J. Biochem. 120, 752-758 (1996)

The *rpoD1* Gene Product Is a Principal Sigma Factor of RNA Polymerase in *Microcystis aeruginosa* K-81

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Received for publication, May 10, 1996

We performed molecular characterization of the RpoD1 protein encoded by the *rpoD1* gene isolated from a cyanobacterium, *Microcystis aeruginosa* K-81. The deduced amino acid sequence (416 aa, 48,871 Da) of RpoD1 exhibited extensive similarity to those of proteins of the eubacterial RpoD family (*Escherichia coli* σ^{70} homologs). We overproduced and purified RpoD1 (54 kDa) from *E. coli*. Biological and biochemical analyses suggested that RpoD1 has a function homologous to that of *E. coli* σ^{70} as follows: (i) the RpoD1 protein complemented an *rpoD* mutant of *E. coli* strain YN543 (*rpoD285*) and (ii) the heterologous RNA polymerase holoenzyme reconstituted from the *E. coli* core enzyme and recombinant RpoD1 was specifically transcribed from *E. coli* promoters. Furthermore, Western blot analysis with antiserum against *Synechococcus* sp. strain PCC 7942 RpoD1 (a principal sigma factor of the σ^{70} type) indicated that *M. aeruginosa* K-81 RpoD1 (σ^{A1}) is the principal σ factor, which is a major component of the σ subunit on exponential cell growth.

Key words: cyanobacteria, RNA polymerase, sigma factor, transcription, Western blot.

The eubacterial RNA polymerase holoenzyme, which is composed of a σ subunit and a core enzyme containing the major subunits, α_2, β, and β', plays a central role in gene transcription (*1*). The core enzyme functions in RNA polymerization but requires the σ subunit for specific transcription initiation at the promoter (*2*). General switching in transcription is mainly due to the modulated promoter selectivity of multiple RNA polymerase holoenzymes combined with a replacement between a common core enzyme and several different σ factors. These σ factors have been classified into two categories, the principal and alternative types. The principal σ factors are responsible for the promoter recognition of housekeeping genes under normal physiological growth conditions, and are conserved as regions 1, 2, 3, and 4 containing consensus amino peptides (*2*). Cyanobacteria (blue-green algae) that perform oxygenic photosynthesis involving two photosystems (PS I and PS II) like higher plants possess multiple σ factor homologs, and genes encoding principal σ factors have been isolated and characterized (*3, 4*). *sigA* and *rpoD1* are the genes encoding the principal sigma factor homologs in *Anabaena* sp. strain PCC 7120 and *Synechococcus* sp. strain PCC 7942, respectively. However, in these cyanobacteria, the functions of other alternative sigma factors remain unclear.

A unicellular cyanobacterium, *Microcystis* (*Synechocystis*) *aeruginosa* K-81, was isolated from Lake Kasumigaura, Ibaraki Prefecture, Japan (*5*), and has been characterized (*6, 7*). The *Microcystis* species cause massive surface blooms in eutrophic water and some strains have been found to produce a peptide-toxin (*8, 9*). Water pollution by *Microcystis* is becoming a serious problem in many countries, including Japan. However, the genetics of *Microcystis* species, particularly gene expression, are poorly understood. Genomic Southern hybridization with an *rpoD* probe designed from highly conserved amino acid sequences in the principal σ factors between *Escherichia coli* and *Bacillus subtilis* (*10*) revealed that *M. aeruginosa* K-81 had multiple *rpoD* homologs (*6*). To define the role of each *rpoD* homolog in gene expression, we started to clone and characterize the *rpoD* homologs (*11*). One of them, *rpoD1* was recently isolated and sequenced, and it was revealed that this gene encodes a principal σ factor homolog (*12*). In this article, we report results indicating that the *rpoD1* gene product (σ^{A1}) has a function homologous to that of *E. coli* σ^{70} in promoter-dependent specific transcription, and that it is the principal σ subunit of RNA polymerase in the log-phase of growth of *M. aeruginosa* K-81.

MATERIALS AND METHODS

Strains and Plasmids—The bacterial strains and plasmids used in this study are listed in Table I.

Overexpression and Purification of Recombinant RpoD1—E. coli JM105 was transformed with pKKA1 carrying *rpoD1* and grown at 37°C in 3 liters of 2×TY medium (*13*) containing ampicillin (75 $\mu g \cdot ml^{-1}$). When the turbidity of the cell culture reached 0.5 absorbance units at A_{660}, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 1 mM to

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Abbreviations: bp, base pairs; kDa, kilo dalton; *rpoD1*, a gene encoding the principal sigma factor of RNA polymerase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; *tac, trp-lac* hybrid.

TABLE I. **Strains and plasmids used in this study.**

Strains and plasmids	Relevant characteristics[a]	Source or reference
Microcystis aeruginosa strain		
K-81	Wild type	(*5*)
Escherichia coli strains		
K-12	Host for purification of RNA polymerase, wild type	(*34*)
YN543	Host for complementation test, *rpoD ts* mutant (*rpoD285*)	(*27*)
JM105	Host for pKK223-3 and its derivatives	(*34*)
JM109	Host for pUC119 derivatives	(*34*)
Plasmids		
pUC119	Cloning vector, Ap^r	(*35*)
pKH19	pUC119+a 7-kb *Hin*dIII fragment containing the EMBL3 right arm and 3′-downstream region of *rpoD1* from ϕEL10; 5′-end of cloned *rpoD1* is at position 1121 (*Sau*3AI site) in Fig. 2	(*12*)
pKXC3	pUC119+a 1.8-kb *Xba*I fragment carrying *rpoD1* without a 3′-region	(*11, 12*)
pKXCΔ106	Plasmid derived from pKXC3 by deletion, containing a 0.99-kb fragment (positions 781 to 1768 in Fig. 2)	This study
pKK223-3	Expression vector, Ap^r	(*25*)
pKKA1	pKK223-3+a 2.2-kb *Eco*RI-*Hin*dIII fragment carrying *rpoD1*	This study
pKΔA5	pKK223-3+a 0.99-kb *Eco*RI-*Xba*I fragment carrying *rpoD1* without a 3′-region	This study

[a] Ap^r, β-lactamase (ampicillin-resistant) gene; *ts*, temperature-sensitive.

overexpress the *rpoD1* gene. To analyze overproduced RpoD1 in the total cellular proteins, 1 ml of cell culture was sequentially harvested at 1 h intervals. Cells were collected by centrifugation and submitted to SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 12.5% acrylamide) (*14*), and then stained with Coomassie Brilliant Blue R250 (Sigma, St. Louis, MO, USA). Five hours after adding IPTG, the remaining cell culture was harvested and RpoD1 was isolated from 16 g (wet weight) of cells as described for the *E. coli* σ^{70} subunit (*15*). This recombinant RpoD1 fraction purified by column chromatography was resolved by SDS-PAGE (10% acrylamide), recovered from the gel (*16*), and used in a reconstitution study.

Purification and Reconstitution of RNA Polymerase—The *E. coli* K-12 RNA polymerase holo- and core enzymes were purified as described by Burgess and Jendrisak (*17*) and Gonzalez *et al.* (*18*), respectively. The *E. coli* σ^{70} subunit was isolated from the holoenzyme purified on an SDS-polyacrylamide gel, renatured, and reconstituted with the purified core enzyme (*14*). The recombinant *M. aeruginosa* K-81 RpoD1 protein (σ^{A1}) isolated from the SDS-polyacrylamide gel was used to reconstitute heterologous RNA polymerase as described for *E. coli* homogeneous RNA polymerase. RNA polymerase of *M. aeruginosa* K-81 was partially purified as described previously (*19*) with modifications, as follows (all steps performed at 4°C). Cells were grown in CB liquid medium (*6*) at 30°C under 2,000-lux continuous white light illumination. The cells (106 g; wet weight) were harvested at the mid-log phase by centrifugation, washed in TGEDM buffer [20 mM Tris-HCl (pH 8.0), 10% glycerol, 0.1 mM EDTA·2Na, 0.1 mM DTT, and 10 mM $MgCl_2$] containing 50 mM KCl and 1 mM PMSF, precipitated, resuspended in 270 ml of the same buffer, and then disrupted by passage twice through a French press at 1,700 kg·cm^{-2}. The cell lysate was centrifuged at 143,000×*g* for 90 min and about 250 ml of supernatant was recovered. A portion of this crude extract was Western blotted. The remainder of the fluid was precipitated by 30 to 60% saturation with ammonium sulfate, dissolved in 10 ml of TGEDM buffer containing 400 mM NaCl, and dialyzed against 100 volumes of the same buffer. The dialysate was loaded onto a 2.6×150 cm Bio-Gel A-1.5 m (Bio-Rad Laboratories, Richmond, CA, USA) column and proteins were eluted with the same buffer. The peak fractions (60 ml) exhibiting enzyme activity were combined, precipitated with ammonium sulfate at 70% saturation, resuspended, and dialyzed against TGEDM buffer containing 50 mM NaCl. A portion of this gel filtration fraction was Western blotted. The remaining dialysate (4 ml) was loaded onto a 1.5×5.5 cm Heparin-Sepharose CL-6B (Pharmacia LKB, Piscataway, NJ, USA) column, washed with 100 ml of the same buffer, and then eluted with a linear gradient of 50 mM to 1,500 mM NaCl in 100 ml of TGEDM buffer. The peak fractions (2 ml) showing enzyme activity were dialyzed against TGEDM buffer (50% glycerol) containing 50 mM NaCl and stored at −80°C. A portion of this Heparin-Sepharose fraction was Western blotted. For N-terminal amino acid sequencing, this fraction was resolved by SDS-PAGE (10% acrylamide), and the 54 kDa protein corresponding to RpoD1 was isolated from the gel according to Hager and Burgess (*16*).

In Vitro RNA Synthesis—During the purification procedure, K-81 RNA polymerase activity was monitored using poly[dA-dT]DNA as a template. An assay mixture (40 μl), comprising 50 mM Tris-HCl (pH 8.0), 0.05 mM EDTA·2Na, 0.5 mM DTT, 10 mM $MgCl_2$, 0.1 mM each ATP/CTP/GTP, 0.1 mM [5,6-^{3}H]UTP (0.925 GBq/mmol; Du Pont, Wilmington, DE, USA), 1.0 μg template DNA (poly[dA-dT]·poly[dA-dT]; Boehringer Mannheim, Sandhoferstrasse, Germany), and 5 μl of RNA polymerase fraction, was incubated at 37°C for 10 min. The synthesized RNA was precipitated with 200 μl of 30% (w/v) CCl_3-COOH, followed by incubation for 10 min at 0°C. The reaction mixture was spotted onto Whatman GF/C filters, washed three times with 5% (w/v) CCl_3COOH, and then dried. The level of radioactivity was determined using a liquid scintillation counter (Beckman, Somerset, NJ, USA). The activity of the purified or reconstituted RNA polymerase was measured by means of the multiple-round assay (*20*) with slight modifications. The assay mixture (40 μl) comprised 50 mM Tris-HCl (pH 8.0), 0.05 mM EDTA·2Na, 0.5 mM DTT, 10 mM $MgCl_2$, 0.1 mM each ATP/CTP/GTP, 0.05 mM [α-^{32}P]UTP (18.5 GBq/mmol; Du Pont), 1 μg template DNA (supercoiled pKK223-3; Table I), and various amounts of RNA polymerase. The mixture was incubated at 37°C for 10 min, and then the reaction was stopped by the addition of stop solution (140 μl) comprising 40 mM EDTA·2Na and 300 μg·ml^{-1} of *E. coli* tRNA. The RNA products (*tac*, 276 nt; RNA-I, 107 nt) were precipitated with 2-propanol and resolved by electrophoresis on a 5% polyacrylamide gel containing 8 M urea, followed by autoradiography. Molecular size markers for RNA (0.16 to 1.77-kb Ladder) were purchased from GIBCO BRL.

Western Blots—Proteins separated by SDS-PAGE (12.5% acrylamide) (*14*) and electrotransferred onto a

nitrocellulose membrane (Hybond-ECL, Amersham) were detected using a polyclonal rabbit antiserum raised against the *Synechococcus* sp. strain PCC 7942 principal sigma factor RpoD1 protein (Tanaka *et al.*, unpublished data) and a goat anti-rabbit IgG conjugated to alkaline phosphatase (BioMakor, Rehovet, Israel), as described (*21*). The molecular size markers for proteins (LMW calibration kit) were from Pharmacia LKB.

Complementation Test—E. coli YN543 (*rpoD285*) cells transformed with plasmid pKKA1 or an expression vector, pKK223-3, were incubated in 2×TY liquid medium containing 75 $\mu g \cdot ml^{-1}$ of ampicillin at 30°C for 1 day. The cultures were diluted 100-fold in fresh medium and then grown at 30°C to an A_{660} of 0.2-0.3. Subsequently, each culture was divided into two, and the temperature of one was raised to 42°C while the other was maintained at 30°C. Culture turbidity and cell viability were measured for 5 h. Viability was estimated by plating diluted cultures on a 2× TY plate. Colonies were counted after 2-day incubation at 30°C.

Other Techniques and the rpoD1 Accession Number—DNA was sequenced by means of dideoxy chain termination (*22*) on single- or double-stranded templates derived from deletion clones from pKXC3 using a Sequenase Version 2.0 kit (United States Biochemical, Cleveland, OH, USA) or a DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The isolated protein (100 pmol) was blotted onto a PVDF immobilon-P membrane (Millipore, Bedford, MA, USA) according to Matsudaira (*23*), and then the N-terminal region was sequenced (476A sequencer, Applied Biosystems). DNA and protein were analyzed using the GENETYX program (SDC Software, Tokyo). The *rpoD1* nucleotide sequence has been deposited in DDBJ under accession No. D50318.

RESULTS

Amino Acid Sequence Alignment—The sequence alignment of the RpoD family (*E. coli* σ^{70} type) with *M. aeruginosa* K-81 RpoD1 is shown in Fig. 1. The amino acid sequence of K-81 RpoD1 exhibited a high degree of similarity to those of σ^{70} homologs. The amino acid residues of conserved regions 1.2, 2, 3, and 4 (*2*) in K-81 RpoD1 were 64, 72, 95, and 97% identical to those of *E. coli* σ^{70}, *B. subtilis* σ^{A}, *Synechococcus* sp. strain PCC 7942 RpoD1, and *Anabaena* sp. strain PCC 7120 SigA, respectively. The

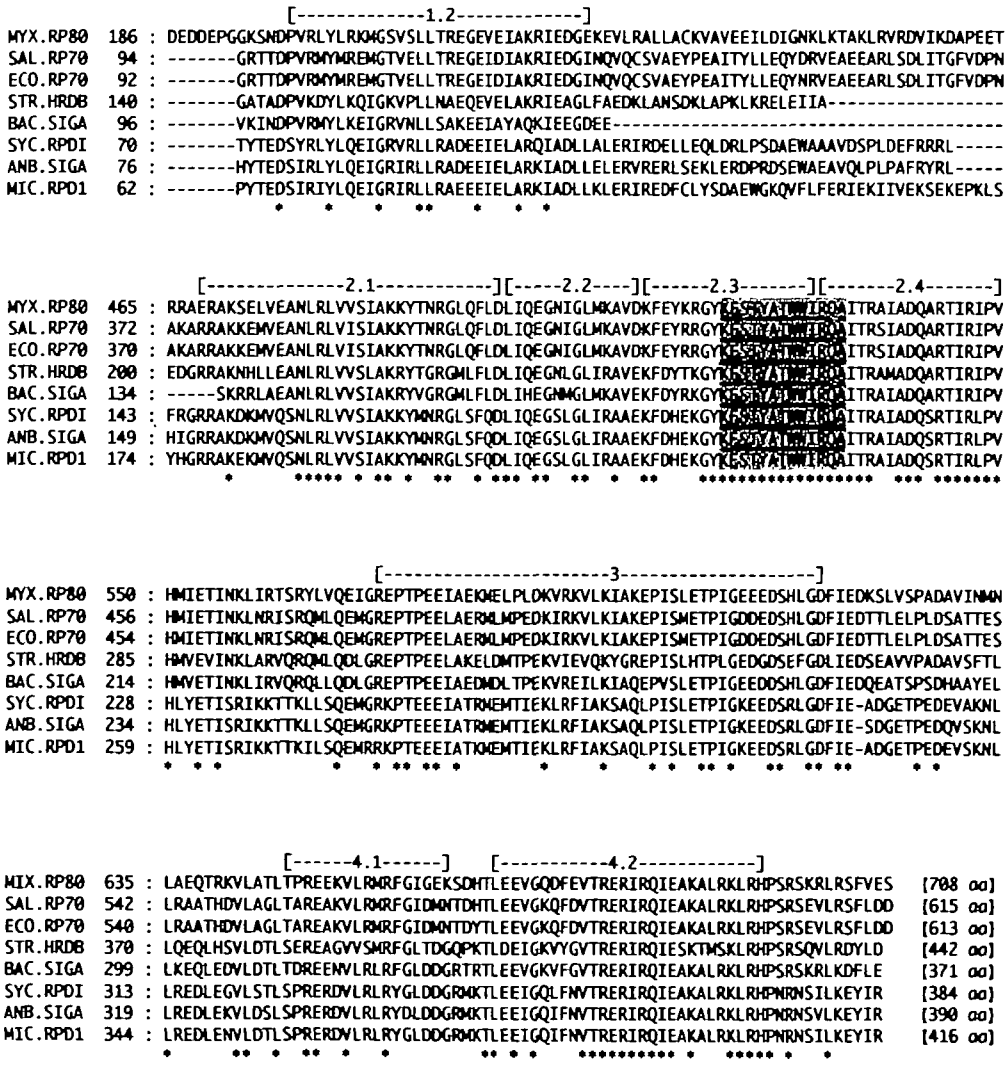

Fig. 1. **Amino acid sequence alignment of the eubacterial RpoD family.** The sequences, using a one-letter code, were translated from the GenBank or DDBJ entries given in parentheses as follows: MYX.RP80, *Myxococcus xanthus* RpoD (M32347); SAL.RP70, *Salmonella typhimurium* RpoD (M14427); ECO.RP70, *Escherichia coli* RpoD (J01687); STR.HRDB, *Streptomyces coelicolor* HrdB (X52983); BAC.SIGA, *Bacillus subtilis* SigA (X03897); SYC.RPD1, *Synechococcus* sp. strain PCC 7942 RpoD1 (D10973); ANB.SIGA, *Anabaena* sp. strain PCC 7120 SigA (M60046); MIC.RPD1, *Microcystis aeruginosa* K-81 RpoD1 (D50318). The number of amino acid residues is shown at the right and the total amino acid number of each protein is also indicated in bold parentheses. The identical amino acid residues in all proteins are indicated by asterisks. Subregions 1.2 to 4.2 are indicated. The sequence of the *rpoD* box is indicated by a shaded box.

K-81 RpoD protein also possessed a complete *rpoD* box sequence (KFSTYATWWIRQA) at the junction of subregions 2.3 to 2.4. We therefore tried to overproduce and purify the RpoD1 protein for further analyses as follows.

Construction of an Expression Vector for RpoD1—To delete the upstream of *rpoD1*, pKXC3 DNA was digested with restriction enzymes *Kpn*I and *Bam*HI, and then treated with exonuclease III (*24*), using a Kilo-deletion kit (TaKaRa, Kyoto) (Fig. 2). After self-ligating the deletion plasmid, we obtained a plasmid, pKXC⊿106 (Fig. 2). A 0.99-kb *Eco*RI-*Hin*dIII fragment (both restriction sites were derived from the multiple cloning site of the pUC119 vector) was isolated from pKXC⊿106, and then introduced into the *Eco*RI-*Hin*dIII site downstream of the *tac* promoter on expression vector pKK223-3 (*24*) to yield pK⊿A5. As plasmid pK⊿A5 lacked the 3′-downstream region of *rpoD1*, we replaced a small *Nco*I-*Hin*dIII fragment from pK⊿A5 with a 1.6-kb *Nco*I-*Hin*dIII fragment carrying the 3′-downstream region of *rpoD1* derived from pKH19 to create pKKA1 (Fig. 2 and Table I).

Overexpression and Identification of the RpoD1 Protein—Figure 3 shows the total cell protein separated by SDS-PAGE (12.5% polyacrylamide) at various times. Host *E. coli* JM105 cells harboring pKKA1 produced a high level of the 54 kDa protein after the addition of IPTG (lanes 3 to 6). When we cultured JM105 cells harboring pKK223-3 in the presence or absence of IPTG, no 54 kDa protein was induced (data not shown). The SDS-PAGE profile showed that the 54 kDa protein is not overproduced before induction with IPTG (lane 2). However, Northern and Western blot analyses revealed that the *rpoD1* transcripts and the 54 kDa protein were detected at low levels in *E. coli* JM105 cells harboring pKKA1 even in the absence of IPTG (data not shown). These results indicate that there was a small amount of leakage of the *rpoD1* transcripts and the 54 kDa protein in the *tac* promoter-operator system (*25*). The overexpressed 54 kDa protein coincided well with the deduced molecular mass of 48,871 for RpoD1 (416 aa) considering discrepancies between the gel mobility and molecular mass (*15, 26*). The overexpressed 54 kDa protein constituted about 10% of the total cellular protein (lane 6), and the overproduction slightly inhibited the growth of the *E. coli* JM105 host (data not shown). The overproduced protein was insoluble in bacterial lysates, presumably because of the formation of intracellular inclusion bodies. This protein was purified from these cells and its NH_2-terminal amino acid sequence was determined. The sequence of the first seventeen NH_2-terminal amino acid residues of the 54 kDa proteins was M-M-Q-A-H-D-V-L-T-L-T-E-P-P-L-D-L, showing perfect agreement with that of the RpoD1 protein deduced from the nucleotide sequence of *rpoD1* (*12*). Thus, we concluded that the purified 54 kDa protein is RpoD1.

Complementation Test—The amino acid sequence of RpoD1 exhibited extensive homology to the principal σ factors found in other bacteria. Therefore, we examined whether or not the *M. aeruginosa* K-81 RpoD1 protein complements an *rpoD ts* mutant of *E. coli* strain YN543 (*27*). We introduced plasmids pKKA1 and pKK223-3 into YN543, and then examined the growth and viability of the transformants without IPTG (Fig. 4). The results of a preliminary experiment indicated that plasmid pKKA1, which contains a *tac* promoter-operator system, can constitutively produce a small amount of RpoD1 protein in YN543 without IPTG induction (data not shown), since the YN543 strain does not have the genetic marker, *lacI*^{q}. The results of the complementation test showed that the turbidity of cells carrying pKKA1 increased after a temperature shift-up to 42°C, and that these cells remained alive (2×10^7 cells·ml^{-1}) for at least 5 h (Fig. 4, left). In contrast, the turbidity of cells carrying pKK223-3 did not increase after the temperature shift-up, and the cell viability significantly decreased (2×10^3 cells·ml^{-1}) (Fig. 4, right). These findings indicated that the K-81 RpoD1 protein complements *E. coli rpoD* mutant strain YN543.

In Vitro Transcription Assay—We confirmed that the recombinant RpoD1 protein functions as a σ factor by means of an *in vitro* transcription assay. The SDS-PAGE profile of the purified RNA polymerase and σ factors used in the assay is shown in Fig. 5. The specific transcription of heterologous RNA polymerase reconstituted with the *E. coli* core enzyme and RpoD1 was examined using *E. coli* promoters (Fig. 6). The *E. coli* holoenzyme (Fig. 6, lane 1)

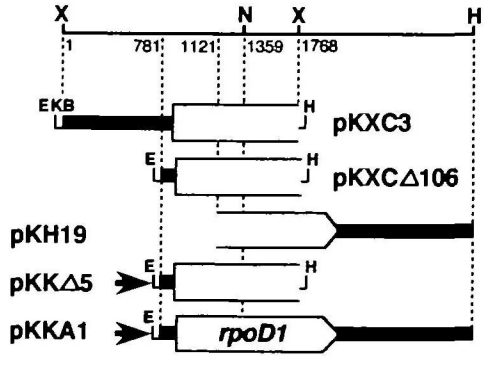

Fig. 2. **Construction of an *rpoD1* expression vector.** A physical map with the restriction enzymes (B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nco*I; X, *Xba*I) and inserts in plasmids are shown. The restriction enzyme derived from the multiple cloning site on each vector is presented a small character. The nucleotide numbers, from the *Xba*I site, are indicated. The *tac* promoter is denoted by an arrow.

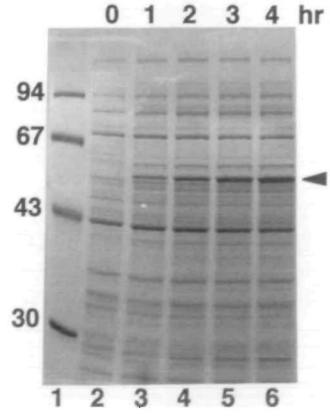

Fig. 3. **Overproduction and identification of the recombinant RpoD1 protein.** A Coomassie Brilliant Blue-stained SDS-polyacrylamide gel demonstrating the overproduction of RpoD1. Lanes 2 to 6 are proteins obtained from cells incubated for 0, 1, 2, 3, and 4 h, respectively, after adding IPTG. The position of RpoD1 is indicated by a filled arrowhead at the right. The positions of the molecular weight markers are shown in kilodaltons (kDa) on the left.

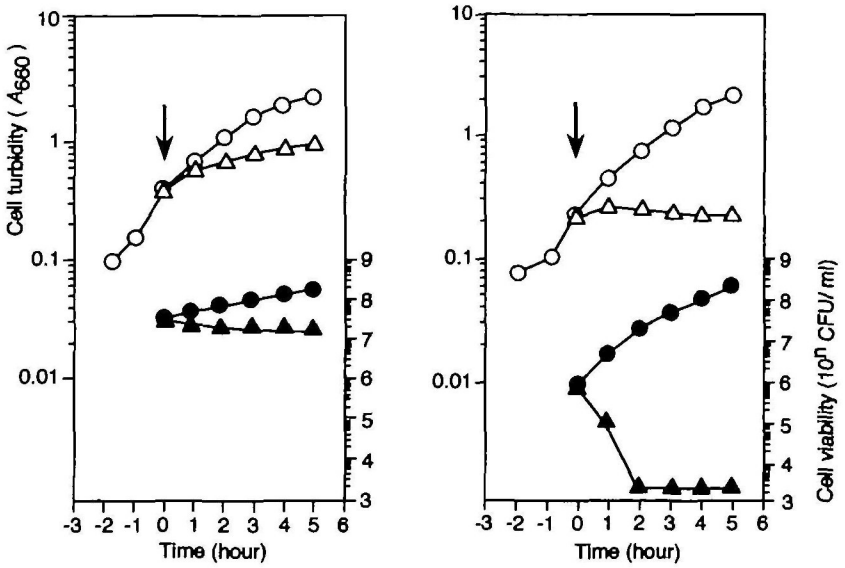

Fig. 4. **Complementation testing of *M. aeruginosa* K-81 RpoD1 in *E. coli rpoD* mutants.** The growth curves and viability of temperature-sensitive mutants of *E. coli* YN543 (*rpoD285*) carrying plasmids pKKA1 (left) and pKK223-3 (right) are shown. Symbols: A_{660} at 30°C (○) and 42°C (△); colony counts at 30°C (●) and 42°C (▲); ↓, temperature shift from 30 to 42°C.

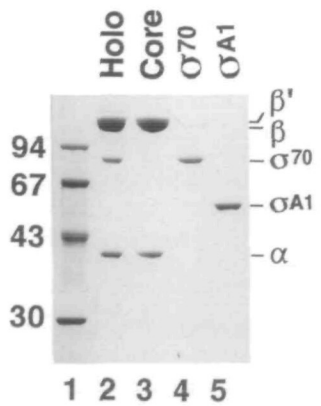

Fig. 5. **SDS-PAGE of the purified RNA polymerases and σ subunits.** The *E. coli* RNA polymerase holoenzyme (lane 2), core enzyme (lane 3), σ^{70} subunit (lane 4), and *M. aeruginosa* K-81 RpoD1 (σ^{A1}) were purified as described under "MATERIALS AND METHODS." Samples (10 pmol each) were resolved by electrophoresis on a 10% SDS-polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue. The positions of molecular size marker proteins (lane 1) are the same as in Fig. 3.

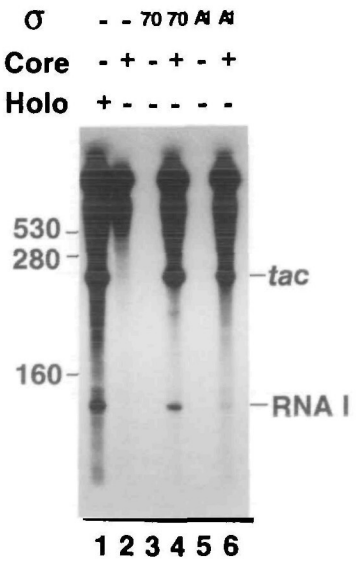

Fig. 6. **Promoter-dependent transcription by reconstituted heterologous RNA polymerase.** Multiple-round *in vitro* transcription was performed using a DNA template (0.3 pmol) containing the *tac* and RNA-I promoters. Each reaction included 1.5 pmol of *E. coli* RNA polymerase holo- or core enzyme. To reconstitute the RNA polymerase holoenzyme, the *E. coli* core enzyme was incubated with a three-fold molar excess of the σ^{70} subunit (4.5 pmol) or the σ^{A1} subunit (4.5 pmol). The presence (+) or absence (−) of proteins, and the composition of each reaction mixture with these proteins are shown at the top. Specific transcripts and the positions of molecular size markers for RNA (nucleotide, nt) are indicated on the right and left, respectively.

and reconstituted *E. coli* holoenzyme (lane 4) synthesized transcripts from *E. coli tac* and RNA-I promoters (*28*) on supercoiled pKK223-3 DNA (*24*). When we used heterologous RNA polymerase (lane 6), both products were also detected. On the contrary, when we used only the *E. coli* core enzyme (lane 2), the σ^{70} subunit (lane 3), or *M. aeruginosa* K-81 recombinant RpoD1 (lane 5), no transcripts were detected from these promoters. These results indicated that the RpoD1 protein (σ^{A1}) has a function homologous to that of the *E. coli* principal sigma factor σ^{70} *in vitro*.

Western Blot Analysis—*M. aeruginosa* K-81 RpoD1 exhibited particularly extensive similarity in amino acid sequence to the cyanobacterial proteins of the RpoD family (Fig. 1). Gene disruption analyses of *Synechococcus* sp. strain PCC 7942 *rpoD1*, *2*, *3*, and *4* also indicated that the *rpoD1* gene was essential for cell growth of the PCC 7942 strain (Tanaka *et al.*, unpublished data). We therefore contemplated that a polyclonal antibody against the PCC 7942 RpoD1 protein could recognize the principal σ factor (*E. coli* σ^{70} type) of *M. aeruginosa* K-81. The SDS-PAGE profile of the partially purified RpoD1 protein from a *M. aeruginosa* K-81 cell lysate in the late log-stage and the purified *M. aeruginosa* K-81 recombinant RpoD1 protein from *E. coli* is shown in Fig. 7(left). Figure 7(right) shows that the antiserum crossreacted not only with *E. coli* σ^{70}

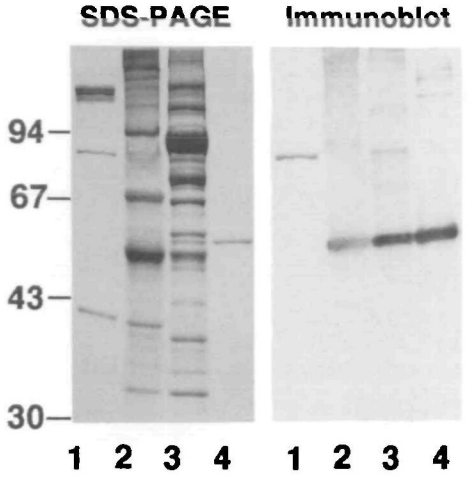

Fig. 7. **Detection of the major sigma factor in *M. aeruginosa* K-81 on Western blotting.** Left panel, Coomassie Blue-stained gel after SDS-PAGE; right panel, immunoblot with rabbit antiserum against the principal σ factor of *Synechococcus* sp. strain PCC 7942. Lanes: 1, *E. coli*, purified RNA polymerase holoenzyme (5 pmol); 2, *M. aeruginosa* K-81, gel filtration column fraction of RNA polymerase (5 μl); 3, *M. aeruginosa* K-81, Heparin-Sepharose column fraction of RNA polymerase (5 μl); and 4, *M. aeruginosa* K-81, purified recombinant RpoD1 (σ^{A1}) protein (5 pmol), respectively. Each fraction was prepared as described under "MATERIALS AND METHODS." The positions of the molecular size markers are the same as in Fig. 3.

(lane 1) but also with K-81 RpoD1 σ^{A1} (lane 4). These results indicated that *M. aeruginosa* K-81 RpoD1 (σ^{A1}) was recognized by this antiserum. As judged on comparison of the signal intensities in lanes 1 and 4 (Fig. 7, right), this PCC 7942 antibody more effectively recognizes K-81 σ^{A1} than *E. coli* σ^{70}. Moreover, this antiserum also crossreacted with a protein in the crude extract (*12*), gel filtration (lane 2), and Heparin-Sepharose fractions (lane 3) of *M. aeruginosa* K-81. The size of these crossreacting proteins (lanes 2 and 3) was identical to that of the RpoD1 protein (54 kDa, lane 4), and no other major band crossreacted with this antiserum. Furthermore, we isolated the protein that crossreacted with this antiserum from the Heparin-Sepharose fraction (Fig. 7, left, lane 3), and its N-terminal amino acid sequence was sequenced. The N-terminal amino acid residues of this protein were identical to those of the RpoD1 (σ^{A1}) protein, indicating that the RpoD1 protein is synthesized in K-81 cells. These findings signify that the RNA polymerase fraction contained σ^{A1} as the major σ factor on exponential cell growth of *M. aeruginosa* K-81.

DISCUSSION

The *in vivo* and *in vitro* investigations showed that the *M. aeruginosa* K-81 RpoD1 (σ^{A1}) protein has a homologous function to that of *E. coli* principal sigma factor σ^{70} (Figs. 4 and 6). In the complementation test, *E. coli* YN543 cells carrying *rpoD285* (*rpoD800*) synthesized RpoD285, which lacks 14 amino acid residues in the N-terminal region (in a stretch of 245 amino acids that separates regions 1 and 2) of σ^{70} (*29*). The RpoD285 protein is a substrate for the proteinase, La, in the *E. coli* host, resulting in that the cells exhibit temperature sensitive growth (*30*). Because YN543 is also a *recA1* mutant, the complementation pattern observed in this study could not be due to homologous recombination between the chromosomal DNA of YN543 and the plasmid DNA carrying *rpoD1*, but due to the actual function of the K-81 RpoD1 protein in YN543. Studies on *in vitro* mRNA synthesis involving a heterologous reconstituted RNA polymerase holoenzyme are interesting. The strategies included supplementing the *E. coli* core enzyme with a σ factor from the filamentous cyanobacterium, *Calothrix* sp. strain PCC 7601, with allowed specific initiation of transcription at a *Calothrix* promoter (*31*), but not with *Chlamydia psittaci* 6BC σ^{66} at a *Chlamydia* promoter (*32*). In this study, homologous reconstituted Eσ^{70} synthesized the respective mRNA products from the *tac* and RNA-I promoters. The heterologous reconstituted RNA polymerase holoenzyme with K-81 RpoD1 efficiently produced the transcript from the *tac* promoter but weakly from the RNA-I promoter (Fig. 6). These functional differences in the promoter-recognition between homogenous and heterologous reconstituted RNA polymerase might be dependent on the amino acid residues for the interaction with the target promoter sequence in each sigma factor and/or the structural changes in a responsive holoenzyme at the protein-complex level.

Western blot analysis indicated that polyclonal antibodies against *Synechococcus* sp. strain PCC 7942 RpoD1 specifically crossreacted with *E. coli* σ^{70} and K-81 σ^{A1} (Fig. 7). This antibody also crossreacted mainly with σ^{A1} on Western blots analysis with a cell free extract of *M. aeruginosa* K-81. If an *rpoD* gene other than *rpoD1* encodes the major factor in *M. aeruginosa* K-81, at least one other major band should have crossreacted with the antibody. However, we did not detect any other major band (Fig. 7), suggesting that σ^{A1} is the principal σ factor on the log-stage growth of *M. aeruginosa* K-81.

In the heterocystous filamentous cyanobacterium, *Anabaena* sp. strain PCC 7120, the *sigA* gene encodes a principal σ factor in the vegetative cells (*3*), while *sigB* and *sigC*, of which transcripts are detectable only under nitrogen limitation, encode putative sigma factors. These *sigB* and *sigC* genes are not essential for cell growth, however, their functions are not yet clear (*33*). On the other hand, the *Synechococcus* sp. strain PCC 7942 *rpoD1* gene encodes principal σ factor, and the other, *rpoD2*, *3*, and *4*, genes are not essential for cell growth (*4*, Tanaka *et al.*, unpublished data). Unicellular *M. aeruginosa* K-81, which has no ability for nitrogen fixation, has at least three *rpoD* homologs (*6, 7*). One of these, the *rpoD1* gene, was isolated (*11*), and its gene product, RpoD1, was characterized as the *E. coli* σ^{70} homolog in this study. Recent studies showed that the K-81 *rpoD1* transcripts and RpoD1 constitutively appeared under light and dark conditions, suggesting that the gene expression of *rpoD1* is not affected by a light-signal (*12*). We should also describe the similarity of the *rpoD* box sequence among the cyanobacterial *rpoD* homologs. PCC 7120 SigA, PCC 7942 RpoD1, and K-81 RpoD1 have a complete *rpoD* box sequence in subregions 2.3 to 2.4 (Fig. 1), whereas PCC 7120 SigB/C and PCC 7942 RpoD2/3/4 have incomplete *rpoD* box sequences, respectively (*4, 33*). This may be an interesting finding for characterization of the principal σ factor homolog. We are now in the process of cloning other *rpoD* homologs from the K-81 strain. Disruption of the *rpoD* gene is useful for clarifying the role

of each *rpoD* homologous gene of *M. aeruginosa* K-81, however, the restriction barrier composed of an extracellular nuclease and restriction endonucleases prevents the uptake of exogenous DNA (unpublished data). At present, we are constructing a restriction barrier-deficient mutant of *M. aeruginosa* K-81, and the strain will be a useful host for transformation.

We wish to thank Y. Nakamura for the generous gift of the *E. coli* YN543 strain. We are also grateful to G.K. Agrawal and R. Rakwal for reading the manuscript.

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