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Construction of Cloning Cartridges for Development of Expression Vectors in Gram-Negative Bacteria

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A cloning cartridge was constructed that can be inserted into a plasmid of choice to form an expression vector in which gene expression is inducible with an inexpensive inducer, sodium salicylate, at low concentrations. This cartridge consists of a 3.6-kb restriction fragment which contains the positive regulatory gene *nahR* from plasmid NAH7, a promoter, P_G, that *nahR* regulates, a multiple cloning site, a transcription terminator, and a gene conferring tetracycline resistance. Within promoter P_G of the cloning cartridge, a sequence of three nucleotides upstream of the ATG sequence encoding the initiation codon was altered to create an *NdeI* recognition site (CATATG) for cloning of the 5′ end of a gene without affecting the distance between the transcription start site and the gene coding region. In addition, the 5′ end of a gene can be converted into an *NdeI* recognition site without altering the amino acid sequence it encodes and then cloned into this cartridge for regulated expression. Several other synthetic restriction sites were also inserted downstream of the *NdeI* site for accepting the 3′ end of a cloned gene. A derivative of this cloning cartridge lacking the *NdeI* sequence was also constructed for cloning and expression of a restriction fragment containing a gene(s) of unknown sequence. Use of the cloning cartridges in a broad-host-range plasmid has allowed successful cloning and inducible expression of several genes in all of the gram-negative bacteria tested to date. Protein production to at least 10% of the total soluble cell proteins was observed from a cloned gene expressed in *Pseudomonas putida*.

An expression vector is an indispensable tool for the study of gene expression in bacterial cells. The need to develop expression vectors is particularly acute for little-studied bacterial strains in which gene expression needs to be assessed. At least two approaches can be used to introduce an expression vector into these bacterial strains. A native plasmid, if it is known, can be converted into an expression vector, or a broad-host-range expression vector can be introduced into these strains. The native plasmid of a poorly studied strain is usually not very well characterized. Genetic elements essential for regulated gene expression must be introduced from other sources for conversion to an expression vector. A number of broad-host-range vectors have been developed for gram-negative bacteria (1, 15, 24). However, only a few of these vectors (3, 10, 16) allow regulated gene expression. New broad-host-range vectors that allow regulated and more efficient gene expression and are more convenient to use still need to be developed. Construction of a DNA restriction fragment carrying all of the elements essential for gene cloning and expression would facilitate the development of expression vectors. Such a cloning cartridge can be inserted into a replicon of either broad host range or narrow host range to convert it into an expression vector.

The regulation of naphthalene catabolic genes carried by the NAH7 plasmid has been well studied (31). The NAH7 plasmid is a naturally occurring plasmid in *Pseudomonas putida* G7 (ATCC 17485). It carries catabolic genes for the degradation of naphthalene to Krebs cycle intermediates. These genes are organized in two operons. The first operon encodes enzymes for the conversion of naphthalene to salicylate (upper pathway), and the second operon encodes enzymes for the oxidation of salicylate to acetylaldehyde and pyruvate (lower pathway). Both operons are activated in the presence of the inducer, salicylic acid or some of its analogs, and the product of the regulatory gene, *nahR*. The *nahR* gene maps immediately upstream of the lower pathway operon and next to the *nahG* gene, which encodes the enzyme salicylate hydroxylase (Fig. 1). The *nahR* and *nahG* genes are transcribed in opposite directions, and their promoters, P_R and P_G, share sequences. While P_G is subject to the positive regulation of NahR protein, P_R directs the synthesis of NahR protein constitutively. The nucleotide sequences of *nahR*, P_R, and P_G have been determined (22–24, 32).

In this report, I describe the construction of a cloning cartridge and its derivative based on the NahR-regulated gene expression system. The two cloning cartridges were tested on a derivative of the broad-host-range plasmid pKT231 (2) for use in gene cloning and expression in a variety of bacterial hosts. Plasmids carrying one or the other cartridge have allowed cloning and inducible expression of several tested genes in all of the gram-negative bacteria tested to date. High-level protein production from a cloned gene was also demonstrated.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. putida* G572 is wild-type G1 cured of the CAM plasmid (26), and *P. putida* G1343 is a Met^- derivative of *P. putida* G572 (29). *P. putida* KT2440 ($r^- m^+$) is a spontaneous restriction-negative derivative of strain mt-2 (2). *Escherichia coli* JM103 has the genotype Δ(*lac-pro*) *thi strA supE endA sbcB15 hsdR4*(F′ *traD36 proAB lacI*^{q}ZΔM15) (17). *E. coli* Y5250 is strain HB101 (*hsdS20 recA13 proA2 leuB6 thi-1* [5]) carrying plasmid pKMY342 (see below). *Pseudomonas mendocina* Y4075 is *P. mendocina* Y4007 carrying pKMY342. *P. mendocina* Y4007 is a toluene-4-monooxygenase (T4MO)-defective mutant of wild-type KR1 generated by treatment with nitrosoguanidine (30). *Aeromonas hydrophila* Y21, *Enterobacter cloacae* Y81, *Klebsiella pneumoniae* Y61, and *Pseudomonas putida* Y2511 are all wild-type strains (isolated and typed at Amgen) carrying pKMY342.

Plasmid pKMY342 was constructed by cloning the 4.7-kb

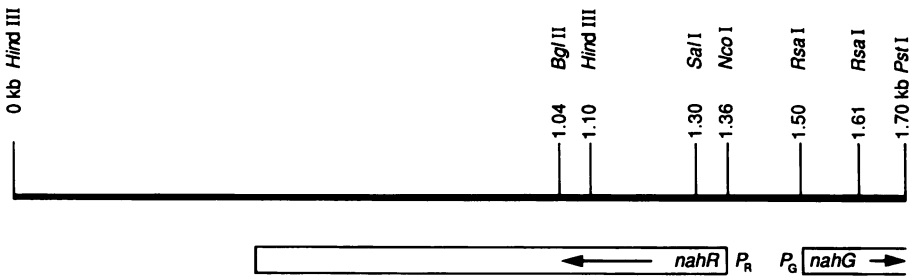

FIG. 1. Restriction map of a region of plasmid NAH7 containing the *nahR* gene and part of the *nahG* gene. P_R and P_G are promoters of *nahR* and *nahG*, respectively. Arrows indicate transcriptional directions.

*Xba*I-*Sac*I fragment of pKMY341 (30) carrying the T4MO gene cluster into the expression vector pKMY319 (see Results and Discussion). Analysis with restriction enzymes demonstrated that pKMY342 contains two copies of the 4.7-kb *Xba*I-*Sac*I fragment joined by a *Sac*I-*Kpn*I-*Xba*I linker derived from the multiple cloning site in pKMY319. Plasmids pKY67 (29), pKMY514, and pKMY515 were used to construct plasmid pKMY517. Plasmid pKY67 is a NAH7 plasmid containing a Tn*5* insertion in the *nahG* gene. Cloning of an ~2.7-kb *Xma*I fragment of pKY67 containing the Tn*5* gene conferring kanamycin resistance and the NAH7 gene *nahH* encoding catechol 2,3-dioxygenase into plasmid pUC19 (28) generated pKMY514. Cloning of the ~1.5-kb *Nco*I-*Xho*I fragment of pKMY514 containing the catechol 2,3-dioxygenase gene (11) into plasmid pCFM1146 (30) (see Fig. 4) produced pKMY515. An *Xba*I site is located upstream of the *Nco*I site in pKMY515, and cloning of the ~1.5-kb *Xba*I-*Xho*I fragment of pKMY515 into pKMY319 generated pKMY517. Plasmid pLuc2 was used in the construction of plasmid pKMY520. It was obtained from E. Fisher at Amgen and was constructed by converting the 5′ end of the luciferase gene from the firefly *Photinus pyralis* (8) into an *Nde*I site and cloning it into the expression vector pCFM4722 (6). Cloning of the ~1.7-kb *Xba*I-*Asp* 718 fragment of pLuc2 containing the luciferase gene into the expression vector pKMY299 (see Results and Discussion) generated pKMY520.

Manipulation of DNA. Enzymatic cleavage, modification and ligation of DNA, and determination of nucleotide sequences were done as described elsewhere (30).

Luciferase assay. Luciferase activity was determined by measuring the light produced in a reaction catalyzed by this enzyme. An aliquot of cell suspension (3 to 10 μl) was mixed with water and 30 μl of assay buffer containing 0.2 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.7) and 50 mM $MgSO_4$ in a total volume of 100 μl in a cuvette placed in a luminometer (Biocounter M2500; Lumac, The Netherlands). The light-emitting reaction was initiated by injection of 100 μl of 1 mM luciferin (Sigma Chemical Co., St. Louis, Mo.) in 5 mM citrate buffer (pH 5.5) and 100 μl of 100 mM rATP in water, and the light produced was displayed in relative light units. The specific activity of luciferase was expressed in relative light units per microgram of protein. The protein concentration was determined as described elsewhere (30).

RESULTS AND DISCUSSION

Construction of a cloning cartridge. The cloning cartridge was designed to contain five elements essential for efficient gene cloning and expression based on the NahR-regulated expression system. These five elements consist of a gene conferring drug resistance, *nahR*, P_G, a multiple cloning site, and a transcription terminator. A recognition site for the frequently cutting restriction endonuclease *Rsa*I is located 3 bp upstream of the *nahG* coding region (Fig. 1). The initial steps in construction of the cloning cartridge involved converting this *Rsa*I site into a *Sca*I restriction site convenient for insertion of a multiple cloning site and placing the gene conferring tetracycline resistance (Tet^r) in *E. coli* plasmid pBR322 (4) next to the P_R region.

Plasmid pKMY256 (30) (Fig. 2) is *E. coli* plasmid pUC19 (28) carrying an ~5.3-kb *Pst*I insert which contains the *nahR* gene, P_G, and ~200 bp of the *nahG* gene. Digestion of pKMY256 DNA with the enzymes *Pst*I and *Sal*I and self-ligation led to the cloning of an ~420-bp *Sal*I-*Pst*I fragment containing P_R and P_G into pUC19. The resulting plasmid was designated pKMY288. In pKMY288, the promoters P_R and P_G are located on an ~200-bp *Sal*I-*Rsa*I fragment (Fig. 2). The *Rsa*I site of this fragment can be ligated to the *Sca*I site of pKMY256 to regenerate the *Sca*I site. Replacement of the ~1.4-kb *Sal*I-*Sal*I-*Sca*I fragment of pKMY256 with the ~200-bp *Sal*I-*Rsa*I fragment of pKMY288 resulted in the deletion of the remaining *nahG* sequence and the conversion of the *Rsa*I site in P_G into a *Sca*I site (Fig. 2). The newly formed plasmid was designated pKMY289.

To place the Tet^r gene next to P_R, the ~470-bp *Bgl*II-*Sca*I fragment of pKMY289 carrying P_R and P_G was used to replace an ~4.4-kb *Bgl*II-*Sca*I fragment in plasmid pBR322 carrying a copy of transposon Tn*5* (20). The resulting plasmid was designated pKMY291 (Fig. 2). A pKMY291 derivative, designated pKMY292, was constructed by deleting the intervening sequence between the *Hin*dIII site within *nahR* and the *Hin*dIII site within the promoter of Tet^r gene (Fig. 2). This step placed the Tet^r gene immediately downstream of P_R.

In the following steps, a multiple cloning site was inserted at the newly created *Sca*I site within P_G and a transcription terminator was inserted at the end of the cloning site. In most bacterial genes, the nucleotide sequence ATG specifies the initiation codon. This trinucleotide and the sequence preceding it can be converted by site-specific mutagenesis into the recognition site of the restriction endonuclease *Nde*I (CATATG) without affecting the amino acid sequence that a gene encodes. To accommodate genes modified in this manner, an *Nde*I site can be similarly created in an expression vector at the junction between the promoter and the gene coding region. Other restriction sites can be introduced downstream of the *Nde*I site for accepting the 3′ end of a cloned gene. In such an expression system, the distance between the transcription start site and the gene coding region is unaltered, regardless of the gene cloned. The

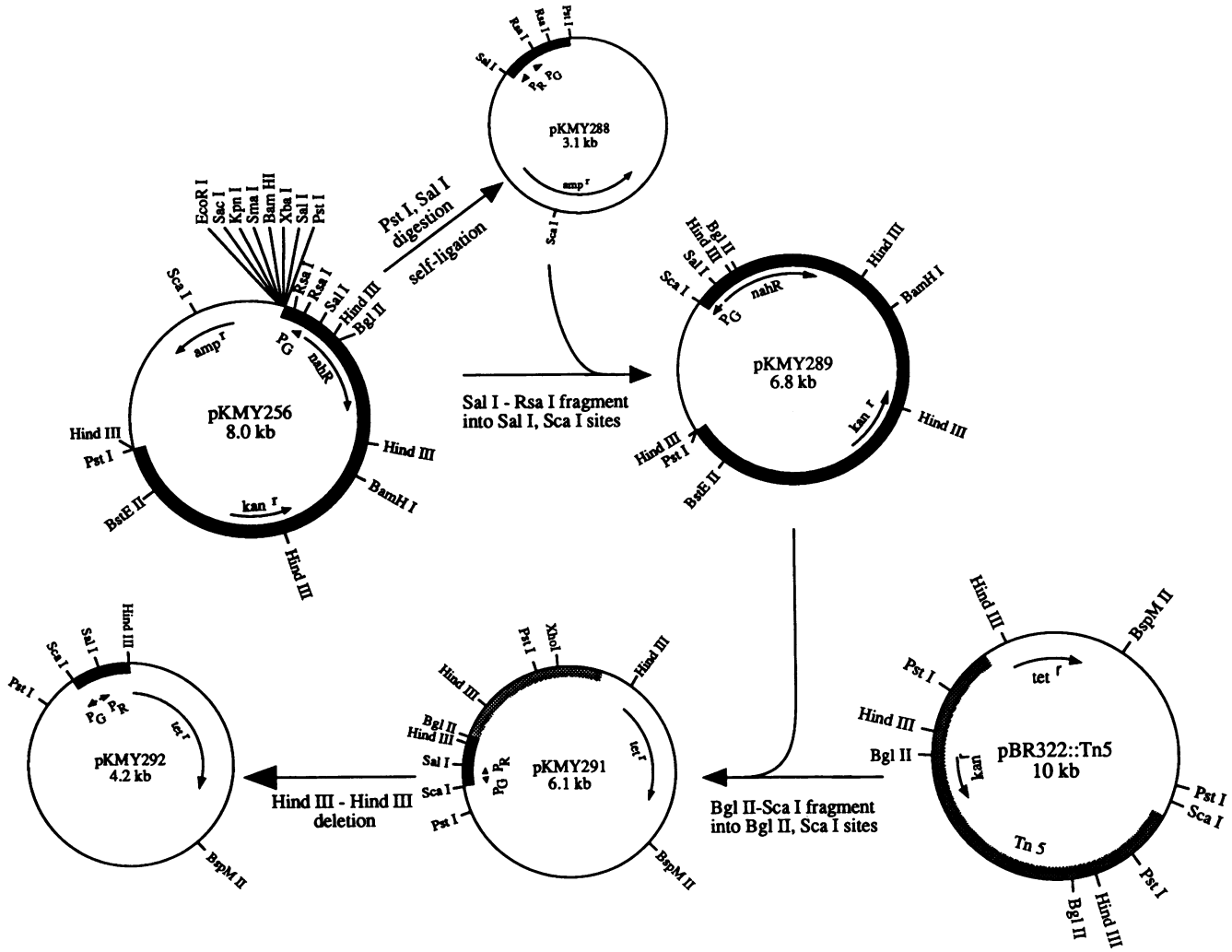

FIG. 2. Initial steps in the construction of a cloning cartridge.

promoter P_R was modified to contain an *Nde*I restriction site at the 3′ end, followed by a number of other unique restriction sites.

An oligonucleotide containing the sequence shown in Fig. 3 was synthesized and ligated into the *Sca*I and *Pst*I sites of pKMY292. Insertion of this sequence converted the *Sca*I site into the *Rsa*I site naturally occurring within P_G, restored the distance between the *Rsa*I site and the coding region, generated an *Nde*I cloning site at the 3′ end of P_G, and placed a number of other cloning sites immediately downstream of the *Nde*I site. The resulting plasmid was designated pKMY293 (Fig. 4).

E. coli plasmid pCFM1146 (30) carries a transcription terminator that can be easily incorporated into other systems. Downstream of the transcription terminator is a *Bgl*II restriction site, and immediately upstream of it is a multiple cloning site that includes an *Eco*RI site, a *Xho*I site, and a number of other restriction sites (Fig. 4). This transcription terminator was placed immediately downstream of the multiple cloning site in pKMY293 in two steps. The *Bsp*MII-*Pst*I fragment of pKMY293 carrying the Tet^r gene, P_R, P_G, and the multiple cloning site was initially cloned into the *Xma*I and *Pst*I sites of plasmid pUC9 (18) to place the *Eco*RI site of pUC9 downstream of the Tet^r gene (Fig. 4). The resulting plasmid was designated pKMY294. In the next step, the *Eco*RI-*Xho*I fragment of pKMY294 was cloned into the *Eco*RI and *Xho*I sites of pCFM1146 to place the transcription terminator immediately downstream of the multiple cloning site of P_G (Fig. 4). The resulting plasmid was designated pKMY295.

The remaining steps established a unique restriction site downstream of the transcription terminator and restored the

```
   RsaI     HpaI        XbaI        SacI       SacII       PstI
5' ACCATATGGTTAACATCGATTCTAGAGGTACCGAGCTCCTCGAGCCGCGGACAGATCTCTGCA 3'
3' TGGTATACCAATTGTAGCTAAGATCTCCATGGCTCGAGGAGCTCGGCGCCTGTCTAGAG    5'
     NdeI       ClaI        KpnI        XhoI        BglII
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FIG. 3. Sequence of the oligonucleotide ligated into the *Sca*I and *Pst*I sites of pKMY292.

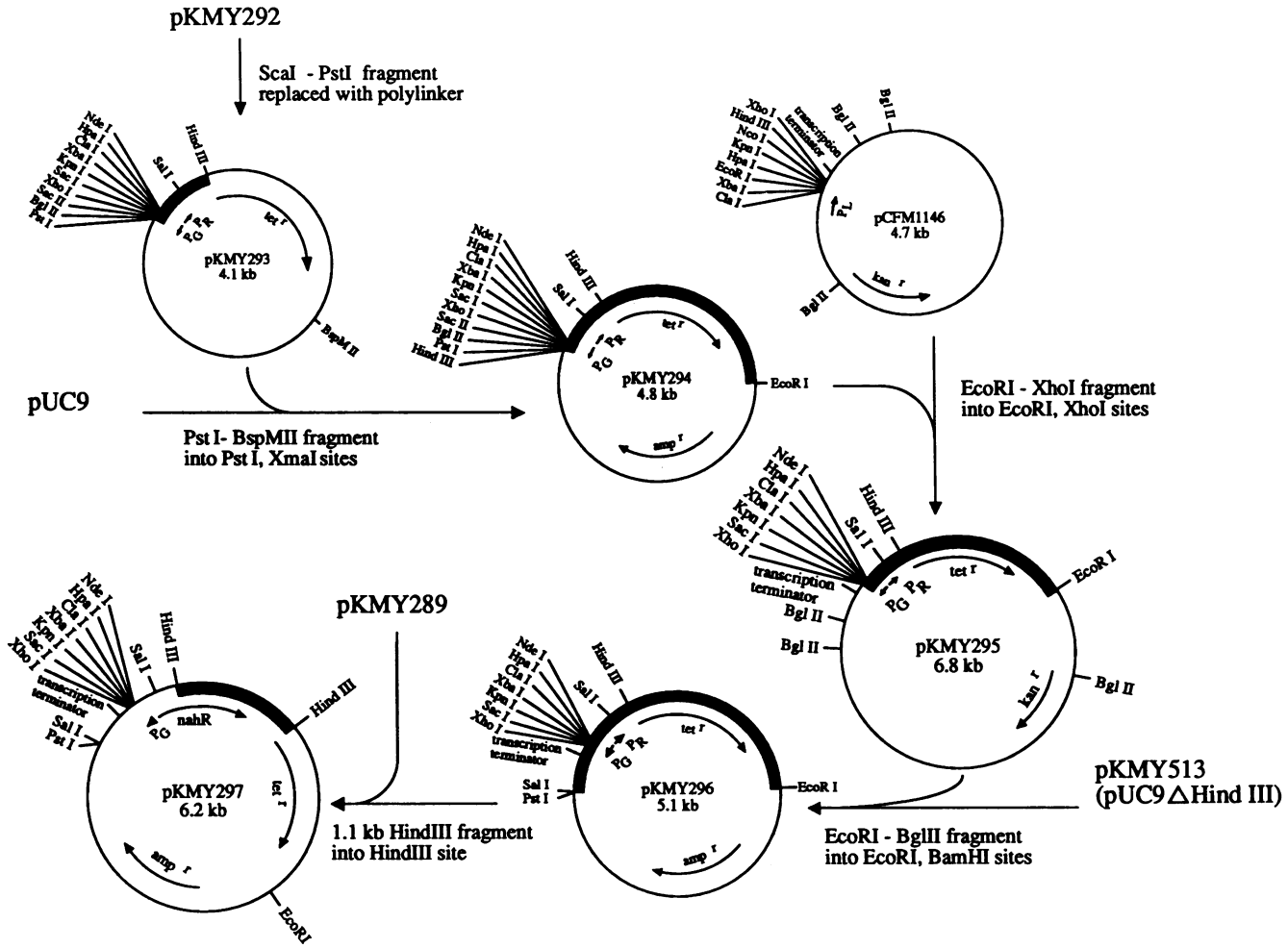

FIG. 4. Subsequent steps leading to the construction of a cloning cartridge in plasmid pKMY297.

nahR gene. In pKMY295, the *Bgl*II site downstream of the transcription terminator needed to be replaced with a restriction site unique in the cloning cartridge for the convenience of transferring the cartridge among replicons. To achieve this end, a pUC9 derivative without a *Hin*dIII site, designated pKMY513, was initially constructed by cleaving pUC9 with *Hin*dIII, followed by end filling and blunt-end ligation (Fig. 4). The *Eco*RI-*Bgl*II fragment of pKMY295 carrying the Tet^r gene, P_R, P_G, the multiple cloning site, and the transcription terminator was cloned into the *Eco*RI and *Bam*HI sites of pKMY513 to eliminate the *Bgl*II site and incorporate a unique *Pst*I site next to the destroyed *Bgl*II site (Fig. 4). The resulting plasmid was designated pKMY296.

In pKMY296, the part of the *nahR* gene downstream of the *Hin*dIII site within *nahR* is still missing. To ensure correct and convenient assembly of the *nahR* gene, an indicator plasmid, designated pKMY512, was constructed which contained the naphthalene dioxygenase gene cluster and its NahR-regulated promoter from plasmid NAH7. In the presence of an inducer, sodium salicylate, and the NahR protein, the naphthalene dioxygenase genes of pKMY512 can be activated, which in turn catalyzes the formation of indigo dye in *E. coli* (9). Two intermediate plasmids, pN400 and pKMY239, were involved in the construction of pKMY512. Plasmid pN400 (obtained from T. Osslund, Amgen) was constructed by cloning the *Pvu*II-*Bgl*II fragment of plasmid pE317 (9) containing the naphthalene dioxygenase genes into the *Sma*I and *Bam*HI sites of plasmid pUC18 (28). Plasmid pKMY239 was constructed by inserting the ~6.4-kb *Sac*I fragment of pN400 containing the naphthalene dioxygenase genes into the *Sac*I site of the broad-host-range plasmid pKMY223 (30). Deletion of the *Bgl*II-*Eco*RI fragment of pKMY239 carrying a portion of the *nahR* gene generated pKMY512. The 1.1-kb *Hin*dIII fragment of pKMY289 (Fig. 2) carrying the portion of the *nahR* gene missing in pKMY296 was inserted into the *Hin*dIII site of pKMY296 to complete construction of the cloning cartridge (Fig. 4). In this step, the desired plasmid was selected for its ability to induce *E. coli* cells harboring pKMY512 to produce indigo in the presence of sodium salicylate. This plasmid was designated pKMY297 (Fig. 4). In pKMY297, a 3.6-kb *Eco*RI-*Pst*I fragment containing the Tet^r gene, *nahR*, P_G, a multiple cloning site, and a transcription terminator was assembled as a cloning cartridge.

Use of the cloning cartridge in construction of broad-host-range expression vectors pKMY299 and pKMY319. To test the 3.6-kb *Eco*RI-*Pst*I fragment from pKMY297 for use as a cloning cartridge in different hosts, this fragment was inserted into a derivative of plasmid pKT231 (2), which was itself derived from the broad-host-range plasmid RSF1010

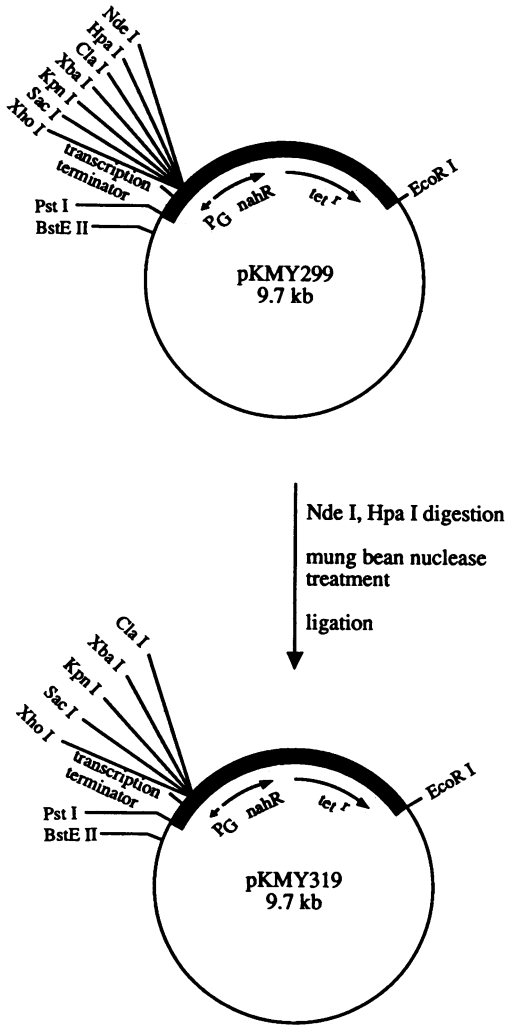

FIG. 5. Derivation of the expression vector pKMY319 from the expression vector pKMY299.

(25). Plasmid pKT231 is a derivative of RSF1010 (2) and served as a source of the RSF1010 replicon. In pKT231, an *Hpa*I site is located ~200 bp from a *Sac*I site, both of which occur in the polylinker of the cloning cartridge. These two sites were removed from pKT231 by digestion of the plasmid DNA with *Hpa*I and *Sac*I, treatment with the Klenow fragment of *E. coli* DNA polymerase I to generate blunt ends, and self-ligation. The resulting plasmid was designated pKMY286. The broad-host-range expression vector pKMY299 was constructed by replacing an *Eco*RI-*Pst*I fragment in pKMY286 with the 3.6-kb *Eco*RI-*Pst*I cloning cartridge (Fig. 5).

In pKMY299, the expected nucleotide sequences at the junction between P_G and the multiple cloning site and at the *Pst*I junction between the cloning cartridge and the RSF1010 replicon are completely confirmed by DNA sequence analysis. The sequence data demonstrated that, as expected, the 5′ end of the synthetic polylinker was ligated at the *Rsa*I site close to the 3′ end of P_G and that the polylinker sequence downstream of the *Xho*I site was replaced by pCFM1146 sequences (Fig. 4). Downstream of P_G, seven unique restriction sites, an *Nde*I site followed by *Hpa*I, *Cla*I, *Xba*I, *Kpn*I, *Sac*I, and *Xho*I sites, can be used for insertion of genes with

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                 * *  * * *
expected   T C A C G A G T A C C A A A C A T C G A T
                                          ClaI

                 * *  * *
observed   T C A C G A C A T C G A T
                          ClaI
```

FIG. 6. Expected and observed sequences around the ligation site in pKMY319. The asterisks mark bases complementary to the 3′ end of the *Pseudomonas aeruginosa* 16S RNA and define a coding region for the putative ribosome-binding site (27).

the 5′ end lying within an *Nde*I recognition sequence. The sequence data also indicated that the *Pst*I end of the cloning cartridge was ligated, as expected, at the corresponding *Pst*I site in RSF1010 DNA starting at nucleotide 7768 (25).

Similar sequence analysis demonstrated, as expected, that the *Eco*RI end of the cloning cartridge was ligated at the corresponding *Eco*RI site in RSF1010 DNA starting at nucleotide 8676 (25). However, the same analysis revealed that the sequence from nucleotides 1 to 1653 in RSF1010 DNA (25) was completely deleted in pKMY299. The deleted region contains the entire *strA* gene and most of the *strB* gene determining streptomycin resistance (25). Restriction patterns of the pKMY286 and pKT231 DNAs suggested that this deletion occurred in plasmid pKT231 used for this study. Further analysis of pKMY286 and pKMY299 DNA with various restriction enzymes did not detect other aberrations in pKMY299. Plasmid pKMY299 can therefore be considered to be an RSF1010 plasmid with nucleotides 1 to 1653 deleted and nucleotides 7768 to 8676 replaced with a 3.6-kb cloning cartridge.

Plasmid pKMY299 was designed for cloning and expression of genes that contain or are engineered to contain an *Nde*I recognition sequence at the 5′ end. For cloning and expression of restriction fragments carrying genes of unknown sequences, the cloning cartridge in pKMY299 was modified. The sequence ATG within the *Nde*I recognition sequence in pKMY299 was removed to prevent false translational initiation in gene expression from P_G. This was achieved by digestion of pKMY299 DNA with *Nde*I and *Hpa*I, treatment with mung bean nuclease to remove the overhang, and self-ligation of the remaining plasmid DNA. The resulting plasmid was designated pKMY319. Sequence analysis confirmed the expected locations of the five remaining cloning sites in pKMY319 in the order *Cla*I, *Xba*I, *Kpn*I, *Sac*I, and *Xho*I. The same analysis revealed that in addition to the AT overhang generated by *Nde*I digestion, the mung bean nuclease removed 8 bp unexpectedly. The expected and observed sequences around the ligation site in pKMY319 are shown in Fig. 6.

One of the important bases encoding a ribosome-binding site was removed in pKMY319. A disruption of this ribosome-binding site might prove advantageous in the use of pKMY319 to express cloned fragments containing genes of unknown sequences. A Shine-Dalgarno sequence, which is normally located only a few base pairs upstream from a gene, is usually cloned on a fragment along with the gene. A second ribosome-binding site encoded by the expression vector might act to reduce expression (21).

Regulated gene expression from plasmids pKMY299 and pKMY319 in gram-negative bacteria. To evaluate the use of pKMY299 and pKMY319 as expression vectors, genes of various origins whose products can be assayed easily were cloned into the plasmids and their expression was studied. An intronless luciferase gene constructed from the firefly *P.*

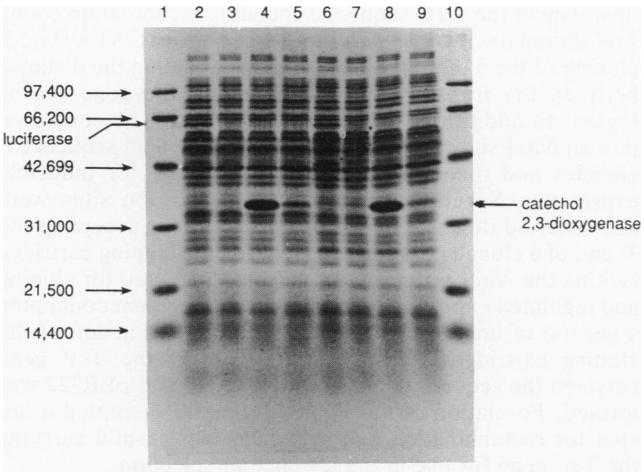

FIG. 7. SDS-PAGE analysis of protein products produced from expression vectors pKMY299 and pKMY319 in *P. putida* G572. Strains harboring plasmid pKMY519 (Y1006) or pKMY520 (Y1009) were grown in 50 ml of PAS (7) containing 0.4% glutamate or L broth (14) in the presence or absence of 0.35 mM sodium salicylate (as an inducer) at 30°C for 13 to 14 h. Cultures of *P. putida* Y1006 and Y1009 were harvested and resuspended into 5 ml of 100 mM potassium phosphate buffer (pH 8.3) containing 10% acetone and 25 mM potassium phosphate buffer (pH 7.8) containing 2 mM EDTA, respectively. The resuspended cells were sonicated, and the cell debris was pelleted by centrifugation in a Beckman (Fullerton, Calif.) JA20 rotor at 15,000 rpm for 30 min. The supernatants (crude extracts) were subject to SDS-PAGE analysis as described elsewhere (30). The gels were stained with Coomassie blue and scanned with a laser densitometer (Ultrascan XL; Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.) to determine relative levels of protein production. For both firefly luciferase and catechol 2,3-dioxygenase, similar percentages of enzyme protein (see text) were produced whether the cultures were grown in PAS medium or L broth. Lanes: 1 and 10, molecular weight standards (30); 2, Y1009 in PAS, induced; 3, Y1009 in PAS, uninduced; 4, Y1006 in PAS, induced; 5, Y1006 in PAS, uninduced; 6, Y1009 in L broth, induced; 7, Y1009 in L broth, uninduced; 8, Y1006 in L broth, induced; 9, Y1006 in L broth, uninduced.

pyralis cDNA and genomic clones (8) was reconstructed to contain an *Nde*I site at the 5′ end. The reconstructed luciferase gene was cloned into pKMY299 at the *Nde*I and *Asp*718 (*Kpn*I) sites (Materials and Methods), and the resulting plasmid, pKMY520, was introduced into *E. coli* and *P. putida* cells. Cells were grown in L broth or phosphate ammonium salts (PAS) as described in the legend to Fig. 7. Expression of the luciferase gene was analyzed in the presence or absence of sodium salicylate (0.35 mM) as an inducer in both hosts. Luciferase activity was determined by measuring the light produced in a reaction catalyzed by this enzyme (Materials and Methods). Production of luciferase protein in *P. putida* was also analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of crude cell extracts. Luciferase production in uninduced *P. putida* cells carrying pKMY520 was barely visible on the SDS-gel (Fig. 7). However, the high sensitivity of the luciferase assay (8) allowed detection of relatively high enzyme activity from uninduced *P. putida* or *E. coli* cells carrying pKMY520 (Table 1). The same assay detected an ~80- to 90-fold induction of the luciferase activity in *E. coli* and *P. putida* (Table 1). The amount of luciferase produced in induced *P. putida* cells carrying pKMY520 represented ~3.7% of the total soluble proteins, as revealed by densitometer tracing of the gel (Fig. 7). These results demonstrated regulated expression of a eukaryotic gene from pKMY299 in two different gram-negative hosts.

TABLE 1. Expression of the luciferase gene of the firefly *P. pyralis* from plasmid vector pKMY299 in *P. putida* and *E. coli*

Plasmid[a]	Host[b]	Luciferase sp act[c] (U/μg of protein)
pKMY520		
Uninduced	*P. putida* G572	4.5×10^4
Induced	*P. putida* G572	4.1×10^6
pKMY520		
Uninduced	*E. coli* JM103	1.9×10^3
Induced	*E. coli* JM103	1.5×10^5

[a] Plasmid pKMY520 is pKMY299 carrying an insert containing the firefly luciferase gene (Materials and Methods).
[b] The bacterial strains are described in Materials and Methods. All cultures were grown in L broth to an optical density at 550 nm of ≈4.5 in the presence or absence of 0.35 mM sodium salicylate (as an inducer) for the luciferase assay (Materials and Methods).
[c] For cells not carrying the luciferase gene, the background value is less than 30 U/μg of protein.

To test the use of pKMY319, restriction fragments carrying the T4MO gene cluster from *P. mendocina* KR1 (30) or the catechol 2,3-dioxygenase gene from plasmid NAH7 (31) were individually cloned into pKMY319 to generate recombinant plasmids pKMY342 and pKMY517 (Materials and Methods). Plasmid pKMY342 was introduced into a number of gram-negative bacterial species, and plasmid pKMY517 was introduced into *P. putida*. Expression of the T4MO gene cluster and the catechol 2,3-dioxygenase gene was measured in the presence or absence of sodium salicylate (0.35 mM) as an inducer. Significantly higher specific activities of both enzymes were observed from induced cultures than from uninduced cultures of all bacterial strains tested (Tables 2 and 3). These results demonstrated the wide use of pKMY319 as an expression vector in obtaining regulated

TABLE 2. Expression of the T4MO gene cluster of *P. mendocina* KR1 from plasmid vector pKMY319 in gram-negative bacteria

Bacterial strain[a]	T4MO sp act[b] (nmol/min/mg)
Aeromonas hydrophila Y21	
Uninduced	0.3
Induced	17
Enterobacter cloacae Y81	
Uninduced	0.9
Induced	23
Escherichia coli Y5250	
Uninduced	0.9
Induced	19
Klebsiella pneumoniae Y61	
Uninduced	1.3
Induced	15
Pseudomonas putida Y2511	
Uninduced	0.8
Induced	27
Pseudomonas mendocina Y4075	
Uninduced	1.2
Induced	19

[a] All bacterial strains contain recombinant plasmid pKMY342, which is pKMY319 carrying an insert containing the T4MO gene cluster from *P. mendocina* KR1 (Materials and Methods).
[b] Determined as described elsewhere (30). Values for toluene-induced and uninduced *P. mendocina* cells were 30 and 0.5 nmol/min/mg, respectively.

TABLE 3. Expression of the catechol 2,3-dioxygenase gene of plasmid NAH7 from NAH7 and plasmid vector pKMY319 in *P. putida*

Plasmid	Host[a]	Catechol 2,3-dioxygenase sp act[b] (μmol/min/mg)
NAH7		
Uninduced	*P. putida* G1343	0.02
Induced	*P. putida* G1343	0.8
pKMY517		
Uninduced	*P. putida* G572	1.7
Induced	*P. putida* G572	20

[a] Both *P. putida* G1343 and *P. putida* G572 are derivatives of wild-type *P. putida* G1 (Materials and Methods).

[b] Determined essentially as described by Sala-Trepat and Evans (19). Preparation of crude extracts for the assay is described in the legend to Fig. 7.

gene expression in gram-negative bacteria. Compared with the level of catechol 2,3-dioxygenase produced from NAH7, a 25-fold overproduction of this enzyme from pKMY517 was observed (Table 3). Production of catechol 2,3-dioxygenase protein in *P. putida* harboring plasmid pKMY517 was also analyzed by SDS-PAGE of crude cell extracts (Fig. 7). Densitometer tracing of the gel revealed that the amount of catechol 2,3-dioxygenase produced represented ~10% of the total soluble cell proteins. These results demonstrated the usefulness of pKMY319 in the overproduction of gene products.

Stability of the cloning cartridges. It was reported that a plasmid carrying the Tet^r gene of the cloning cartridges constructed in this work was not stably inherited in *P. putida* or *E. coli* in the absence of selection (1, 13). Recent evidence suggested that a short sequence within the promoter of the Tet^r gene forms a hot spot for recombination (12) and destabilizes the plasmid (13). This sequence contains a *Hin*dIII site, and disruption of the *Hin*dIII recognition sequence or the sequence in its vicinity stabilized the plasmid carrying the Tet^r gene (13). In the construction of the cloning cartridges, a nucleotide sequence upstream of the *Hin*dIII site in the Tet^r gene promoter was replaced with the *nahR* sequence (Fig. 4). The sequence substitution generated a new hybrid promoter for the Tet^r gene and stabilized the plasmid carrying either of the cloning cartridges.

The hybrid promoter allowed the use of the Tet^r gene as a selection marker in all of the bacterial strains tested (Tables 1 to 3). To test the stability of the plasmid carrying either of the cloning cartridges, *P. putida* KT2440 and G572 (Materials and Methods) harboring pKMY299 or pKMY319 were grown in L broth in the absence of tetracycline for over 50 generations. Each of the cultures was streaked on L agar for single colony formation, and 100 colonies from each culture were tested for tetracycline resistance on L agar supplemented with tetracycline (50 μg/ml). All of the colonies tested remained tetracycline resistant. This result suggested that use of the Tet^r gene in the form carried by the cloning cartridges did not lead to the elimination of the cartridges or a plasmid carrying either of the cartridges in the absence of selection.

Conclusions. A 3.6-kb restriction fragment was assembled in this study for use as a cloning cartridge. It consists of the positive regulatory gene *nahR* from plasmid NAH7, a promoter, P_G, that *nahR* regulates, a multiple cloning site, a transcription terminator, and a gene conferring tetracycline resistance (Tet^r). Within P_G, a sequence of three nucleotides upstream of the ATG sequence encoding the initiation codon was altered to create an *Nde*I recognition site (CATATG) for cloning of the 5′ end of a gene without affecting the distance between the transcription start site and the gene coding region. In addition, the 5′ end of a gene can be converted into an *Nde*I site without altering the amino acid sequence it encodes and then cloned into this cartridge for inducible expression. Several other synthetic restriction sites were also inserted downstream of the *Nde*I site for accepting the 3′ end of a cloned gene. A derivative of the cloning cartridge lacking the *Nde*I sequence was also constructed for cloning and regulated expression of a restriction fragment containing a gene(s) of unknown sequence. In the construction of the cloning cartridges, a hybrid promoter of the Tet^r gene between the sequences of plasmids NAH7 and pBR322 was formed. Formation of the hybrid promoter disrupted a hot spot for recombination and stabilized the plasmid carrying the Tet^r gene for use in the absence of selection.

On a broad-host-range replicon, the cartridges have allowed cloning and inducible expression of the luciferase gene from the firefly *P. pyralis*, the T4MO gene cluster from *P. mendocina* KR1, and the catechol 2,3-dioxygenase gene from plasmid NAH7 in all of the gram-negative bacteria tested to date. Production of catechol 2,3-dioxygenase protein to a level representing ~10% of the soluble cell protein was observed. Therefore, use of the cloning cartridges demonstrated that (i) they can be individually inserted into a replicon of choice and convert it into an expression vector and (ii) a gene cloned into the expression vector can be induced with the inexpensive inducer sodium salicylate at low concentrations in a variety of gram-negative bacteria.

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