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Molecular Cloning of the *Pseudomonas* Carboxypeptidase G_2 Gene and Its Expression in *Escherichia coli* and *Pseudomonas putida*

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The gene coding for carboxypeptidase G_2 was cloned from *Pseudomonas* sp. strain RS-16 into *Escherichia coli* W5445 by inserting *Sau*3A-generated DNA fragments into the *Bam*HI site of pBR322. The plasmid isolated, pNM1, was restriction mapped, and the position of the gene on the 5.8-megadalton insert was pinpointed by subcloning. The expression of carboxypeptidase in *E. coli* was 100-fold lower than in the *Pseudomonas* sp. strain. When the cloned gene was subcloned into the *Pseudomonas* vector pKT230 and introduced into *Pseudomonas putida* 2440, a 30-fold increase in expression over that obtained in *E. coli* was observed. High expression (up to 5% soluble protein) was obtained in *E. coli* by subcloning a 3.1-megadalton *Bgl*II fragment into the *Bam*HI site of pAT153. The increased expression was orientation dependent and is presumed to be due to transcriptional readthrough from the Tc promoter of the vector. Production of carboxypeptidase was shown to be induced (two-fold) by the presence of folic acid, and the mature protein was shown to be located in the periplasmic space of *E. coli*.

The carboxypeptidase G class of enzymes hydrolyze the C-terminal glutamate moiety from folic acid and analogs such as methotrexate (MTX), polyglutamate derivatives of folic acid, subfragments such as *p*-aminobenzoylglutamate, and specific small peptides with C-terminal glutamate residues (18).

Carboxypeptidase G_2 (CPG_2) produced by *Pseudomonas* sp. strain RS-16 differs from the original carboxypeptidase G described by Levy and Goldman (19) and from the more closely related carboxypeptidase G_1 (CPG_1) (21) in both physical and kinetic properties, but like CPG_1, has application in cancer chemotherapy. CPG_2 is a Zn^{2+}-containing dimer of 2 × 42,000 daltons and has high affinities (K_m values of 10^{-5} to 10^{-6} M) for both 5-methyltetrahydrofolate, the predomonant circulatory form of folate in mammals, and the folic acid antagonist MTX, which is widely used in cancer chemotherapy (5). The enzyme may be used directly for the plasma depletion of reduced folates, essential as cofactors in purine and particularly pyrimidine biosynthesis. CPG_2 has been shown to inhibit the development of the Walker 256 carcinoma in vivo (R. F. Sherwood, C. Wiblin, and T. Atkinson, unpublished data) in a manner similar to that of the closely related CPG_1, which inhibits both the Walker 256 and L1210 (leukemic) cancers (4). The enzyme may also be used to remove MTX from circulation in patients in whom prolonged exposure to high doses of MTX leads to toxicity (10). Animal studies have shown >70% recovery of mice given up to 1 g of MTX per kg when treated with 50 to 250 U of CPG_2 per kg h after MTX administration (R. F. Sherwood and C. Wiblin, unpublished data). The enzyme has now been successfully used in the United Kingdom in a number of patients suffering from MTX toxicity (R. F. Sherwood, unpublished data).

CPG_2 is currently produced at 400-liter fermentation scale from *Pseudomonas* sp. strain RS-16, with yields between 200 and 300 U/liter, representing <0.1% soluble protein. Conventional genetic approaches have failed to increase the levels of this enzyme in fermentations. Large quantities of enzyme are required for a full evaluation of the clinical potential of the enzyme. This report describes the primary cloning of the gene coding for CPG_2 in *Escherichia coli* and the construction of an enhanced expression vector which directs the synthesis of up to 5% of its soluble protein as CPG_2.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used were *E. coli* W5445 (*pro leu thi thr supE44 lacY*

tonA hsdR hsdM Str^r), *Pseudomonas putida* 2440 (r$^-$), and *Pseudomonas* sp. strain RS-16. The plasmids employed wee pBR322 (6), pAT153 (29), and pKT230 (1).

Media and culture conditions. *E. coli* was routinely cultured in L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl). Solidified medium (L agar) consisted of L broth with the addition of 2% (wt/vol) Bacto-Agar (Difco Laboratories). Antibiotic concentrations used for the selection of transformants were 50 μg of ampicillin per ml, 15 μg of tetracycline per ml, and 30 μg of kanamycin per ml in the case of *E. coli* and 150 μg of kanamycin per ml in the case of *P. putida*. Expression studies with *E. coli* were conducted in 2YT liquid medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl) containing 1% (wt/vol) glucose and 0.05% (wt/vol) folic acid where appropriate. Pseudomonads were grown in the minimal salts solution of Levy and Goldman (19) but with 10 g of glutamate per liter as a carbon and nitrogen source instead of MTX. The minimal medium employed for *E. coli* was M9 medium (24).

Purification of DNA. Plasmids were purified from chloramphenicol-amplified cultures (11) by Brij lysis (12) and subsequent cesium chloride-ethidium bromide density gradient centrifugation (14). A rapid, small-scale plasmid isolation technique (9) was also employed for screening purposes. Chromosomal DNA from the donor *Pseudomonas* sp. strain was prepared essentially as described by Marmur (20).

Restriction, ligation, and transformation methods. Restriction endonucleases and DNA ligase were purchased from Bethesda Research Laboratories and used in the buffers and under the conditions recommended by the supplier. Transformation of *E. coli* was performed essentially as described by Cohen et al., (13); *P. putida* was transformed by the method of Bagdasarian and Timmis (2).

Agarose gel electrophoresis. Digests were electrophoresed in 0.8% agarose slab gels (10 by 20 by 0.5 cm) on a standard vertical system (Raven) with Tris-borate-EDTA buffer (23). Electrophoresis of undigested DNA was at 125 V, 50 mA for 3 h; digested DNA was electrophoresed at 15 V, 10 mA for 16 h. Fragment sizes were estimated by comparison with fragments of λ DNA digested with *Hin*dIII and λ DNA cut with both *Hin*dIII and *Eco*RI. Fragments were isolated from gels by electroelution (22).

Determination of carboxypeptidase activity. Bacteria were grown in 1-liter batch cultures, and 100-ml samples were taken at various stages in the growth phase. Samples were cooled on ice, centrifuged at 13,000 × *g* for 10 min, resuspended, and frozen in 5 ml of 0.1 M Tris-hydrochloride (pH 7.3) containing 0.2 mM $ZnSO_4$. The cells were disrupted with an MSE Ultrasonic Disintegrator (20 kc/s, 2 amps) for three 30-s intervals on ice. Cell debris was removed by centrifugation at 10,000 × *g* for 5 min. CPG_2 activity was determined by the method of McCullough et al. (21). A 1-ml reaction cuvette containing 0.9 ml of 0.1 M Tris-hydrochloride (pH 7.3) plus 0.2 mM $ZnSO_4$ and 0.1 ml of 0.6 mM MTX was equilibrated at 37°C. Enzyme extract was added to the test cuvette, and the decrease in absorbance at 320 nm was measured with a Pye-Unicam SP1800 double-beam spectrophotometer. Enzyme activity per milliliter of extract was calculated by using an extinction coefficient for MTX of 8,300. One unit represents the hydrolysis of 1 μmol of MTX per min at 37°C. Protein concentration was determined by the method of Bradford (7).

Cell fractionation techniques. Bacterial cultures were grown in the low phosphate medium of Neu and Heppel (26), supplemented with 100 μg of ampicillin per ml, to an optical density at 450 nm of 1.0. A 40-ml amount of culture was centrifuged at 5,000 × *g* for 10 min, washed in 5 ml of 10 mM Tris-hydrochloride (pH 7.0), and suspended in 0.9 ml of 0.58 M sucrose–0.2 mM dithiothreitol–30 mM Tris-hydrochloride (pH 8.0). Conversion to spheroplasts was achieved by the addition of 20 μl of lysozyme (2 mg/ml) and 40 μl 0.1 M EDTA and incubation at 23°C for 10 min (27). The spheroplasts were placed on ice, and 0.1 ml of 30% (wt/vol) bovine serum albumin added, followed by 5 ml of sucrose-Tris buffer. Sedimentation of the spheroplasts was achieved by centrifugation at 5,000 × *g* for 10 min, and the supernatant was retained as the periplasmic fraction. The pellet was suspended in 5 ml of 10 mM Tris-hydrochloride–0.2 mM dithiothreitol (pH 7.0) and sonicated at 20 kc/s, 2 amps for 15 s. Remaining whole cells were removed by centrifugation at 1,000 × *g* for 10 min. Centrifugation at 100,000 × *g* for 1 h at 4°C separated the soluble (cytoplasmic) proteins from the particulate (membrane-bound) proteins. The membrane pellet was suspended in 1 ml of 10 mM Tris-hydrochloride–0.2 mM dithiothreitol (pH 7.0).

CPG_2 was assayed as described above. Alkaline phosphatase was assayed by the method of Miller (24), NADH oxidase was assayed by the method of Osborn et al. (27), and glyceraldehyde-3-phosphate dehydrogenase was assayed by the method of Suzuki and Harris (28).

RESULTS

To isolate the gene for CPG_2, chromosomal DNA prepared from the *Pseudomonas* host was partially digested with *Sau*3A, and fragments of between 6 and 8 megadaltons (Md) were isolated from agarose gels by electroelution. The sized DNA was ligated with alkaline phosphatase-treated, *Bam*HI-cut pBR322 and transformed into *E. coli* W5445, and Ap^r transformants were selected. Of the 3,500 Ap^r colonies obtained, approximately 70% were Tc^s. Utilization of a rapid plasmid isolation technique on 50 Ap^r Tc^s transformants demonstrated that >90% of the gene bank harbored plasmids of the expected size. As a further check on the authenticity of the gene bank, the individual clones were screened for the acquisition of a Leu$^+$ phenotype. Two such clones were identified. Both carried a plasmid capable of transforming *leuB* (β-isopropylmalate dehydrogenase) *E. coli* mutants to prototrophy.

Acquisition of a functional CPG_2 gene should enable *E. coli* to utilize folic acid as a carbon source. The 2,400 gene bank clones were screened for the ability to grow on minimal medium containing folate as the sole source of carbon (i.e., Fol$^+$). A single Fol$^+$ clone was

detected and shown to harbor a plasmid capable of transforming plasmid-minus W5445 to the Fol^+ phenotype. Classical restriction mapping of this plasmid (pNM1) was undertaken (Fig. 1), which revealed the presence of a 5.8-Md insert of pseudomonad DNA within pBR322.

Subcloning. The position of the CPG_2 gene within the 5.8-Md insert was pinpointed by subcloning of various restriction enzyme fragments into pBR322 (Fig. 2). A functional CPG_2 gene was shown to be present on a 3.1-Md *Bgl*II fragment of the pNM1 insert but did not occur on *Xho*I or *Sph*I fragments. The *Bgl*II fragment was cloned into the *Bam*HI site of pBR322 to give pNM11. A further reduction in the size of pNM11 was achieved by digesting with *Sal*I and religating the resultant three fragments. All the Fol^+ deletion plasmids obtained had lost a 1.41-Md *Sal*I fragment but retained the 0.95-Md *Sal*I fragment (i.e., pNM111). In addition, plasmids in which the smaller 0.95-Md *Sal*I fragment had become inserted in the opposite orientation to the parent plasmid (pNM11) were Fol^-. Taken together, these subcloning results indicate that the CPG_2 gene lies between the *Bgl*II site at 4.14 and the *Sal*I site at 6.03 on pNM1. Furthermore, the gene contains *Sph*I (5.17), a *Sal*I (5.07), and at least one *Xho*I (4.56 and/or 5.56) site. The exact position of the gene is currently being determined by DNA sequencing.

Orientation of the cloned gene. The observation that the CPG_2 gene resided on a *Bgl*II fragment of pNM1 allowed the isolation of recombinant plasmids carrying the inserted DNA in both orientations. The two plasmids, pNM21 and pNM22 (Fig. 3a), both transformed *E. coli* to Fol^+, indicating that a pseudomonad promoter was present on the fragment. However, cells carrying plasmid pNM21, in which the *Bgl*II fragment was cloned in the opposite orientation to pNM1, exhibited more rapid growth with folic acid as the sole carbon source. This difference was clearly visible on agar medium, where colonies developed concentric yellow halos of precipitated pteroic acid, the insoluble product of folate hydrolysis.

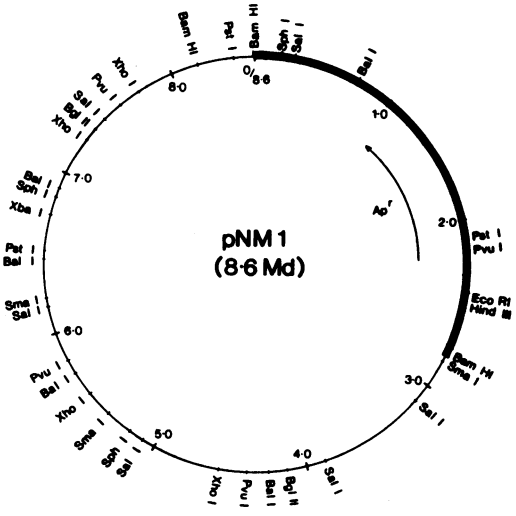

FIG. 1. Restriction map of pNM1. The heavy line represents pBR322-derived DNA.

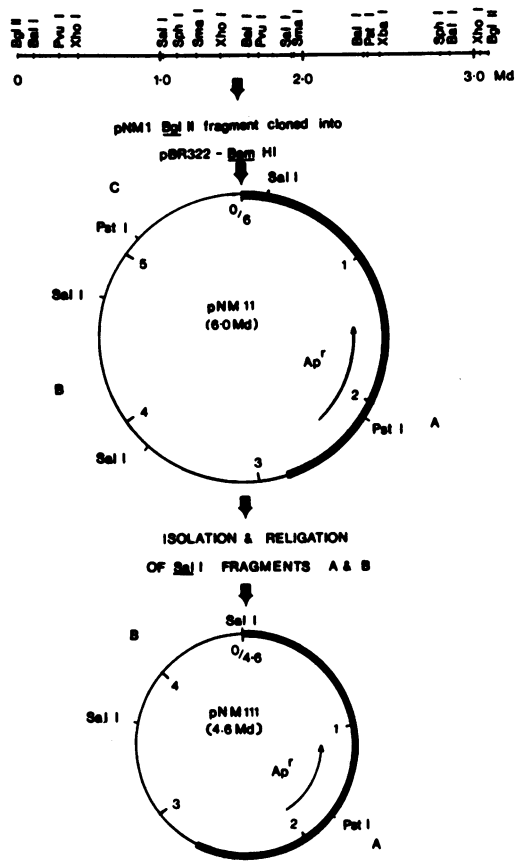

FIG. 2. Derivation of the Fol^+ subclones pNM11 and pNM111. The 3.1-Md *Bgl*II fragment of pNM1 was cloned into the *Bam*HI site of pBR322 to give pNM11. For simplicity the asymmetric *Pst*I site of the *Bgl*II fragment has been included on the pNM111 map to indicate the orientation of the insert. Plasmid pNM111 was derived from pNM11 essentially by deleting a 1.46-Md *Sal*I fragment (B). The heavy line represents pBR322.

Confirmation that pNM21 gave enhanced expression of CPG_2 over pNM22 was obtained by assaying enzyme production during batch growth of cells containing either plasmid (Table 1). The expression of CPG_2 from the plasmids pNM22 and pNM1 was 2.5 U/liter of culture, representing 0.01% soluble protein. In contrast, expression from pNM21 was 3,000 to 3,500 U/liter of culture, which represented 4.7% soluble protein. As the cloned gene is inserted into the *Bam*HI site of pAT153, the observed higher

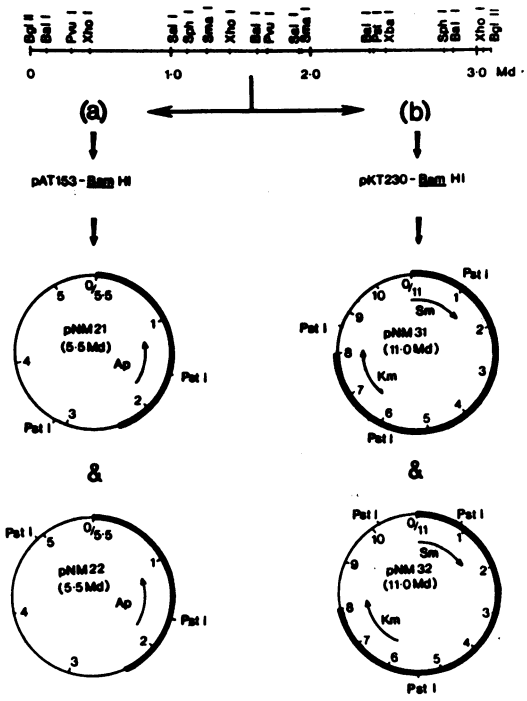

FIG. 3. Construction of the Fol$^+$ plasmids pNM21, pNM22, pNM31, and pNM32. The plasmids were obtained by cloning the 3.1-Md *Bgl*II fragment of pNM1, in both orientations, into the *Bam*HI site of (a) pAT153 to give pNM21 and pM21 and (b) pKT230 to give pNM31 and pNM32. The orientation of the *Bgl*II fragment within these vectors is indicated by the position of the asymmetric *Pst*I site. The heavy line represents (a) pAT153 and (b) pKT230.

expression of pNM21 is almost certainly due to transcriptional readthrough from the Tc promoter of pAT153. The low expression of CPG_2 carried on plasmids pNM1 and pNM22 is consistent with the view that *Pseudomonas* promoters function poorly in *E. coli* (2, 8, 16, 17). In the presence of folic acid there was a consistent twofold increase in the specific activity of enzyme measured in cell sonicates. However, even in the presence of inducer, the levels of CPG_2 produced from its own promoter in *E. coli* were only 1% of the level in the *Pseudomonas* sp. strain.

Expression of the cloned gene in *P. putida*. The observation that the CPG_2 gene was expressed in *E. coli* regardless of the orientation of the gene within the vector suggested that the promoter region of the CPG_2 gene had been cloned with the structural gene. The low expression of CPG_2 within *E. coli* from its natural promoter (pNM1, pNM22, pNM111) confirmed other findings that *Pseudomonas* promoters are poorly recognized by *E. coli* RNA polymerases (2, 8, 16, 17). It would be expected that if the gene were introduced back into a pseudomonad cellular environment, then improved expression from the *Pseudomonas* promoter would result. The 3.1-Md *Bgl*II fragment was therefore subcloned into the *Pseudomonas* cloning vector pKT230 at its single *Bam*HI site. Two plasmids were obtained, pNM31 and pNM32 (Fig. 3b), representing the two possible orientations of the cloned gene. These plasmids were transformed into *P. putida* 2440 by the method of Bagdasarian and Timmis (1). Pseudomonad cells carrying both plasmids were cultured in minimal salts medium, and enzyme production was monitored.

Yields of 500 to 1,000 U/liter of culture were obtained regardless of gene orientation within the plasmid. Specific activity of the enzyme in cell sonicates was 1.5 to 4.0 U/mg of protein, representing 0.3 to 0.7% soluble protein compared with <0.05% soluble protein in donor strain RS-16. In contrast, *E. coli* W5445 cultures carrying either plasmid yielded 12 to 40 U of enzyme per liter at a specific activity of <0.07 U/mg (<0.01% soluble protein).

Periplasmic localization of CPG_2. There is evidence that CPG_2 is located in or near the periplasmic space of *Pseudomonas* sp. strain RS-16. Pteroic acid, the product of CPG_2 hydrolysis of folic acid, is extremely insoluble and is found predominantly outside the cell in both liquid and solid media. Exogeneous pteroic acid is also seen in *E. coli* cultures containing the cloned gene when folic acid is present in the medium. This is clearly demonstrated by the halo of precipitated pteroic acid observed around colonies carrying plasmids in which expression of CPG_2 is from Tc promoter of pBR322 (e.g., pNM21).

The localization of CPG_2 produced by *E. coli* cells carrying pNM21 was examined by the

TABLE 1. CPG_2 by *E. coli* W5545 containing plasmids pNM1, pNM21, and pNM22[a]

Culture age (h)	CPG_2 sp act (U/mg of soluble protein)					
	pNM1		pNM22		pNM21	
	Fol$^-$	Fol$^+$	Fol$^-$	Fol$^+$	Fol$^-$	Fol$^+$
3	0.008	0.005	0.010	0.019	13.9	23.3
4	0.009	0.011	0.015	0.013	12.3	26.9
5	0.007	0.019	0.016	0.016	11.5	25.6
6	0.005	0.024	0.014	0.023	13.7	24.1
7	0.015	0.029	0.024	0.043	13.2	20.6
8	0.013	0.028	0.024	0.046	13.0	23.6

[a] Cells were grown in 2YT medium supplemented with 1% (wt/vol) glucose and where appropriate 0.05% (wt/vol) folic acid. The generation time was 56 to 66 min. Culture samples were disrupted by sonication and centrifuged at 15,000 × *g*, and the enzyme activity in the supernatant was determined. The original plasmid isolate is pNM1; pNM21 and pNM22 are two subclones composed of pAT153 and a *Bgl*II 3.1-Md subfragment from pNM1, cloned in either orientation at the *Bam*HI site of pAT153.

separation of cellular proteins into cytoplasmic, periplasmic, and whole membrane fractions. As a control, levels of three marker enzymes, alkaline phosphatase (periplasmic), glyceraldehyde-3-phosphate dehydrogenase (cytoplasmic), and NADH · O_2 oxidoreductase (membrane bound), were also determined. Table 2 shows that 97% of the CPG_2 activity occurs in the periplasm, equivalent to the marker periplasmic enzyme alkaline phosphatase.

CPG_2 synthesized in *E. coli*. The specific activity of CPG_2 in crude cell extracts of *E. coli* carrying pNM21 was 50-fold higher than equivalent extracts from *Pseudomonas* sp. strain RS-16. To determine whether the cloned gene product in *E. coli* had the same properties as CPG_2 from the pseudomonad, enzyme was purified from *E. coli* carrying pNM21 by a modification of the method of Baird and co-workers (3). The specific activity of homogeneous CPG_2 from *E. coli* was 535 U/mg of protein, which compares to 550 U/mg of protein from strain RS-16. CPG_2 purified from *E. coli* clone pNM21 coelectrophoresed with CPG_2 from *Pseudomonas* sp. strain RS-16 at a subunit molecular weight value of 42,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. K_m values determined by using MTX as substrate were 7.4×10^{-6} and 8.0×10^{-6} M, respectively, for the enzyme from pNM21 and RS-16. Antiserum raised against the *Pseudomonas* enzyme indicated immunological identity between *E. coli* and *Pseudomonas* CPG_2, as a confluent precipitation line was formed on Ouchterlony double diffusion analysis (Fig. 4).

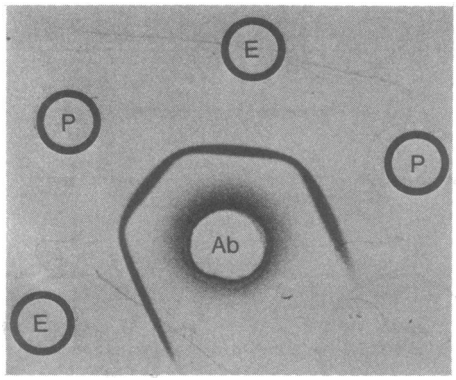

FIG. 4. Double diffusion Ouchterlony assay of the *Pseudomonas* and cloned CPG_2. Samples were located in a 1% (wt/vol) agarose gel in barbitone buffer and incubated at room temperature in a humid atmosphere for 20 h. The gel was washed in saline, dried, and stained with Coomassie blue R250. The centre well (Ab) contains rabbit anti-CPG_2 serum, whereas the outer wells contain purified (single-band sodium dodecyl sulfate-polyacrylamide gel electrophoresis) CPG_2 derived either from *Pseudomonas* sp. strain RS-16 (P) or *E. coli* W5445 carrying pNM21 (E). CPG_2 antibody was prepared by injecting 3 mg of purified enzyme in incomplete Freunds adjuvant into a rabbit on day 1. A repeat dose was given on day 14, and the rabbit was bled on day 28. After removal of clot, the serum was used at a 1:2 dilution.

DISCUSSION

The recombinant CPG_2 plasmid (pNM1) isolated from the gene bank directed the synthesis of low levels of the enzyme (100-fold lower than the *Pseudomonas* sp. strain), confirming other findings that *Pseudomonas* genes are poorly expressed in *E. coli* (2, 8, 15–17). However, when a 3.1-Md *Bgl*II subfragment of pNM1 carrying the gene was cloned into the *Bam*HI site of pAT153, high levels of gene expression, up to 5% of the soluble protein, were found. This was dependent on the orientation of the fragment and hence the direction of transcription. High expression was probably determined by readthrough from the Tc promoter of pAT153. A similar finding has been reported by Inouye et al. (17), who found that readthrough from the Tc promoter of pBR322 caused a 100-fold increase in the expression of the pseudomonad-derived catechol 2,3-oxygenase (*xylE* gene) also cloned at the *Bam*HI site. Orientation-dependent high expression has also been found with other cloned *Pseudomonas* genes (2, 15, 16). The effect of orientation relative to the Tc promoter of pAT153 on CPG_2 expression indicates that the direction of transcription of the gene proceeds clockwise on the pNM21 map.

TABLE 2. Localization of CPG_2 in *E. coli* carrying pNM21[a]

Fraction	% Enzyme activity[b]			
	CPG_2	AP	GAPDH	NADHOX
Periplasmic	97.0	97.1	6.8	0.25
Cytoplasmic	2.6	2.3	93	8.4
Membrane bound	0.4	0.6	0.2	89.1

[a] Localization experiments were performed as described in the text employing the spheroplast technique of Neu and Heppel (25).

[b] AP, Alkaline phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NADHOX, NADH · O_2 oxidoreductase.

The observation that expression of the CPG_2 gene also occurred in the reverse orientation within the vector suggested that the CPG_2 promotor had also been cloned. This was substantiated by cloning the 3.1-Md *Bgl*II fragment into the *Pseudomonas-E. coli* shuttle vector, pKT230. Enzyme levels of CPG_2 in *P. putida* 2440 were 500 to 1,000 U/liter of culture regardless of the orientation of the gene within pKT230, and expression from the CPG_2 promoter, based on enzyme yield, was 30-fold higher in *P. putida* than in *E. coli*. The presence of the

CPG_2 regulatory region on the 3.1-Md *Bgl*II fragment was also indicated by the apparent twofold induction of CPG_2 production in *E. coli* in the presence of folic acid. A similar low level of induction of a pseudomonad gene cloned in *E. coli* has been described for one of the TOL enzymes, benzylalcohol dehydrogenase, from *P. putida* (17).

The DNA sequence of the CPG_2 gene is currently being determined. The task has been aided by the reduction in size of the DNA insert carrying the gene (i.e., from 5.8 Md in pNM1 to 1.9 Md in pNM111), the demonstration that the gene contains *Sph*I, *Sal*I, and *Xho*I sites, and the deduced direction of transcription of the gene (i.e., counterclockwise on the pNM111 map, as the insert is in the opposite orientation to pNM21). The location of CPG_2 in the *Pseudomonas* sp. strain has not been determined, as the production of spheroplasts of this strain is difficult due to its relative insensitivity to lysozyme. In *E. coli* the cloned enzyme was clearly shown to reside in the periplasmic space, indicating that the DNA sequence of the gene encodes a signal peptide at the N-terminal region of the protein. Sequencing of the CPG_2 promoter region may help to answer the question as to why *Pseudomonas* genes in general exhibit a low level of expression in *E. coli*.

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