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Purification of the regulatory protein AlgR1 and its binding in the far upstream region of the *algD* promoter in *Pseudomonas aeruginosa*

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ABSTRACT **A regulatory protein AlgR1, previously suggested to be a member of a two-component sensory transduction system because of its homology to OmpR and NtrC and its ability to allow activation of the *algD* promoter under conditions of high osmolarity, has been hyperproduced in *Escherichia coli* after deletion of the upstream region including part of the Shine–Dalgarno sequence of the *algR1* gene and its subsequent cloning under the *tac* promoter. The AlgR1 protein is purified as a monomer, and the sequence of the nine N-terminal amino acids of the monomer matches with that predicted from the DNA sequence of the *algR1* gene. The purified AlgR1 protein binds to two separate DNA fragments of the *algD* upstream region. DNase protection experiments identify these two DNA segments as 14-mer sequences centered at −382 and −458 regions, which contain a common CCGTTCGTC sequence in them. While the presence of at least one AlgR1 binding site is important for the activation of the *algD* promoter, the presence of both of the binding sites in the upstream region leads to a higher level of activation.**

Alginate is a polysaccharide produced by *Pseudomonas aeruginosa* cells during infection in the cystic fibrosis lung (1). Under normal environmental conditions, *P. aeruginosa* retains all of the alginate biosynthetic (*alg*) genes but does not produce alginate because one of the critical steps in the pathway—namely, conversion of GDP-mannose to GDP-mannuronate—is blocked because of the absence of the enzyme GDP-mannose dehydrogenase (GMD), which the gene *algD* encodes. Both the *algD* gene and its control region have been sequenced (2). In nonmucoid (non-alginate-producing) *P. aeruginosa* cells, the *algD* promoter is not functional, while in mucoid cells isolated from the cystic fibrosis lung, the *algD* promoter is fully active. The activation of the *algD* promoter is mediated by a number of regulatory proteins such as AlgR1 (1, 3), AlgR2 (4), AlgR3 (5), etc., and the activation process is greatly enhanced in the presence of specific environmental factors such as nitrogen starvation (1), high osmolarity (6), ethanol-induced dehydration (7), etc. The regulatory genes *algR1*, *algR2*, and *algR3* also have been sequenced (3–5). The sequence of *algR1* shows homology with those of other regulatory genes such as *ompR*, *ntrC*, etc. (1, 3). Indeed, in *Escherichia coli*, OmpR can functionally substitute for AlgR1 in the activation of the *algD* promoter in a high-osmotic environment (6). The *algD* promoter resembles *ompC*/*ompF* promoters in sequence homology (8). It also resembles a number of other enteric promoters in having a RpoN (σ^{54})-recognizing motif (8). In this paper, we describe the purification of AlgR1 and its binding at multiple sites in the upstream regions of the *algD* promoter, which is similar to the binding of OmpR at multiple sites of the *ompF* promoter (9) or of NtrC to multiple loci of the *glnAp2* promoter (10, 11). In spite of this organizational similarity and the amino acid sequence homology between these proteins, the DNA targets are different, suggesting that there are likely to be interesting differences in the mode of activation of all three promoters.

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MATERIALS AND METHODS

Plasmids. A list of plasmids is given in Table 1.

DNA Manipulation and Sequencing. Transformation, cloning, and other DNA manipulations were carried out by standard methods (12). The nucleotide sequences were determined for both strands by the dideoxy chain-termination method (13) with single-stranded DNAs generated from pUC118 and pUC119 clones (14).

Purification of AlgR1. Competent cells of *E. coli* MV1184 (14) were transformed with pJK223R1 and grown in 2XTY broth (12) supplemented with glucose (0.1%) and ampicillin (50 μg/ml) at 37°C. A 5-ml overnight culture was inoculated into 500 ml of the same medium in a 2-liter Erlenmeyer flask. After 2 hr of cultivation, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM. The culture was incubated for an additional 4 hr before harvesting. Cells from 4 liters of culture were resuspended in 40 ml of TMBG buffer [50 mM Tris·HCl, pH 7.6/5 mM $MgCl_2$/5 mM 2-mercaptoethanol/10% (vol/vol) glycerol] containing 1 mM phenylmethylsulfonyl fluoride. Sonication was used to make cell-free extracts. Cell debris was removed by centrifugation at 150,000 × *g* for 1 hr. The supernatant was applied to a 2.6 × 10 cm heparin-agarose (type I; Sigma) column equilibrated with TMBG buffer. After washing with TMBG buffer, the column was eluted with a 300-ml linear gradient of 0–1.2 M NaCl prepared in TMBG buffer. The AlgR1-containing fractions eluted at ≈0.7 M NaCl were pooled (90 ml). The pooled fraction was dialyzed against TMB buffer (50 mM Tris·HCl, pH 7.6/5 mM $MgCl_2$/5 mM 2-mercaptoethanol) overnight. Precipitation formed during dialysis was recovered by centrifugation at 12,000 × *g* for 10 min and dissolved in 4 ml of TMB buffer containing 1 M NaCl. One-tenth milliliter of the protein sample was applied to a Superose 12 HR10/30 FPLC (fast protein liquid chromatography) column (Pharmacia) equilibrated with TMB buffer containing 1 M NaCl. Elution was performed with the same buffer at a flow rate of 0.2 ml/min. AlgR1 was eluted in a fraction corresponding to about M_r 28,000, and the sample was used for further experiments.

Gel Retardation Assay. Binding of the AlgR1 to DNA was carried out in 20 μl of the reaction mixture containing 0.01 pmol of ^{32}P-labeled DNA fragment (see Fig. 4), 0.5 μg of poly(dI-dC), and 20–60 ng of AlgR1 in 20 mM Tris·HCl, pH 8.0/100 mM NaCl/0.5 mM dithiothreitol/11.25% glycerol. The DNA binding reaction was started by adding AlgR1 and performed at room temperature for 10 min. The samples were immediately loaded onto a polyacrylamide gel containing 5% acrylamide, 0.13% methylenebisacrylamide, 44.5 mM Tris,

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Table 1. Plasmids used for gel retardation and footprint analysis

Plasmid	Inserted restriction fragment	Inserted sites of pUC118
pJK1182HB	*Hin*dIII–*Bcl* I, 1.7 kb	*Hin*dIII, *Bam*HI
pJK1182PB	*Pvu* II–*Bcl* I, 1047 bp	*Hin*cII, *Bam*HI
pJK1182SB	*Stu* I–*Bcl* I, 883 bp	*Hin*cII, *Bam*HI
pJK1182BB	*Bal* I–*Bcl* I, 772 bp	*Hin*cII, *Bam*HI
pJK1182NB	*Nsi* I–*Bcl* I, 628 bp	*Pst* I, *Bam*HI

All plasmids are derivatives of pUC118 and contain the 1.7-kb *Hin*dIII–*Bcl* I fragment carrying a part of the *algD* gene and its upstream region or restriction digestion-deleted fragments of the *Hin*dIII–*Bcl* I fragment.

44.5 mM H_3BO_3, and 1 mM EDTA. After application of the running buffer (44.5 mM Tris/44.5 mM H_3BO_3/1 mM EDTA), gels were dried and exposed to XAR-5 film (Kodak).

DNase I Footprinting. The DNase I protection experiments were done as described by Galas and Schmitz (15) with the following modifications. DNase I reaction buffer was 20 mM Tris·HCl, pH 7.9/3 mM $MgCl_2$/5 mM $CaCl_2$/80 mM NaCl/0.1 mM dithiothreitol/0.1 mM EDTA/50 μg of bovine serum albumin per ml. DNase I solution (10 μg/ml) was freshly diluted from the stock solution (1 mg/ml in 0.1 M NaCl). AlgR1 was incubated with the DNA (100 μl) for 10 min at 25°C prior to the addition of 1 μl of DNase I solution for 40 sec at 25°C. The DNase I reaction was stopped, DNA was precipitated, samples were resuspended in 8 M urea/44.5 mM Tris/44.5 mM H_3BO_3/1 mM EDTA/0.025% bromophenol blue/0.025% xylene cyanol and applied to the sequencing gel.

RESULTS

Overproduction of the AlgR1 Protein in *E. coli*. The AlgR1 protein could not be overproduced in *E. coli* or *P. aeruginosa* harboring a high-copy-number plasmid containing the *algR1* gene or when the *algR1* structural gene with its Shine–Dalgarno sequence but lacking most of its promoter sequence was cloned behind a strong promoter such as *tac*. A relatively long distance between the Shine–Dalgarno sequence and the translational start codon [11 base pairs (bp), Fig. 1*B*] of the *algR1* gene causing inefficient translation appeared to be the major hindrance to the hyperproduction of AlgR1 from its own promoter and from *tac*. Therefore, we constructed the plasmid for the overexpression of *algR1* gene as shown in Fig. 1*A*. Essentially, the Shine–Dalgarno sequence was changed by using the DNA sequence of the multicloning site of pUC19 (14); subsequently, the *algR1* gene with the new Shine–Dalgarno sequence (Fig. 1) was subcloned under the *tac* promoter of pKK223-3, resulting in pJK223R1. The new Shine–Dalgarno sequence is located 5 bp upstream of the *algR1* start codon (Fig. 1*B*). When *E. coli* MV1184 was transformed with pJK223R1 and cultivated in the presence of isopropyl β-D-thiogalactopyranoside, a strong protein band appeared on the sodium dodecyl sulfate (SDS)/polyacrylamide gel (Fig. 2). The apparent molecular weight from the SDS/PAGE was estimated to be about 30,000, which corresponds well to that (27,588) calculated from the amino acid sequence deduced from the primary sequence of the *algR1* gene (1, 3). The DNA-binding ability of the MV1184/pJK223R1 cell extract to the *algD* upstream DNA segment by the gel retardation assay (data not shown) implied that the overproduced protein was AlgR1.

Purification of AlgR1. The AlgR1 protein was purified as described. About 80–90% pure AlgR1 protein was obtained (Fig. 3). After gel filtration, the protein band on the SDS/polyacrylamide gel was eluted, and its N-terminal amino acid sequence was determined. The sequence was found to be Met-Asn-Val-Leu-Ile-Val-Asp-Asp-Glu, which is the same as that deduced from the nucleotide sequence of *algR1* (3). It

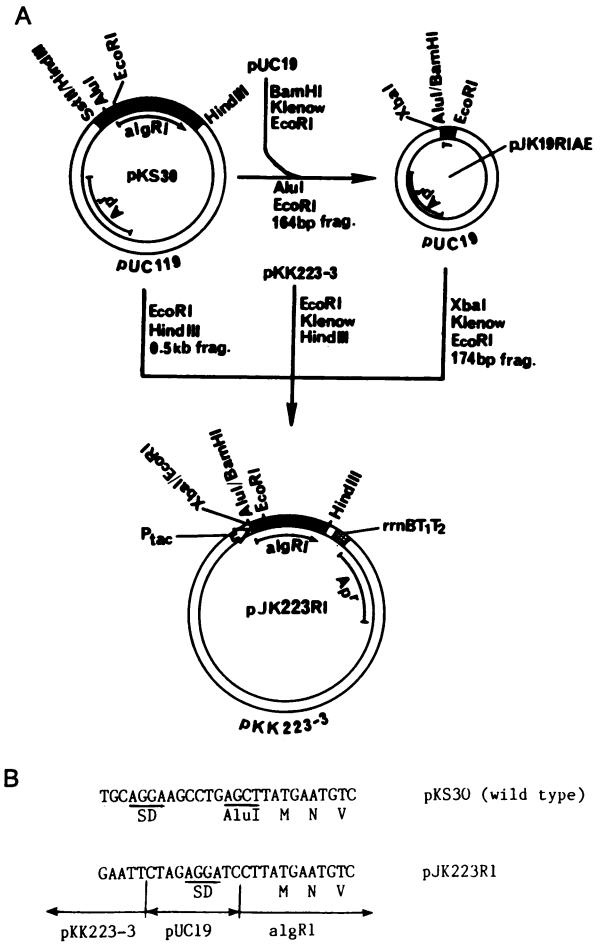

FIG. 1. (*A*) The scheme of the construction of pJK223R1. The donor plasmid for *algR1* gene, pKS30, is the derivative of pUC119 (14) with a 1-kilobase (kb) *Sst* II–*Hin*dIII fragment containing *algR1*. The 164-bp *Alu* I–*Eco*RI fragment of pKS30, which carries the first 162 bp of the *algR1* open reading frame and 2 bp of the upstream sequence was subcloned to *Eco*RI and "polished" ("overhangs" removed) *Bam*HI sites of pUC19 (16), resulting in pJK19R1AE. The 174-bp *Xba* I–*Eco*RI fragment of pJK19R1AE (the *Xba* I site was polished) and the 0.5-kb *Eco*RI–*Hin*dIII fragment of pKS30 were ligated with the backbone of *Hin*dIII/*Eco*RI-digested pKK223-3 (17) (*Eco*RI site was polished) to obtain pJK223R1. Open boxes denote sequences of pUC119, pUC19, and pKK223-3; solid boxes denote the *algR1* gene and adjacent sequences. The open reading frame and the transcriptional direction of *algR1* are shown with an arrow. Ap^r, β-lactamase gene; P_{tac}, *tac* promoter; $rrnBT_1T_2$, *rrnB* ribosomal terminator. Only restriction sites used are shown. (*B*) DNA sequences around the start codon of *algR1* in pKS30 and pJK223R1. Three N-terminal amino acids of AlgR1 are shown under the nucleotide sequences by one-letter symbols. Also, the origins of the nucleotide sequence are presented under the nucleotide sequence. SD, the Shine–Dalgarno sequence.

is noteworthy that most of AlgR1 aggregates at low salt concentration. For example, 85% of AlgR1 was found in the precipitate during dialysis after heparin-agarose column chromatography. The precipitate could be dissolved in a buffer with high salt concentration (for example, 1 M NaCl in TMB buffer).

Gel Retardation Assay. Initial gel retardation experiments showed that AlgR1 bound to both an 0.8-kb *Hin*dIII–*Stu* I fragment (nucleotide position −399 to about −1200, the transcriptional start site being numbered +1) and a 495-bp *Stu* I fragment (nucleotide position +95 to −398) of the *algD* upstream region. So we examined DNA binding of AlgR1 to these regions in detail by making deletions of the upstream region. We subcloned smaller restriction fragments of the

1.7-kb *Hin*dIII–*Bcl* I DNA segment (nucleotide position +484 to about −1200) in pUC118 to conveniently label fragments with ^{32}P (Table 1). The fragments containing various *algD* upstream regions with the flanking sequence of pUC118 were labeled with ^{32}P by Klenow fragment and applied to the gel retardation assay. The electrophoretic mobilities of fragments B (nucleotide position −399 to −562) and C (nucleotide position −288 to −398) were retarded on the gels upon addition of AlgR1 (Fig. 4, lanes B and C), but those of other fragments were not (Fig. 4, lanes A, D, and E). Since AlgR1 tends to form aggregates at low salt concentration, only a part of AlgR1 may be in active form for DNA binding. That is probably the reason why increasing AlgR1 concentration did not lead to the increase in intensity or mobility of the shifted bands in the gel retardation assay (Fig. 4, lanes B and C).

Footprinting. The gel retardation experiments showed that there are at least two binding sites of AlgR1 in the region from 288 bp to 562 bp upstream of the *algD* transcriptional start point as described above. To determine precise sites for AlgR1 binding, a DNase I protection experiment was performed. In agreement with the results of the gel retardation assay, two sites were found to be protected against DNase I cleavage by AlgR1 (Fig. 5*A*). These two sites have sequences of CCGTTCGTCTGCAA (−389 to −376) and CCGTTCGTCCCTCC (−465 to −452). It is interesting to note that there is a common homologous sequence of CCGTTCGTC in the two AlgR1 binding sites (Fig. 5*B*).

Cooperativity and Importance of the AlgR1 Binding Sites. Recent transcriptional fusion studies (20) using a promoterless *xylE* gene under the control of the *algD* promoter indicate that when both the AlgR1 binding sites centering on positions −382 and −458 are present upstream of the *algD–xylE* fusion construct (harboring up to 1.1 kb of the upstream region), the specific activity of catechol 2,3-dioxygenase (product of the *xylE* gene, C230) is high (about 25–28 units/mg of protein). In deletion derivatives harboring the −432 region at its 5′ end (where the terminal AlgR1 binding site at −458 is lost because of the deletion), the catechol 2,3-dioxygenase specific activity is reduced to 15.7 units/mg of protein. However, in derivatives harboring only up to the −332 region at the 5′ end (where both the −382 and −458 AlgR1 binding sites are deleted), the catechol 2,3-dioxygenase specific activity is reduced drastically to 2.6 units/mg of protein. Thus it appears that while the second AlgR1 binding site centered at the −458 region is stimulatory to the activation process, the AlgR1 binding site centered at the −382 region is critical for the activation of the *algD* promoter.

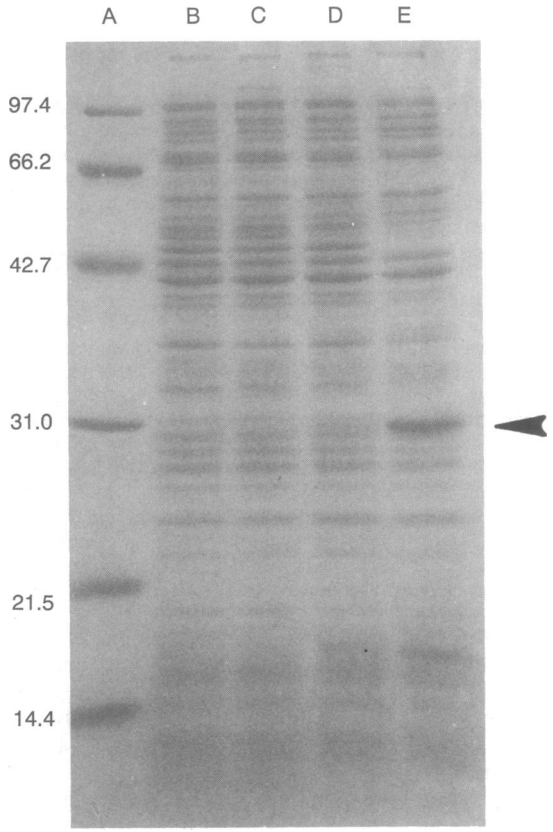

FIG. 2. Overproduction of AlgR1 in *E. coli* MV1184 harboring pKK223-3 (lanes B and C) or pJK223R1 (lanes D and E) were analyzed by SDS/polyacrylamide gel electrophoresis (18). Strains were grown in the presence (lanes C and E) or absence (lanes B and D) of 0.5 mM isopropyl β-D-thiogalactopyranoside. Lane A contains the molecular mass markers (shown in kDa).

DISCUSSION

In this report, we describe the purification of the regulatory protein AlgR1, which activates the *algD* promoter containing the σ^{54} RNA polymerase holoenzyme recognition sequence. Although the amino acid sequence of AlgR1 has been deduced from its DNA sequence (1, 3), the confirmation of such a sequence was not possible because of the lack of purification of AlgR1. The N-terminal amino acid sequence of purified AlgR1 matched perfectly with that predicted from the DNA sequence, suggesting that AlgR1 indeed belongs to the class of OmpR- and NtrC-type of regulatory proteins involved in prokaryotic signal transduction mechanism (21). AlgR1 has previously been shown to be interchangeable with OmpR in the activation of the *algD* promoter in *E. coli* in a high osmotic environment (6). The molecular mass of AlgR1 is 27.6 kDa, while those of OmpR and of NtrC are 28 kDa (22) and 54 kDa (23), respectively. Thus, AlgR1 resembles OmpR in size rather than NtrC. It is important to point out that AlgR1 isolated and purified by the procedure described in this report appears to be a monomer on gel filtration with 1 M NaCl. Even though the genetic data suggested that OmpR may exist as a multimer (24), the molecular weight of native OmpR protein was found to be 27,000 when purified as a

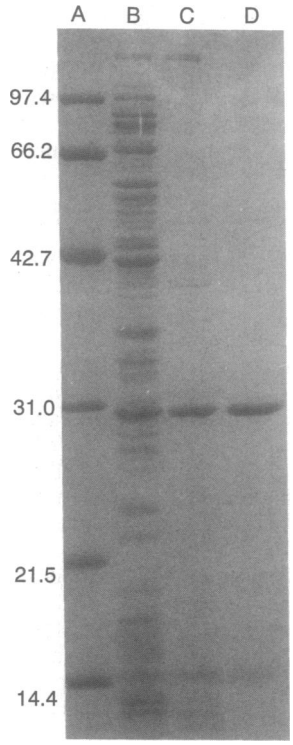

FIG. 3. Purification of the AlgR1 protein. Identification of AlgR1 and determination of purity were carried out on SDS/polyacrylamide gel. Samples applied were: the molecular weight markers (lane A; shown in kDa), the cell-free extract of MV1184 harboring pJK223R1 (lane B), the sample after heparin-agarose chromatography (lane C), and the sample after Superose 12 filtration (lane D).

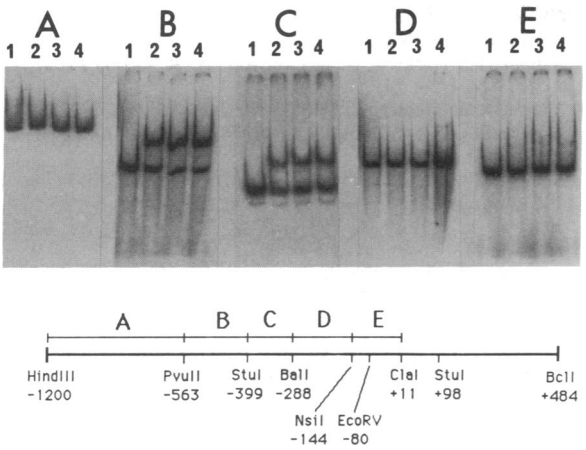

FIG. 4. Binding of AlgR1 to the *algD* upstream region. (*Lower*) The 1.7-kb *Hin*dIII–*Bcl* I fragment carrying the upstream region and a part of the *algD* open reading frame. The mRNA start site is numbered +1. The fragments of *Hin*dIII–*Pvu* II (A), *Pvu* II–*Stu* I (B), *Stu* I–*Bal* I (C), *Bal* I–*Nsi* I (D), and *Nsi* I–*Cla* I (E) with the flanking sequence of the pUC118 multicloning site were isolated from pJK1182HB, pJK1182PB, pJK1182SB, pJK1182BB, and pJK1182NB (Table 1), respectively, and labeled with ^{32}P by using the Klenow fragment. (*Upper*) Each of ^{32}P-labeled DNA fragment (0.01 pmol) was incubated with the following amounts of AlgR1 and analyzed on polyacrylamide gel; none (lane 1), 20 ng (lane 2), 40 ng (lane 3), and 60 ng (lane 4).

monomer (22). Thus, both AlgR1 and OmpR may show dimeric or multimeric forms within the cells under certain physiological conditions, but as isolated, both of them exhibit monomeric forms in high-salt solutions.

The gel retardation experiments of the *algD* upstream sequences with purified AlgR1 demonstrated binding to two discreet DNA fragments with high affinity (Fig. 4). A low-affinity binding could also be detected with fragment E (Fig. 4). Subsequent DNase I footprinting experiments showed clearly that AlgR1 specifically binds to the sequence CCGTTCGTCN$_5$, which happens to be present in two copies in the upstream region of the *algD* promoter. The distance of the binding sequences from the *algD* transcription start site is about −382 and −458 bp, which is somewhat further than other upstream activator sequences that bind prokaryotic regulatory proteins such as OmpR, NtrC, NifA, XylR, etc. For example, the OmpR binding sequence upstream of the *ompF* promoter contains three randomly repeated sequences at −100 to −91, −90 to −81, and −80 to −71 and another sequence at −51 to −42 (9). NtrC is known to bind predominantly to three sites near the *glnA* promoter at −140, −110 and −88 (10, 11). In the nitrogen-fixation system of *Klebsiella pneumoniae*, NifA protein binding occurs predominantly at the −125, −116, and −72 sites of the *nifU* promoter, although an additional site at −90 has also been identified (25). NifA is also known to bind with the upstream activator sequence of the *nifH* promoter at −123 to −136, where strong binding requires the presence of NifL protein under nitrogen limiting conditions. Growth in the presence of NH_4^+ diminished the interaction of NifA with the upstream activator sequence even in the presence of NifL, suggesting that environmental conditions may modulate protein–protein interactions for strong or reduced binding with the upstream activator sequence (26). We have shown (4) that AlgR2 works in a cooperative manner to activate the *algD* promoter in the presence of AlgR1. The nature and extent of the AlgR1 binding to the CCGTTCGTCN$_5$ sequence in the presence and absence of AlgR2 under a variety of conditions and the importance in the activation process of (*i*) the spacing between the AlgR1 binding sites and the promoter or (*ii*) the orientation of the AlgR1 binding sites need to be determined.

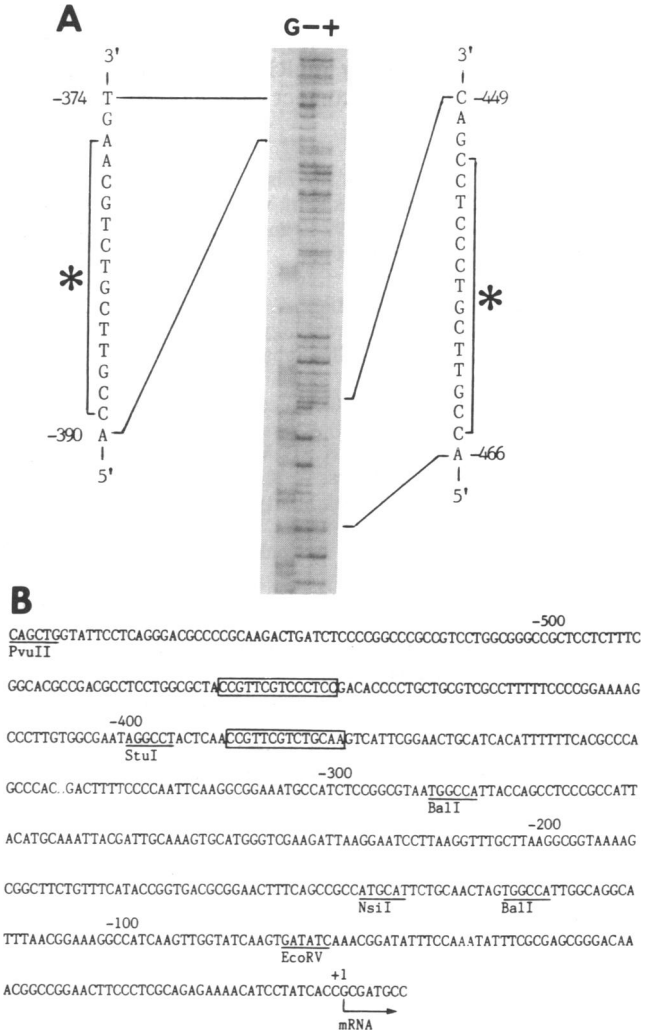

FIG. 5. DNase I footprinting. (*A*) The 506-bp *Hin*dIII–*Eco*RV fragment carrying the 486-bp *Pvu* II–*Eco*RV fragment of the *algD* upstream region with the flanking sequence of the pUC118 multicloning site was isolated from pJK1182PB, and labeled with ^{32}P, and the labeled DNA fragment was treated with DNase I in the absence or presence of AlgR1 (480 ng) as described and applied to the sequencing gel. "G-reaction" was performed as reported by Maxam and Gilbert (19). The nucleotide sequences are written on both sides of the lanes. Asterisks show the areas protected by AlgR1. (*B*) Nucleotide sequence of the *algD* upstream region, determined as described. The transcriptional start site is numbered +1. The nucleotide sequence downstream from position −167 has been reported by Deretic *et al.* (2). The regions protected in the footprinting analysis are boxed.

An interesting feature of the presence of AlgR1 binding sequences is that such a binding sequence with a single mismatch is also present within the *algD* gene (nucleotides 1740–1748, CTGTTCGTC; ref. 2). The implication of the presence of such a sequence within the *algD* gene is not clear. Sequences for binding various repressors are present within the structural genes in the *lac* (27) and the *gal* operons (28) in *E. coli*. The presence of regulatory DNA elements upstream or downstream of promoters is believed to allow loop formation of the intervening DNA, thus enabling the DNA-bound regulatory protein and RNA polymerase to contact each other. DNA looping has indeed been demonstrated by negative regulation and electron microscopy between repressors bound to operators in the *gal* operon (29) and between activator protein NtrC and the RNA polymerase at the *glnA*

promoter (30). It would be interesting to see if AlgR1 molecules binding at the two upstream sites of the *algD* promoter would allow DNA looping by AlgR1–AlgR1 contact and/or AlgR1–RNA polymerase σ^{54} contact for functional activation of the *algD* gene.

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