JOURNAL OF BACTERIOLOGY, June 1986, p. 739–745
0021-9193/86/060739-07$02.00/0

Vol. 166, No. 3

Nucleotide Sequence of a DNA Segment Promoting Transcription in *Pseudomonas putida*

SACHIYE INOUYE,[1] YOSHIO ASAI,[1]† ATSUSHI NAKAZAWA,[1] AND TERUKO NAKAZAWA[2]*

Department of Biochemistry, School of Medicine,[1] and School of Allied Health Sciences,[2] Yamaguchi University, Ube, 755 Japan

Received 5 August 1985/Accepted 24 February 1986

A DNA segment that promotes gene expression in *Pseudomonas putida* was identified in pTN8, a mutant plasmid of an RP4-TOL recombinant. A promoter on the segment was cloned with a promoter-probe vector containing the *xylE* gene of the TOL plasmid. The *xylE* gene was expressed under the control of the promoter, and the gene product catechol 2,3-dioxygenase was constitutively synthesized. As analyzed by an S1 nuclease protection assay, the amount of mRNA produced in *P. putida* was more than that in *Escherichia coli*. Fine S1 nuclease mapping and reverse transcriptase mapping revealed three tandem transcription start sites in both *P. putida* and *E. coli*. The nucleotide sequence preceding the transcription start sites was determined; a part of this sequence contained a sequence homologous to *E. coli* promoter sequences. A tentative consensus sequence for *P. putida* constitutive promoters is proposed.

The TOL plasmid of *Pseudomonas putida* mt-2 encodes a whole set of enzymes that degrade toluene, *m*-xylene, and *p*-xylene (30). The synthesis of these enzymes is induced by substrates or their metabolites. The genes for the enzymes are organized into two operons; the first operon (*xylCAB*) specifies the upper pathway enzymes, and the second operon (*xylDLEGF*) specifies the lower pathway enzymes (5, 7, 8, 14). Two regulatory genes, *xylR* and *xylS*, positively control these operons (5, 9, 10).

Previously, we found that the TOL plasmid was not stably maintained in *Pseudomonas aeruginosa* at high temperatures (21). However, when RP4 was introduced into the same cell, a temperature-resistant clone that grew on *m*-toluate agar was obtained. Thus, a recombinant plasmid between RP4 and TOL was obtained and designated pTN1 (22). The plasmid specified constitutive low expression of the *xylE*, *xylG*, and *xylF* genes, while it specified inducible high expression of the *xylD* gene (23).

P. putida cells carrying pTN1 grew poorly on M9 agar containing *m*-toluate and produced spontaneous segregants which grew more rapidly. One such segregant (designated pTN2) showed inducible synthesis of whole *xyl* gene products similar to that of the wild-type TOL plasmid (22, 23). The restriction cleavage map of the TOL region of pTN2 (23) was identical to that of the corresponding region of the wild-type plasmid pWW0 (4, 5). Thus, we have been using the pTN2 plasmid for analysis of the expression of *xyl* genes in *P. putida* and *Escherichia coli*. Compared with the pTN2 plasmid, the pTN1 plasmid had an additional 3.6-kilobase-pair (kbp) insert in a region upstream from the *xylE* gene and downstream from the *xylD* gene (23). The 3.6-kbp insert might be identical to the insertion sequence (3.4 kbp) described by Lehrbach et al. (15), which originated from a part of pWW0 outside of the TOL determinant region. It is plausible that the insertion of the 3.6-kbp segment first occurred in pTN1 and brought about constitutive low expression of the genes. Then, deletion of the segment from pTN1 would have occurred, leading to the wild-type phenotype of pTN2.

On the other hand, we previously isolated another type of segregant from pTN1 that grew well on M9 agar containing *m*-toluate; this was designated pTN8. This plasmid showed constitutive high expression of the *xylE*, *xylG*, and *xylF* genes in *P. putida* (24). A new transcription start site might have been generated in a region upstream from the *xylE* gene on pTN8. Because we have been interested in the promoter structure of *Pseudomonas* genes, attempts were made to clone and analyze the putative promoter on pTN8.

In this report we present evidence for the occurrence of a promoter on pTN8. The restriction map of pTN8 was indistinguishable from that of pTN1; both pTN8 and pTN1 had the 3.6-kbp insert at the same position. From S1 nuclease and reverse transcriptase mapping, the transcription start site was located on the insert. The nucleotide sequence around the start site on pTN8 was determined.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1.

Media and culture conditions. L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) was used, and 1.5% agar was added to make L agar plates. When needed, ampicillin (100 μg/ml for *E. coli* and 1 mg/ml for *P. putida*) or streptomycin (10 μg/ml for *E. coli* and 500 μg/ml for *P. putida*) was added to L agar plates. Incubations were carried out at 27°C for *P. putida* and 37°C for *E. coli*.

Enzymes and reagents. Restriction endonucleases were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), New England BioLabs, Inc. (Beverly, Mass.), and Takara Shuzo (Kyoto, Japan) and used under the conditions recommended by suppliers. T4 DNA polymerase, T4 polynucleotide kinase, and T4 ligase were products of Takara Shuzo. S1 nuclease was purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.), and reverse transcriptase was purchased from Life Sciences, Inc. (St. Petersburg, Fla.). [γ-^{32}P]ATP (specific activity, >5,000 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, Ill.).

* Corresponding author.
† Present address: Biological Science Laboratories, Lion Corp., Tazima 202, Odawara, 256 Japan.

TABLE 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Characteristics	Source or reference
P. putida mt-2		
TN2100	Prototroph	25
TN1126	*met trp*, TN2100 derivative	13
E. coli		
20SO	*thi lac mal mtl ara xyl rpsL*	1
C600	F⁻ *leu fhuA thi thr lacY supE*	Laboratory stock
GM31	*thr leu dcm his ara thi lac gal xyl mtl rpsL fhuA tsx supE*	17
Plasmids		
pTN1	RP4-TOL recombinant, *xylE*	22
pTN2	Spontaneous derivative of pTN1	22
pTN8	Spontaneous derivative of pTN1	24
Rlb679A	Sm^r Ap^r	2
pTS1045	Promoter-probe vector	This study
pTS1059	Recombinant of R1b679A and pTN8	This study
pTS1137	Recombinant of pTS1059 and pTS1045	This study
pTS1138	Recombinant of pBR322 and pTS1137	This study

Enzyme assay. Procedures for preparation of crude extract, assay of catechol 2,3-dioxygenase, and protein determination have been described previously (23). Levels of catechol 2,3-dioxygenase in vivo were estimated by spraying with 0.1 M catechol solution. Colonies of the cells producing the enzyme became yellow because of 2-hydroxymuconic semialdehyde, the product of the enzyme reaction.

DNA manipulation. The procedures for plasmid DNA preparation, restriction endonuclease cleavage, ligation, transformation, DNA fragment isolation, and gel electrophoresis have been described previously (10).

S1 nuclease and reverse transcriptase mapping and DNA sequencing. Preparation of crude RNA, S1 nuclease mapping, and reverse transcriptase mapping were performed as described previously (11, 12). The amounts of S1 nuclease and reverse transcriptase used were 1,000 and 24 U, respectively. DNA sequencing was done by the method of Maxam and Gilbert (18).

RESULTS

Restriction map of pTN8. The plasmid pTN8 was found to have the same restriction map as pTN1, from which pTN8 was derived. The maps of the *xylDLEFG* operon in pTN2, pTN1, and pTN8 are summarized in Fig. 1. Both pTN8 and pTN1 apparently had the same 3.6-kbp insert within the *xylL* region (7). The insert contained three *Bst*EII sites and one *Kpn*I site.

Cloning of promoter region of pTN8. To examine the sequence on pTN8 which caused high constitutive expression of *xylE*, pTN8 DNA was digested with *Eco*RI, and the 10.5-kbp fragment was isolated. The fragment contained most of the *xylDLEGF* operon and the 3.6-kbp insert (Fig. 1 and 2). Plasmid Rlb679A, a broad-host-range multicopy plasmid belonging to IncP4 (2), was digested with *Eco*RI, ligated with the 10.5-kbp *Eco*RI fragment, and introduced into *E. coli* C600. From ampicillin- and streptomycin-resistant transformants, clones that developed a yellow color after they were sprayed with catechol solution were selected. Plasmid pTS1059 was isolated from one of these clones.

Plasmid pTS1045 is a promoter-probe vector constructed in our laboratory from Rlb679A, pACYC177 (3), and pTS87 (11) (Fig. 2) (details will be published elsewhere). Two *Bam*HI sites separated by 1.2 kbp were located in the upstream region of the *xylE* structure gene (8, 20). If a DNA fragment containing a promoter replaced the 1.2-kbp *Bam*HI fragment, the *xylE* gene would be expressed. The 5.8-kbp *Pvu*II fragment of pTS1059 was isolated which contained the 3.6-kbp insert but not the *xylE* gene. The fragment was partially digested with *Sau*3A and ligated with *Bam*HI-cleaved pTS1045. After transformation of *E. coli* C600, pTS1137 was obtained from an ampicillin- and streptomycin-resistant transformant which showed an intense yellow color after it was sprayed with catechol solution. Restriction enzyme analysis revealed that a 1.6-kbp segment containing a *Kpn*I site was cloned in pTS1137.

Specific activities of catechol 2,3-dioxygenase synthesized in *P. putida* and *E. coli* carrying various plasmids are shown in Table 2. The *xylE* gene expression directed by pTN8 was four times higher in *P. putida* than in *E. coli*. The cells carrying pTS1059 or pTS1137 synthesized catechol 2,3-dioxygenase more than those carrying pTN8 in both bacteria. This is probably a reflection of the plasmid copy number. The replication origin of pTN8 was derived from RP4, whereas pTS1059 and pTS1137 were from Rlb679. When the plasmids listed in Table 2 were purified by the rapid boiling method (16) and estimated by ethidium bromide staining after gel electrophoresis, the copy numbers of the plasmids were essentially the same in both *P. putida* and *E. coli*. *P.*

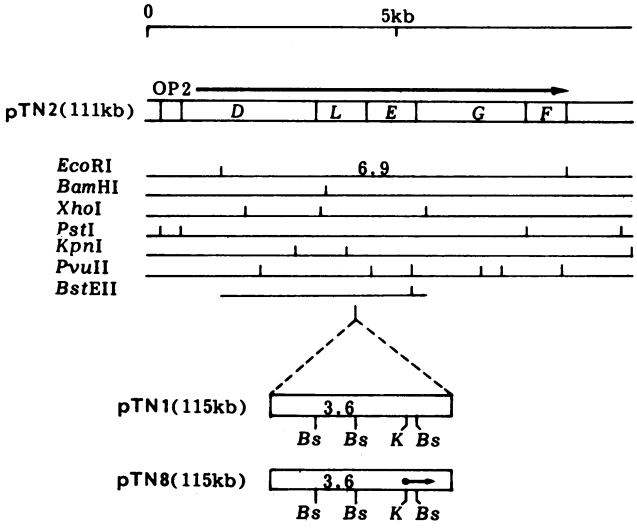

FIG. 1. Restriction enzyme cleavage maps of the *xylDLEGF* operon of pTN2, pTN1, and pTN8. OP2, operator-promoter region of the *xylDLEGF* operon. The arrow at the top shows the direction and extent of *xylDLEGF* transcription. The *xylL* gene was located from the results of Harayama et al. (7). Numbers show the size of the fragment in kilobase pairs. The lower part of the figure shows the inserted region of pTN1 and pTN8. The cloned circle with an arrow indicates the promoter and the direction of transcription. Restriction cleavage sites *Kpn*I (*K*) and *Bst*EII (*Bs*) are indicated. The distance between the *Kpn*I site in the insert and the nearest *Bst*EII site is 170 bp.

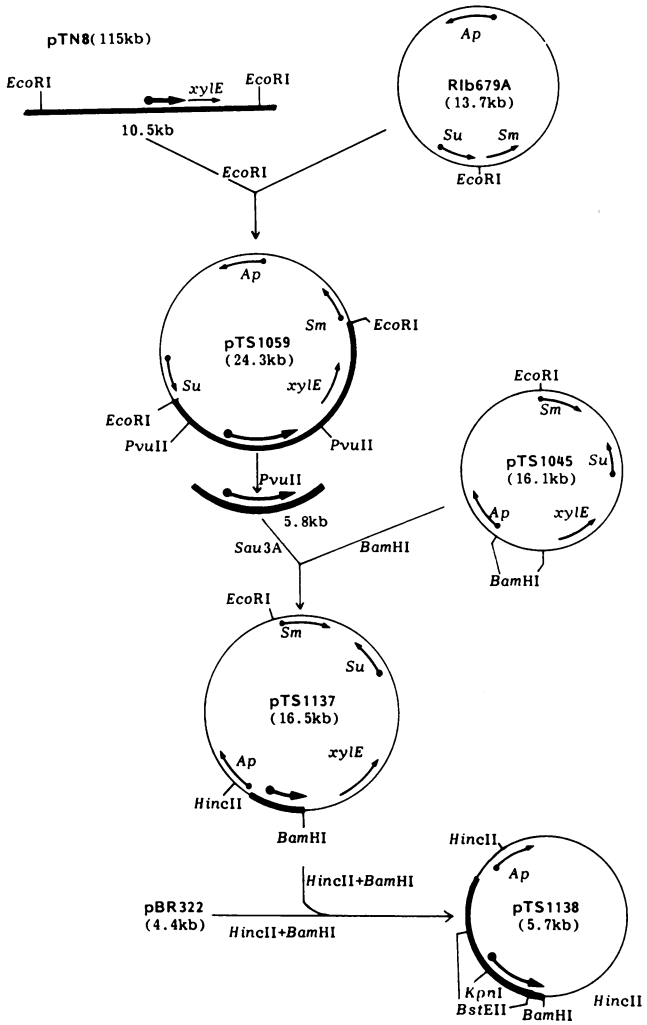

FIG. 2. Construction of recombinant plasmids carrying promoter activity. Construction of these plasmids is described in the text. Thick lines represent the TOL region, and thin lines represent vector regions. Small closed circles indicate promoters, and arrows indicate directions of transcription.

putida cells carrying pTS1137 produced the enzyme more than those carrying pTS1059, whereas *E. coli* carrying pTS1137 produced the enzyme less than those carrying pTS1059.

TABLE 2. Specific activities of catechol 2,3-dioxygenase in *P. putida* and *E. coli* cells carrying plasmids[a]

Plasmid	Sp act of catechol 2,3-dioxygenase (mU/mg)[b] in:			
	P. putida TN2100	*P. putida* TN1126	*E. coli* 20SO	*E. coli* C600
pTN1	240		20	
pTN2	270		4	
pTN8	2,340		625	
pTS1059		4,210		2,270
pTS1045		53		29
pTS1137		6,230		1,020

[a] Cells were grown in L broth in the absence of inducer at 27°C for *P. putida* and at 37°C for *E. coli*.

[b] One milliunit corresponds to the formation of 1 nmol of 2-hydroxymuconic semialdehyde per min at 27°C.

S1 nuclease protection analysis. The in vivo transcription start site on the cloned fragment was analyzed in *P. putida* and *E. coli* by S1 nuclease mapping. To obtain a probe for S1 nuclease mapping, the promoter region in pTS1137 was recloned onto pBR322 (Fig. 2, bottom). Both pTS1137 and pBR322 were cleaved with *Hin*cII and *Bam*HI and then ligated. After transformation of *E. coli* GM31, pTS1138 was obtained from an ampicillin-resistant transformant and used for S1 nuclease mapping, reverse transcriptase mapping, and DNA sequencing. A detailed restriction map of the promoter region of pTS1138 is shown in Fig. 3. The 1.1-kbp *Bst*EII fragment (S1 nuclease probe A) was purified and labeled at the 5′ ends. A single-stranded fragment was isolated and hybridized with RNAs prepared from *P. putida* and *E. coli* cells. The hybridized materials were digested with S1 nuclease, and protected DNA fragments were electrophoresed. Three bands corresponding to 160, 135, and 120 nucleotides were detected when RNA preparations from cells of *E. coli* C600(pTS1137) or *P. putida* TN1126(pTS1137) were used (Fig. 4a, lanes 4A, 4B, 7A, and 7B). The amounts of mRNA in *P. putida* seemed to be more than those in *E. coli*, especially for the shorter mRNA species mRNA-2 and mRNA-3. The patterns of the protected fragments did not change with two different quantities of S1 nuclease, i.e., 500 and 1,000 U, and were reproducible. When RNA from the cells of *P. putida* or *E. coli* carrying pTS1059 was subjected to the assay, the same pattern was obtained (data not shown). On the other hand, no band was detected with RNA from cells carrying pTS1045 or from cells without the plasmid. Protection by RNA was not observed when the other strand of the *Bst*EII fragment was used as a probe (data not shown). These results indicate that transcription starts at the region 160, 135, and 120 nucleotides upstream from the right *Bst*EII site (Fig. 3) in both *P. putida* and *E. coli*.

To examine the transcription initiation site in pTN8, RNA was prepared from *P. putida* TN2100 carrying pTN8 and also from the cells carrying pTN1 or pTN2. The DNA probe was the single-stranded 280-base *Bst*NI-*Bst*EII fragment (Fig. 3, S1 probe B) labeled at the *Bst*EII site. When an RNA preparation from TN2100(pTN8) was subjected to the assay, three protected bands were detected (Fig. 4b, lane 5). The positions of these bands were the same as those with RNA from TN2100(pTN1137) (Fig. 4b, lane 4). On the other hand, no band was detected when RNA from cells carrying pTN1, pTN2, and pTS1045 was used (Fig. 4b, lanes 6 through 8).

Determination of transcription start site. The precise start site was determined by coelectrophoresis of S1 nuclease-protected fragments on a sequence gel with chemically cleaved reaction products of the 340-base-pair (bp) *Taq*I fragment (Fig. 5a). A single-stranded DNA of the *Bst*NI-*Taq*I fragment (Fig. 3, S1 probe C) labeled at the *Taq*I site was hybridized with RNA from TN1126(pTS1137) or C600(pTS1137). Essentially the same protected bands appeared with both RNA preparations, although the amount of protected DNA was much less with *E. coli* RNA. Ladders were seen in protected bands, which were reported to be inherent to the S1 nuclease mapping assay.

Reverse transcriptase mapping was also carried out to assign the start site. The *Bst*EII fragment was purified, labeled at the 5′ ends, and cleaved with *Taq*I. The 70-bp *Taq*I-*Bst*EII fragment labeled at the *Bst*EII site (Fig. 3, primer) was isolated, hybridized with an RNA preparation of *P. putida* carrying pTS1137, and extended with reverse transcriptase. The extended DNA was electrophoresed in a sequence gel together with chemically cleaved products of

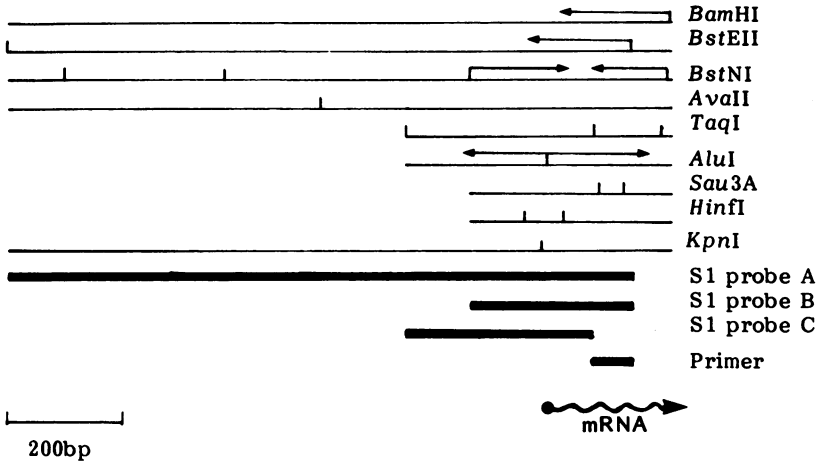

FIG. 3. Restriction map with sequencing strategy of the promoter region of pTS1138 and DNA fragments used for S1 nuclease and reverse transcriptase mapping. Restriction cleavage sites are represented by short vertical lines. Thin arrows show the extent and direction of sequence analysis. Thick lines show the DNA fragments used as probes for S1 nuclease mapping (S1 probes A, B, and C) and the fragment used as a primer for reverse transcriptase mapping (primer). The closed circle with a wavy arrow indicates the promoter and the direction of transcription.

the *Ava*II-*Bst*EII fragment labeled at the *Bst*EII site. Three distinct bands were observed, two of which were prominent (Fig. 5b, lane 1). Essentially the same results were obtained with an RNA preparation of *E. coli* carrying pTS1137, although the intensity of the bands was different (data not shown). When the results of S1 nuclease mapping and reverse transcriptase mapping were compared, the DNA fragment extended with reverse transcriptase corresponded to the central main bands of each ladder obtained by S1 nuclease mapping. After a correction of 1.5 bases in determining the length of the extended fragment (6), the three bands corresponded to adenines at positions 130, 149, and 162 (Fig. 6).

Nucleotide sequence of the promoter region. In Fig. 6 is shown the nucleotide sequence of the 335-bp *Bst*NI-*Bam*HI fragment. The sequence was determined by the strategy shown in Fig. 3. The transcription start sites indicated by arrowheads (Fig. 6) were adopted from the results of reverse

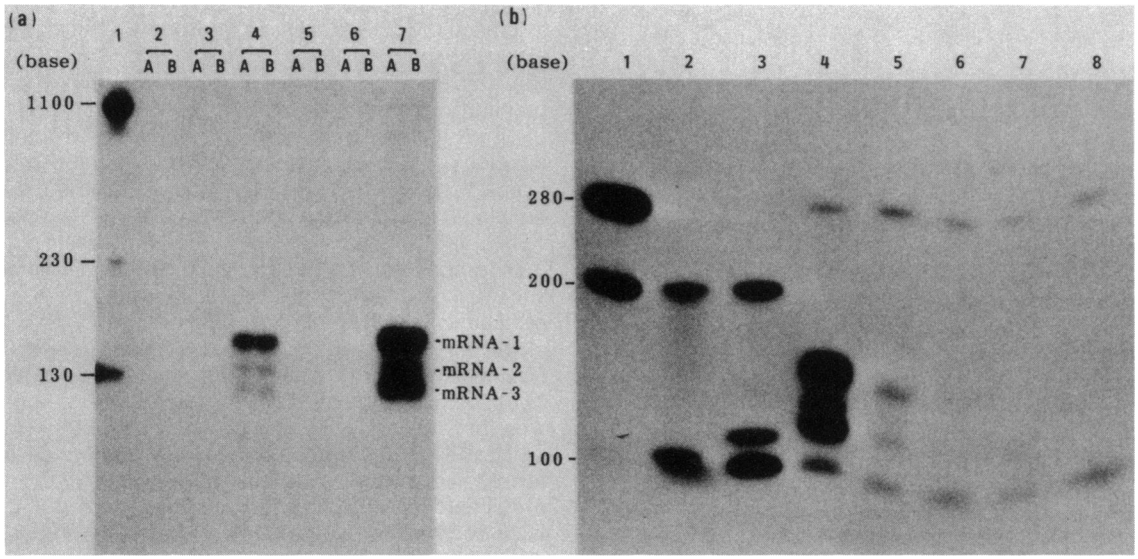

FIG. 4. S1 nuclease mapping analysis of transcripts in *P. putida* and *E. coli*. (a) The DNA probe was a single-stranded, 1,100-base *Bst*EII fragment (Fig. 3, S1 probe A) labeled at the right *Bst*EII site. The protected DNA fragments were electrophoresed on a 8% polyacrylamide–8 M urea gel. Lane 1 is marker DNA fragments. RNA preparations used in lanes 2 to 7 were obtained from cells of C600, C600(pTS1045), C600(pTS1137), TN1126, TN1126(pTS1045), and TN1126(pTS1137), respectively. Amounts of S1 nuclease added were 500 U (lanes A) or 1,000 U (lanes B). (b) The DNA probe was a single-stranded, 280-base *Bst*NI-*Bst*EII fragment (Fig. 3, S1 probe B) labeled at the *Bst*EII site. The protected DNA fragments were electrophoresed on 5% polyacrylamide–8 M urea gel. Lanes 1 to 3 are marker DNA fragments. RNA preparations used in lanes 4 to 8 were obtained from cells of TN1126(pTS1137), TN2100(pTN8), TN2100(pTN1), TN2100(pTN2), and TN1126(pTS1045), respectively. A band corresponding to 280 nucleotides probably represents the probe DNA protected by the contaminated antisense strand. A 100-nucleotide band was not found in other experiments.

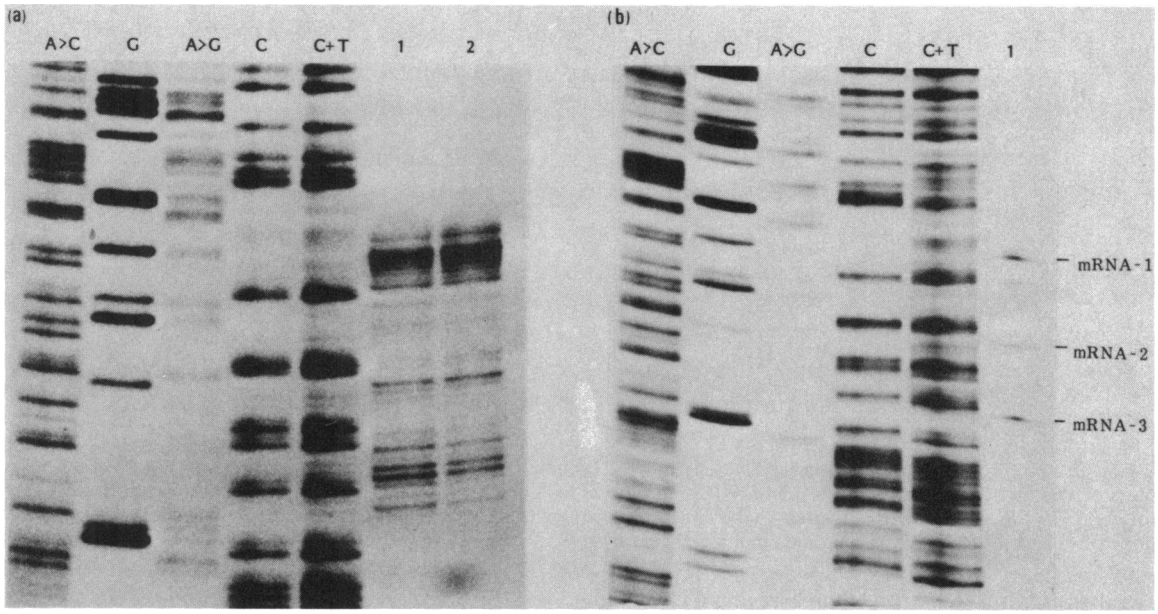

FIG. 5. Determination of the transcription start site by S1 nuclease and reverse transcriptase mapping. (a) S1 nuclease mapping. The DNA probe used was a single-stranded *Bst*NI-*Taq*I fragment (Fig. 3, S1 probe C) labeled at the *Taq*I site. Protected DNA fragments (lanes 1 and 2) were electrophoresed together with chemically cleaved products for sequencing the *Taq*I fragment labeled at the right *Taq*I site (Fig. 3) (lanes A>C to C+T). RNA preparations were obtained from cells of TN1126(pTS1137) (lane 1 in panels a and b) and C600(pTS1137) (lane 2). The amount of RNA preparation from *E. coli* C600(pTS1137) used was four times more than that from *P. putida*. (b) Reverse transcriptase mapping. The DNA primer was a *Taq*I-*Bst*EII fragment (Fig. 3, primer) labeled at the *Bst*EII site. The extended products were subjected to electrophoresis (lane 1), together with chemically cleaved products for sequencing the *Ava*II-*Bst*EII fragment labeled at the *Bst*EII site (lanes A>C to C+T). RNA was extracted from TN1126(pTS1137).

transcriptase mapping. In the region preceding the first start site (mRNA-1), there was a sequence homologous to the −10 and −35 consensus sequence of *E. coli* promoters (26, 27), although they were located about 5 bp downstream from the ordinary sites. No consensus sequence was found in the region preceding the second (mRNA-2) and the third (mRNA-3) start sites. To determine whether the transcription initiation signal was in the 70-bp *Hin*fI fragment, the fragment was isolated from pTS1138, treated with T4 DNA polymerase, and ligated to *Bam*HI-cleaved and T4 DNA polymerase-treated pTS1045. The resulting plasmid directed production of the same levels of catechol 2,3-dioxygenase as pTS1137 in either *P. putida* or *E. coli* (data not shown). These results indicate that all information necessary for transcription initiation is included in the 70-bp *Hin*fI fragment.

Downstream from the transcription start sites there was an initiation codon from which a continuous reading frame existed, at least up to the *Bam*HI site at the end, and a sequence AGGAG was found, which was complementary to the 3′ ends of the 16S rRNA of both *E. coli* and *P. aeruginosa* (Fig. 6) (29).

DISCUSSION

We cloned a promoter from an RP4-TOL plasmid, pTN8, and determined the nucleotide sequence around the promoter. It was located on a 1.1-kbp *Bst*EII fragment of pTS1138 in which a unique *Kpn*I site was present (Fig. 3). The possibility that rearrangement of the pTN8 promoter occurred during *Sau*3A digestion and religation was unlikely for the following reasons: (i) the 70-bp *Hin*fI fragment containing the promoter had no *Sau*3A site, (ii) the 170-bp *Kpn*I-*Bst*EII fragment in the promoter region was found in both pTS1138 and pTN8 (Fig. 1 and 6), (iii) S1 nuclease

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BstNI                                                          50
CCTGGCGCCTCGGAGGAGGCCTGTATATCGGTATCCGCTGCGGACGTTGC
GGACCGCGGAGCCTCCTCCGGACATATAGCCATAGGCGACGCCTGCAACG

                                                    HinfI   100
ATAACCGCCACAGGCGCCACCACAGGATTTGCTCATGATACGACTCCACT
TAGTGGCGGTGTCCGCGGTGGTGTCCTAAACGAGTACTATGCTGAGGTGA

-35(P1)        KpnI     -10(P1)                        150
TGAACAATGTTGTGGTACCATTTAAAACTATAAAGCTACTATAAGGTCAA
ACTTGTTACAACACCATGGTAAATTTTGATATTTCGATGATATTCCAGTT
                                mRNA-1          mRNA-2
        HinfI                                          200
TAGAGTAAAGAATCCGTTGGGGAGGAGGCTGATGCGCATTGGTCAGTTGG
ATCTCATTTCTTAGGCAACCCCTCCTCCGACTACGCGTAACCAGTCAACC
        mRNA-3
                              Sau3A                    250
CGCAGTTGGTAGGGGTCGAAACACAGACGATCCGCGTCTATGAACAGCAG
GCGTCAACCATCCCCAGCTTTGTGTCTGCTAGGCGCAGATACTTGTCGTC

                  Sau3A          BstEII                300
GGCTTGTTGCCGCCGCCTGATCGGCAGGACAACGGTTACCGTGTCTATAC
CCGAACAACGGCGGCGGACTAGCCGTCCTGTTGCCAATGGCACAGATATG

                                                        350
CGAGAAACACGTTAAGCGGCTGGCCTTTATTCGATGCCTGCCGCATCCTG
GCTCTTTGTGCAATTCGCCGACCGGAAATAAGCTACGGACGGCGTAGGAC

BamHI
GATCC
CTAGG
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FIG. 6. Nucleotide sequence of the promoter region in the 3.6-kbp insert in pTN8. Nucleotides are numbered from the *Bst*NI site. Arrowheads indicate the transcription start sites determined by reverse transcriptase mapping. Sequences which are homologous to the consensus sequence of *E. coli* promoters (−35 and −10 regions) are boxed. Restriction sites are shown with brackets above the sequence. The underlined bases are complementary to the 3′ ends of the 16S rRNA of *P. aeruginosa* and *E. coli*. The AUG codon is overscored and underlined.

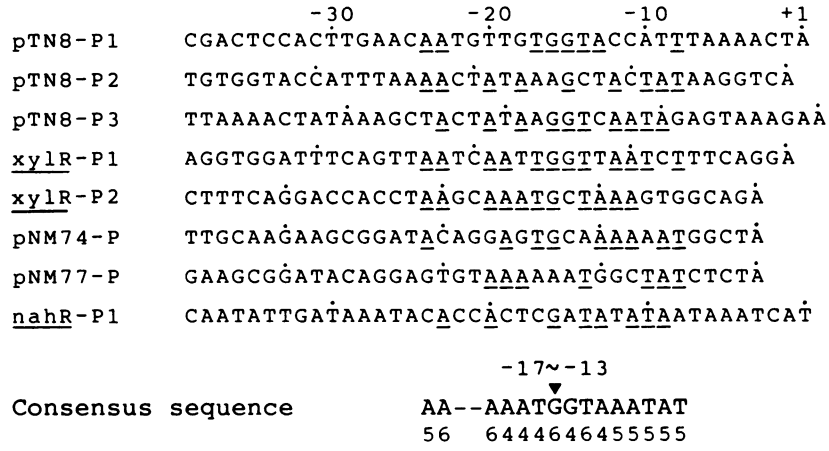

FIG. 7. Constitutive promoters of *P. putida*. pTN8-P1, pTN8-P2, and pTN8-P3 are the promoters shown in Fig. 6. *xylR*-P1 and *xylR*-P2 are promoters of *xylR* (12), and pNM74-P and pNM77-P are promoters of constitutive mutants of the *xylDLEGF* operon (19). *nahR*-P1 is the promoter of *nahR* (28). Bases which are identical to the consensus sequence are underlined. Numbers below the consensus sequence indicate the frequency of occurrence of each nucleotide among eight promoters.

mapping analysis with RNAs from cells carrying pTN8 and pTS1137 showed the same pattern (Fig. 4b).

As determined by the levels of catechol 2,3-dioxygenase (Table 2) or the amounts of mRNA (Fig. 4), the promoter appeared to be more active in *P. putida* than in *E. coli*, although the possibility remained that mRNA was more unstable in *E. coli* than in *P. putida*.

From the results of S1 nuclease and reverse transcriptase mapping, the transcription was found to start at three sites which proceeded in the same direction. In *E. coli* cells carrying pTS1137, mRNA-1 was prominent, whereas in *P. putida* cells other mRNA species, especially mRNA-3, were also abundant (Fig. 4 and 5). These results might be related to the nucleotide sequence preceding the transcription start sites; a sequence similar to the −10 and −35 consensus sequence of *E. coli* promoters was found only in the region of the first start site (Fig. 6). It was not clear, however, that the sequence was actually recognized as a promoter, because its location was too close to the transcription start site.

The nucleotide sequences preceding the start sites for constitutive transcription in *P. putida* determined so far are summarized in Fig. 7. The *xylR* gene which positively controls *xyl* operons has two initiation sites for constitutive transcription in both *P. putida* and *E. coli* (13). Plasmid pNM74 and pNM77 are constitutive mutants of the *xylDLEGF* promoter described by Mermod et al. (19). The *nahR* gene positively regulates the *nah* and *sal* operons (28). Based on these data a tentative consensus sequence AA--AAATGGTAAATAT was deduced for *P. putida* promoters. The position of G in the center was at nucleotides −17 to −13 of the transcription start site. The sequence contained a sequence similar to the −10 region (TATAAT) of *E. coli* promoters (27). The −35 region (TTGACA) of *E. coli* promoters was not found except in pTN8-P1. It should be noted that the consensus sequence proposed in Fig. 7 was not present in the promoter region of inducible operons such as *xylCAB*, *xylDLEGF*, *nah*, and *sal* (11, 12, 28).

Plasmid pTN8 is a spontaneous mutant of pTN1 which was selected because it grows well on *m*-toluate agar. A simple inversion of the 3.6-kbp insert of pTN1 to form pTN8 is unlikely, because the *Kpn*I and *Bst*EII sites within the insert did not change after the mutation (Fig. 1). Thus, the mutation from pTN1 to pTN8 might result from a change in a limited region. The restriction map of *Kpn*I and *Bst*EII of the 3.6-kbp insert in pTN1 (Fig. 1) was very similar to that of the 3.4-kbp insert in pWW0–673 (14, 15), suggesting that the inserts are identical. Analysis on pTN1, including determination of the nucleotide sequence of the corresponding region, is in progress.

ACKNOWLEDGMENTS

We thank S. Iyobe for the plasmid Rlb679A.

This work was supported by grants-in-aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan. A part of the work was carried out under a contract with the Science and Technology Agency in Japan.

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