproduction and partial purification of FarR

The 32 kDa FarR protein was the only pGS701-specific product detected in protein-stained SDS-PAGE gels of induced cultures of JRG2904 compared to vector-containing controls. It was amplified to 25% of total cell protein in cultures induced at 37°C, but was totally insoluble. However, it accumulated to 18% of total cell protein after induction at 25°C and as much as one third was soluble. This FarR fraction amounted to 10% of the protein in the soluble cell-free extract and it corresponds to an enrichment of at least 100-fold, because no band having the same mobility could be detected in comparable extracts of the vector-containing strain. The FarR protein was enriched a further 2.5-fold in two chromatographic steps, which removed most of the contaminants (Fig. 2; Table 1). Elution of the 32 kDa protein correlated exacty with the gel-retardation activity (see section 3.2). Unfortunately, FarR had a great tendency to precipitate, which is reflected in the poor yield (0.2%) and the failure to achieve a purity greater than 25% (Table 1). The final product contained very few contaminants, but it could not be concentrated beyond 40 μg protein/ml (8-fold) without precipitation, even in the presence of detergents such as Genapol, Triton X-100, Tween-20 and Tween-80 at up to 0.5% w/v. The DNA-binding activity of the purified material disappeared

Table 1
Purification of FarR from *E. coli* JRG2904

Fraction	Total protein (mg)	Purity (%)	FarR protein (mg)	Recovery (%)	Specific activity[a] (U/μg protein)
Cell-free extract	176.0	10.1	17.8	100.0	2.4[b]
Heparin	5.9	21.6	1.26	7.1	5.1
Cation exchange	0.16	25.0	0.04	0.2	6.0

[a] One unit of DNA-binding activity (U) is defined as the amount that completely retards 1 pmol of *farR* promoter DNA under standard gel retardation conditions.

[b] The specific activity of a comparable extract of the vector-containing wild-type strain was estimated as <0.024 U/μg protein.

during attempts at further purification, but declined by only 50% when stored for 2 months at 4°C.

3.2. DNA-binding studies with purified FarR protein

Good DNA-binding activity was observed in gel retardation experiments with the 2.2 kb *Eco*RI–*Hin*dIII fragment of pGS702 containing the *farR* promoter, P_{farR} (Fig. 3A). The apparent dissociation constant (K_d) based on the amount of FarR that binds half of the P_{farR} fragments under test conditions, was 1×10^{-9} M, assuming that two monomers bind to each site and allowing for the impurity of FarR. Comparable preparations were purified from induced cultures of strains containing the expression vector (pET16b) with no *farR* coding region. These preparations contained all of the proteins that contaminate partially-purified FarR but totally lacked DNA-retardation activity. This confirms that the observed DNA-binding activity is specific to FarR. The extremely remote possibility that the activity arises as a secondary consequence of FarR overproduction is very unlikely, especially as it would also have to copurify with FarR.

A total of 120 compounds including all of the citric-acid-cycle intermediates and 20 amino acids, were tested for their ability to abolish FarR-binding to the P_{farR} fragment. Only the long-chain fatty acids, C12:0 to C18:0 (Fig. 3B) and the corresponding acyl-CoA derivatives, behaved like potential coeffectors. The most effective members of each group, based on the concentrations needed for half-maximal FarR release, were the C16:0 compounds, palmitic acid (1.50 mM) and palmitoyl-CoA (0.35 mM). No release was observed with the corresponding C12:0 to C16:0 methyl esters and primary alcohols, or with the C14 to C18 unsaturated fatty acids (myristoleic, palmitoleic, oleic, linoleic and linolenic). The observed specificity suggests that the coeffectors are not simply acting as detergents and this was supported by the fact that Triton X-100 and Tween-20 (≤2% w/v) had no effect on DNA-binding by FarR.

In analogous gel retardation experiments with DNA fragments containing the citric-acid-cycle and PDH-complex promoters illustrated in Fig. 1, faint but reproducible retarded bands were detected with purified FarR and fragments containing P_{lpd}, P_{gltA} and P_{sdh}, but not with those containing P_{pdh}, P_{ace} and P_{suc}, or with protein samples from the vector control (not shown).

3.3. Identification of the FarR operator sequence

Further digests of pGS702 (P_{farR}), pGS704 (P_{lpd}) and pGS708 (P_{gltA} and P_{sdh}) were used in gel retardation studies to locate the FarR binding sites with greater precision. The P_{farR} site was traced to a 350 bp *Bcl*I–*Hin*cII fragment (Fig. 1) which was later incorporated in pGS709 for use in DNaseI footprinting (see below). Likewise, the P_{lpd} site was located in a 550 bp *Rsa*I fragment containing the *aceF-lpd* intergenic region, and the site in pGS708 was traced to a 250 bp *Eco*RV–*Bam*HI fragment containing DNA that lies just downstream of the *glt*A promoters [13] (Fig. 1).

The FarR binding site in P_{farR} was sought by DNaseI footprinting with the 360 bp *Eco*RI–*Hin*dIII fragment of pGS709 (Fig. 4). Two AT-rich sites, each associated with two hypersensitive positions, were protected (Fig. 4A). The best protected or primary site, overlaps the predicted *farR* promoter in a region that contains several TATTT and $^{G}/_{T}$TATT motifs, but no regions of hyphenated dyad symmetry of the type often

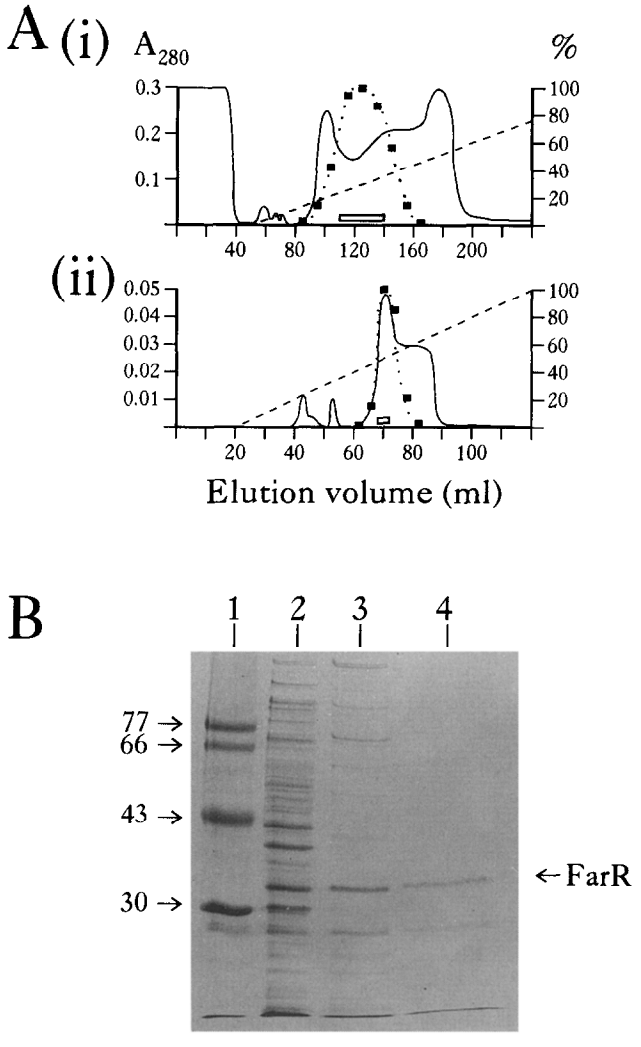

Fig. 2. Purification of FarR. (A) Typical elution profiles for (i) heparin-agarose and (ii) cation exchange chomotography (see 2.2 and Table 1) are shown with: absorbance at 280 nm (—); the ammonium sulphate gradient, % buffer B (----); and DNA-binding activity expressed as a percentage of the most active fraction (■···■). The open bar denotes the fractions retained after each step. (B) SDS-PAGE analysis of samples from each stage of the purification: lane 1, molecular weight markers (kDa); 2, cell-free extract; 3, heparin-agarose chromatography; 4, cation exchange chromatography.

associated with operator sites (Fig. 4B). However, the primary site is located in a 21-bp segment containing two 10-bp sequences that differ at only two positions, ^{29}TGTATTGTAT^{38} and ^{40}TGTATTATTT^{49} ^{74}TGGTTAAATT^{83} and ^{84}CGTATTAATG^{93}, form a direct repeat associated with a secondary binding site that overlaps the ribosomal binding site (Fig. 4B). The four sequences are identical to a TGTATTA$^{A}/_{T}$TT consensus at seven or more positions, and it would appear that FarR binding sites contain two such tandemly-repeated sequences. The three hypersensitive sites at positions 64, 66 and 67, are interesting (Fig. 4). They could be due to DNA-bending caused by interactions between FarR molecules bound at each of the flanking DNA-binding sites, but it is not known whether this is physiologically significant or simply consequence of FarR's known tendency to aggregate. No FarR-protected regions could be detected in DNaseI footprints of the weakly-

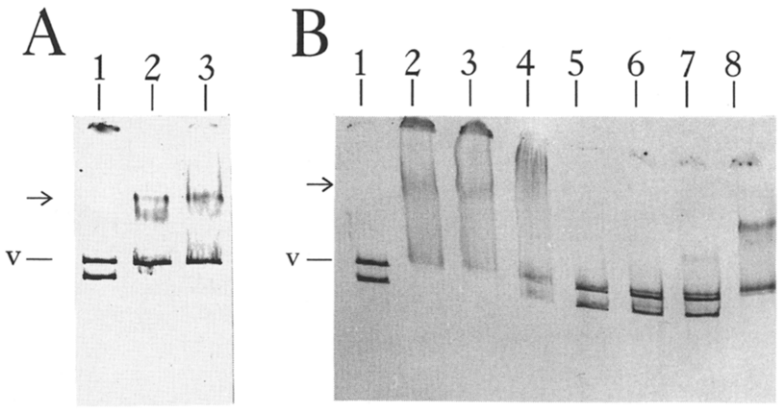

Fig. 3. FarR gel retardation studies with the *farR* promoter fragment. (A) End-labelled DNA (0.1 pmol) from *Eco*RI–*Hin*dIII digested pGS702 was incubated with purified FarR: 1, no FarR protein; 2, 5 ng; 3, 50 ng. (B) Potential coeffectors (5 mM) were incubated with 5 ng FarR protein and end-labelled P_{farR} as above: 1, no FarR; 2, FarR; 3, FarR + capric acid (C10:0); 4, FarR + lauric acid (C12:0); 5, FarR + myristic acid (C14:0); 6, FarR + palmitic acid (C16:0); 7, FarR + stearic acid (C18:0); 8, FarR + arachidic acid (C20:0). The vector fragment (v), derives from pUC119 and the retarded P_{farR} fragments (2.2-kb) are arrowed.

retarded P_{lpd} and P_{gltA} fragments promoter, possibly because FarR could not be supplied in a sufficiently concentrated form. The relevant fragments contain single sequence motifs that resemble the consensus at seven or more positions: TTTGTTGTTT, overlapping the −35 hexamer of P_{lpd}; GGTATTACTT, at +23 to 32 in the *lpd* transcript: and TTTTTTATTT, 80 bp upstream of the translational start in the *gltA* transcript [6,13].

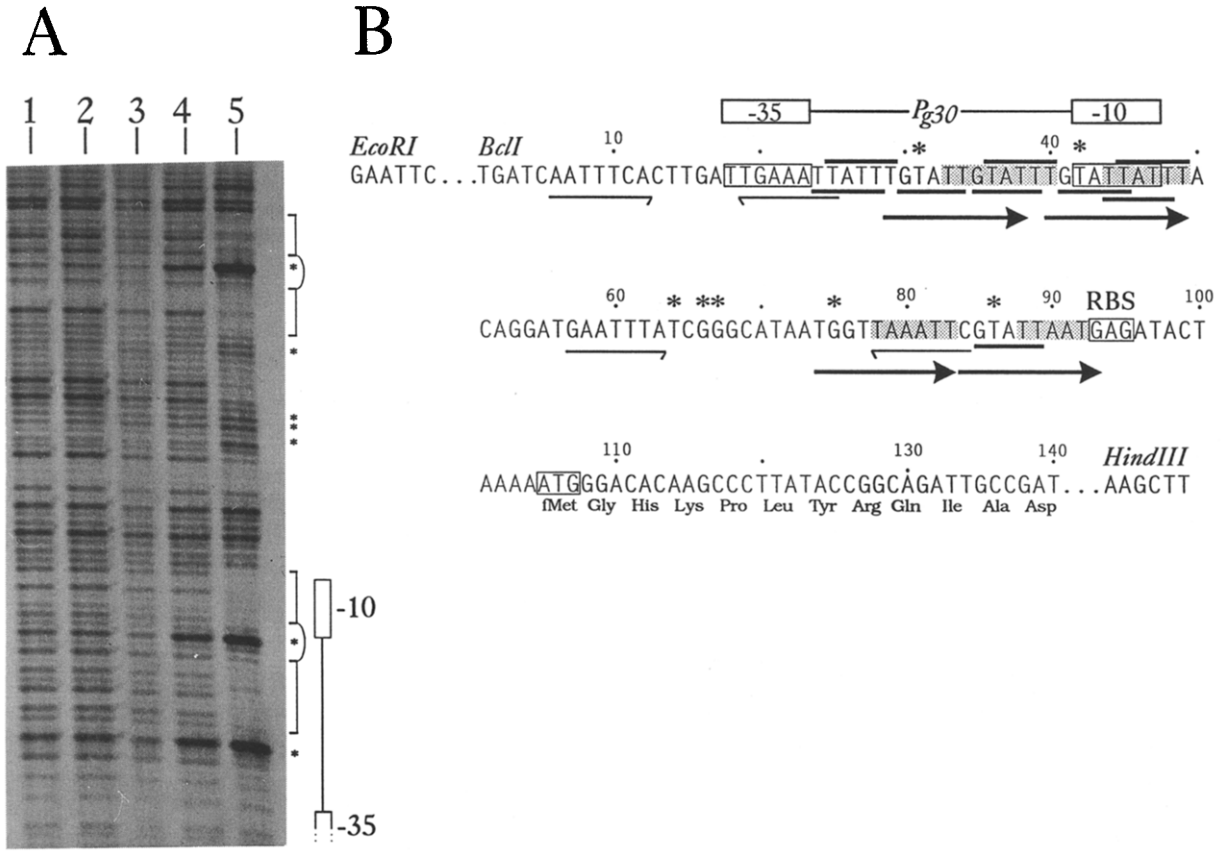

Fig. 4. DNaseI footprint analysis and nucleotide sequence of the *farR* promoter region. (A) DNA, labelled on the coding strand at the upstream *Eco*RI site was incubated with FarR protein: lane 1, no protein; 2, 4 ng; 3, 15 ng; 4, 30 ng; 5, 100 ng; prior to digestion with DNaseI and fractionation in a 5% acrylamide-urea gel with a dimethylsulphate digest of the same DNA fragment. (B) The sequence is numbered from the *BclI* site used in the initial cloning [5], just downstream of the vector *Eco*RI site labelled in pGS709 digests. The −10 and −35 elements of the predicted *farR* promoter [5], the ribosome binding site (RBS), the FarR-protected regions (bracketed or shaded) and the hypersensitive sites (asterisked), indicated. The direct repeats at the FarR binding sites approximating to the TGTATTA^{A}/$_{T}$TT consensus (bold arrows), other repeated motifs, TATTT (overlined) and ^{G}/$_{T}$TATT (underlined), and potentially significant regions of dyad symmetry (converging arrows), are also indicated.

3.4. Conclusion

The FarR protein was purified to 25% homogeneity from a soluble source that was enriched by at least 100-fold. Specific binding at two sites containing tandem repeats similar to TGTATTA^{A}/$_{T}$TT, was detected in the *farR* promoter region. Their locations indicate that FarR negatively autoregulates its own synthesis in response to long-chain fatty acid deficiency, by binding at these operator sites. The coeffector specificity of FarR resembles that of the FadR protein of *E. coli*, which represses fatty acid degradation genes, activates a fatty acid biosynthesis gene (*fabA*), and is thought to have a role in regulating the glyoxylate cycle operon, *aceBAK* [14]. FadR is released from its operators by fatty acyl-CoA thioesters (≥C12), palmitoyl-CoA being the most effective [15]. The FadR binding-site consensus, **ACCTGGTCAGACGTGTG** [14], contains a repeated **AC-TG**, but bears no resemblance to the FarR-site. The weak retardations observed with FarR and P_{lpd} and P_{gltA} fragments could mean that FarR modulates expression of the citric acid cycle in response to long-chain fatty acid synthesis, but further work will be needed to define the nature and physiological significance of these interactions, and to identify other potential members of the FarR regulon.

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