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High pressure conditions stimulate expression of chloramphenicol acetyltransferase regulated by the *lac* promoter in *Escherichia coli*

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Abstract: Recombinant plasmids with the chloramphenicol acetyltransferase (CAT) structural gene behind several kinds of promoters were tested for expression in *Escherichia coli* during growth at atmospheric pressure (0.1 MPa) and at high pressure (30 MPa). Expression of the CAT gene from the *lac* promoter was remarkably activated (approx. 78-fold) by high pressure in the absence of the inducer isopropyl-β-D-thiogalactopyranoside (IPTG). The stimulation of the CAT activity by the *lac* promoter at high pressure did not simply result from an increased plasmid copy number, because the CAT activities from the other promoters and β-lactamase activities were unaffected at high pressure.

Key words: Escherichia coli; *lac* Promoter; High pressure; Chloramphenicol acetyltransferase; Gene expression

Introduction

Regulation of gene expression has been studied for many years, yet few studies have been published showing regulation by pressure. Welch et al. [1] reported that several heat shock proteins in *Escherichia coli* were induced at high pressure, and these authors suggested that temperature-regulated genes in *E. coli* may also be controlled by pressure. Barophilic bacteria isolated from the deep-sea also showed gene expression regulated by high pressure [2–4].

We are investigating the molecular mechanisms of gene regulation in microorganisms isolated from deep-sea samples to determine how they have become adapted to high hydrostatic pressure conditions. In this study, as a simple model to examine these mechanisms, we have tested the relationship between gene expression and pressure conditions using the *E. coli* system.

In this paper, the chloramphenicol acetyltranserase (CAT) gene was used as a reporter gene in *E. coli*, and the relationship between gene expression and high pressure condition was

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examined using various promoters to express the CAT gene.

Materials and Methods

Bacterial strains and plasmids

E. coli JM109 (*rec*A1 *sup*E44 *end*A1 *hsd*R17 *gyr*A96 *rel*A1 *thi* Δ(*lac-pro*AB) F'[*tra*D36 *pro*AB$^+$ *lac*I^q*lac*ZΔM15]) was used as host [illegible] the plasmids [5]. The plasmids pCM4 and pCM[illegible] (Pharmacia Co.) were used as the source of [illegible] promoterless CAT structural gene [6]. The pl[illegible] mids pBR322 containing the tetracycline (*tet*) promoter and the β-lactamase (*amp*) promot[illegible] [7], pUC13 containing the *lac* promoter [5], an[illegible] pKK223–3 containing the *tac* promoter [8] we[illegible] used to place the CAT gene downstream of t[illegible]

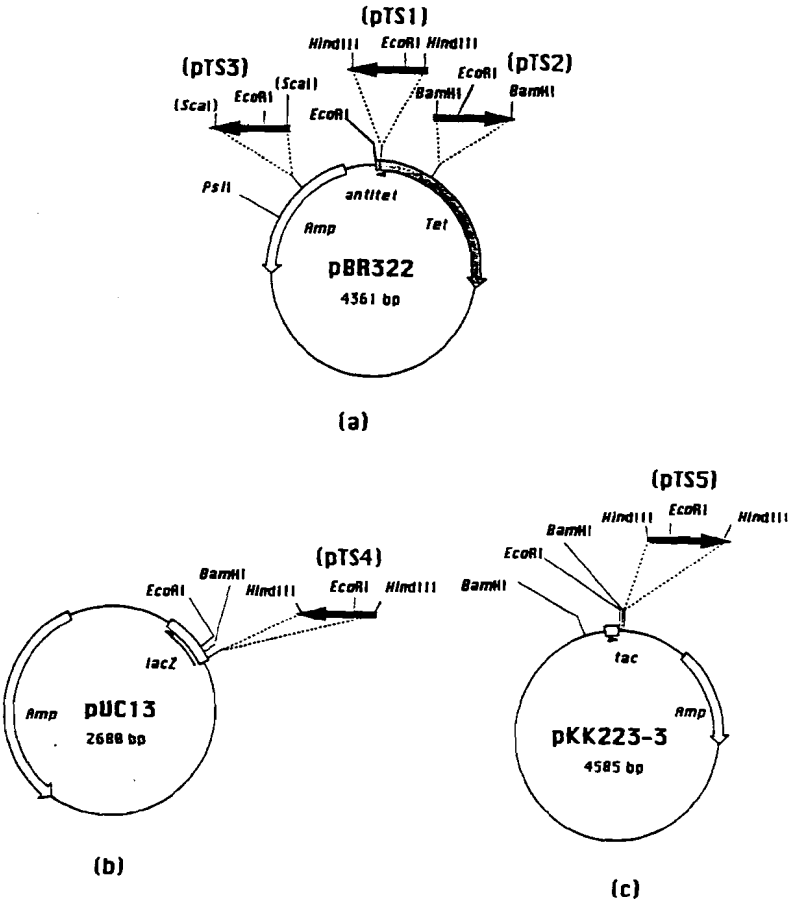
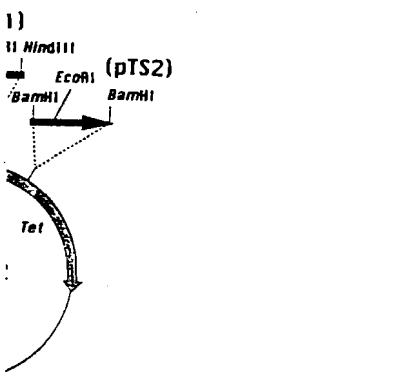
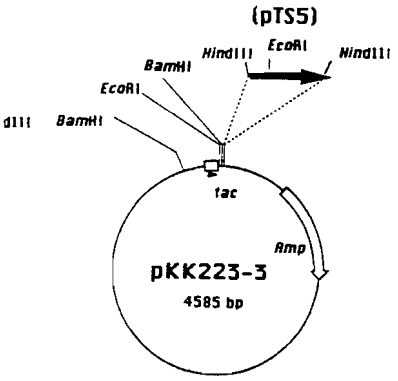


Fig. 1. A circular map of pBR322 (a), pUC13 (b), and pKK223–3 (c) showing the positions of various CAT cartridge (0.8 kb) inserts. The thin line represents the vectors and white and grey arrow boxes show ampicillin resistance gene (*Amp*) and tetracycline resistance gene (*Tet*), respectively, and their directions of transcription. The small white box and arrow heads represent the promoter regions of the vectors, and the black arrow box represents the CAT gene (CAT) cartridge.

Fig. 2. Growt[illegible]

*pro*AB$^+$ *lac*I^q*lac*ZΔM15]) was used as host for the plasmids [5]. The plasmids pCM4 and pCM7 (Pharmacia Co.) were used as the source of the promoterless CAT structural gene [6]. The plasmids pBR322 containing the tetracycline (*tet*) promoter and the β-lactamase (*amp*) promoter [7], pUC13 containing the *lac* promoter [5], and pKK223-3 containing the *tac* promoter [8] were used to place the CAT gene downstream of the respective promoter. Also, the plasmids pBR329 [9] and pACYC184 [10] containing the CAT gene expressed by the anti-tetracycline (*antitet*) and origi CAT (*cat*) promoters from Tn9, respectively, were used.





(c)

) showing the positions of various CAT cartridge (0.8 kb) inserts. oxes show ampicillin resistance gene (*Amp*) and tetracycline cription. The small white box and arrow heads represent the box represents the CAT gene (CAT) cartridge.

Construction of the recombinant plasmids

Preparation of plasmid DNA and recombinant DNA work were performed as described by Sambrook et al. [11]. Recombinant plasmids containing the CAT gene downstream of promoter sequences were constructed as shown in Fig. 1. CAT cartridges were purified from pCM7 digest with *Hin*dIII, pCM4 digested with *Bam*HI, and lled-in *Hin*dIII CAT-cartridge by using Gene Clean Kit (Bio101, Co.). The plasmids pBR322 digested with *Hin*dIII, *Bam*HI, or *Sca*I (blunt end), pUC13 digested with *Hin*dIII, and pKK223-3 digested with *Hin*dIII were dephosphorylated and ligated with appropriate CAT cartridges. The recombinant plasmids, pTS1 and pTS2 constructed from pBR322 and *Hin*dIII CAT-cartridge (counterclockwise, downstream of *antitet*-promoter) and *Bam*HI CAT-cartridge (clockwise, downstream of *tet*-promoter), respectively, pTS3 from pBR322 and blunted CAT-cartridge (counterclockwise, downstream of *amp*-promoter), pTS4 from pUC13 and *Hin*dIII CAT-cartridge (counterclockwise, downstream of *lac*-promoter), and pTS5 from pKK223-3 and *Hin*dIII CAT-cartridge (clockwise, downstream of *tac*-promoter) were obtained after selecting for chloramphenicol-resistant transformants in *E. coli* JM109.

Expression study of the CAT gene

E. coli, carrying the recombinant plasmid, was cultivated in LB medium with oxygen-saturated fruorinert (25% volume of medium) and suitable antibiotic (ampicillin; 50 μg ml^{-1}, kanamycin; 50 μg ml^{-1}, tetracycline; 25 μg ml^{-1}, or chloramphenicol; 20 μg ml^{-1}) for 14 h at 37°C at atmospheric pressure (0.1 MPa) and at high pressure (30 MPa) using a pressure vessel (titanium; Rigosha Co., Tokyo). A 0.1% (v/v) inoculum of each overnight culture was transferred to fresh medium in polypropylene tubes (5 ml sterilized Kraio-tube) at 0°C, the tubes were sealed with parafilm and then cell incubation was started at 37°C immediately at each pressure. After cultivation, the cells were collected by centrifugation (8000 rpm for 15 min), washed with 1 M KCl, 0.1 M Tris · HCl, pH 7.8 buffer, suspended in 0.1 M Tris · HCl, pH 7.8 buffer, and sonicated at 0°C. Supernatants were prepared by centrifugation (14000 rpm for 5 min in 1.5 ml Eppendorf tubes).

Assay of protein concentration, CAT activity, and SDS-polyacrylamide gel electrophoresis

Protein concentration in crude extracts was determined using the Protein Assay Kit (Bio Rad, Co.) with bovine serum albumin (BSA) as the

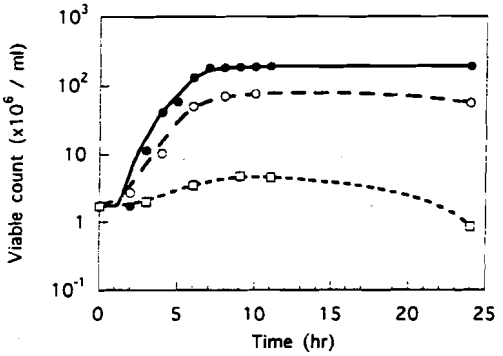


Fig. 2. Growth of strain JM109 in LB medium at several pressures. Viable cell counts were measured by determination of colony forming number. *E. coli* cells were grown at atmospheric pressure (●), 30 MPa (○), and 50 MPa (□).

protein standard. CAT activity was assayed by standard methods [12]. One unit of CAT activity was defined as 1 nmol 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) reduced per min at 37°C [13]. β-Lactamase activity was assayed by the procedure of Sawai et al. [14]. SDS-PAGE was carried out by the method of Laemmli [15]. 12% Ready Gel (Bio Rad. Co.) and Low Molecular Weight (LMW) Calibration Kit (Pharmacia. Co.) were used as protein standard markers.

Results and Discussion

Effect of pressure on growth of the host strain E. coli JM109

When the host strain *E. coli* JM109 was grown at atmospheric pressure and high pressure, almost no effect on growth was detected until 30 MPa pressure. No increase of cell numbers occurred at 50 MPa as shown in Fig. 2, although the optical density increased under this condition. The *E. coli* JM109 cells were able to divide under a pressure of 30 MPa with a doubling time of around 1.08 h, as compared with 0.86 h at atmospheric pressure (0.1 MPa). When *E. coli* cells were incubated at 50 MPa the increase in optical density at 660 nm was due to cell elongation, and not cell division [16]. Because 50 MPa caused severe inhibition of cell growth and was likely to change levels of gene expression as an indirect result of this pronounced effect, we decided to study expression the CAT gene at lesser pressures. The 30 MPa pressure condition was used for growth of the *E. coli* transformants.

When *E. coli* JM109 carrying plasmid was cultivated at high pressure, the copy number of the plasmid per cell showed a tendency to increase. The extent of increase with plasmids such as pBR322 or pUC vectors at 30 MPa was two-three-fold as detected by a simple plasmid preparation method [17]. The quantity of plasmid DNA increased almost directly in proportion to the specific activity of expressed β-lactamase encoded on the plasmid.

Effect of pressure on gene expression directed by the lac-promoter

The recombinant plasmid pTS4 carrying a CAT cartridge behind the *lac* promoter of vector pUC13 (Fig. 1b) was introduced into *E. coli* JM109. A transformant was cultivated at atmospheric pressure (0.1 MPa) and at 30 MPa, and CAT activity and β-lactamase activity were assayed. As shown in Table 1, CAT activity in cells grown at high pressure without IPTG was tremendously increased (about 78-fold) compared with the level expressed at atmospheric pressure, while β-lactamase activity increased only 2.7-fold at high pressure. The extent of increase in CAT activity was almost equal to that obtained by addition of IPTG which acts as a gratuitous inducer of the *lac*-promoter at atmospheric pressure.

The cell extracts from *E. coli* JM109 carrying pTS4 grown under these conditions were applied to a 12% SDS-polyacrylamide gel and subjected to electrophoresis. As shown in Fig. 3, a 25 kDa protein corresponding to the CAT protein was expressed very strongly at high pressure in the absence of IPTG (lane 2). A band of identical mobility appears at atmospheric pressure in the presence of IPTG (lane 3).

Expression of CAT activity encoded by other recombinant plasmids in E. coli grown at atmospheric pressure and high pressure

As shown in Table 2, the CAT activities encoded by other recombinant plasmids (Fig. 1a,c) and expressed from various promoters were mea-

Table 1

Comparison of CAT activity encoded by the plasmid pTS4 expressed in *E. coli* JM109 grown at 0.1 MPa and 30 MPa

Plasmid	Gene	Enzyme activity (U/mg)		Ratio [a]
		0.1 MPa	30 MPa	
pTS4 (−IPTG [b])	*lac*-CAT	64.3	4992	77.6
	β-lactamase	68.6	185	2.7
pTS4 (+IPTG)	*lac*-CAT	6327	15474	2.4
	β-lactamase	75.5	209	2.7

[a] Ratio shows the ratio of the specific enzyme activity (unit/mg of protein) at 30 MPa compared with 0.1 MPa.
[b] −IPTG: no IPTG added; +IPTG: 1 mM IPTG was added.

Fig. 3. Gel electrophor 1–4, protein carrying the (lane 1) and without IPT (phosphoryl. kDa. ...on

Table 2

Comparison E. coli JM10

Plasmid	
pTS	
pTS2	
pTS3	
pTS5 (−IPTG)	
pTS5 (+IPTG)	
pBR	
pACYC184	T

study expression the CAT gene at lesser pressures. The 30 MPa pressure condition was used for growth of the *E. coli* transformants.

When *E. coli* JM109 carrying plasmid was cultivated at high pressure, the copy number of the plasmid per cell showed a tendency to increase. The extent of increase with plasmids such as pBR322 or pUC vectors at 30 MPa was two- to three-fold as detected by a simple plasmid preparation method [17]. The quantity of plasmid DNA increased almost directly in proportion to the specific activity of expressed β-lactamase encoded on the plasmid.

Effect of pressure on gene expression directed by the lac-promoter

The recombinant plasmid pTS4 carrying a CAT cartridge behind the *lac* promoter of vector pUC13 (Fig. 1b) was introduced into *E. coli* JM109. A transformant was cultivated at atmospheric pressure (0.1 MPa) and at 30 MPa, and CAT activity and β-lactamase activity were assayed. As shown in Table 1, CAT activity in cells grown at high pressure without IPTG was tremendously increased (about 78-fold) compared with the level expressed at atmospheric pressure, while β-lactamase activity increased only 2.7-fold at high pressure. The extent of increase in CAT activity was almost equal to that obtained by addition of IPTG which acts as a gratuitous inducer of the *lac*-promoter at atmospheric pressure.

The cell extracts from *E. coli* JM109 carrying pTS4 grown under these conditions were applied to a 12% SDS-polyacrylamide gel and subjected to electrophoresis. As shown in Fig. 3, a 25 kDa protein corresponding to the CAT protein was expressed very strongly at high pressure in the absence of IPTG (lane 2). A band of identical mobility appears at atmospheric pressure in the presence of IPTG (lane 3).

Expression of CAT activity encoded by other recombinant plasmids in E. coli grown at atmospheric pressure and high pressure

As shown in Table 2, the CAT activities encoded by other recombinant plasmids (Fig. 1a,c) and expressed from various promoters were measured in *E. coli* JM109 grown at 0.1 MPa and 30 MPa. The increase in activity of β-lactamase encoded on the plasmid was shown to be around 1.5–3.0 at high pressure due to the increased plasmid copy number in *E. coli* at high pressure. CAT activities from *E. coli* carrying the plasmids pTS1, pTS2, pTS5 in the presence and absence of IPTG, and control plasmids pBR329 showed almost the same level of increase at high pressure as β-lactamase activities. CAT activities from the plasmid pTS3 and another control plasmid pACYC184 also showed no substantial effects resulting from the change in pressure. These results show that gene expression directed by the promoters encoded on the vector pBR322, and *tac* promoter encoded on the expression vector pKK223-3 is not affected at increased pressure, until a condition of 30 MPa is reached.

Our present study clearly shows that pressure activates the *lac* promoter region, and gene expression controlled by this promoter region was induced by high pressure. This is the first evidence of an increase of gene expression directed by the *lac* promoter in *E. coli* at high pressure. More detailed studies of mechanisms responsible for increased gene expression under high pressure are now in progress.

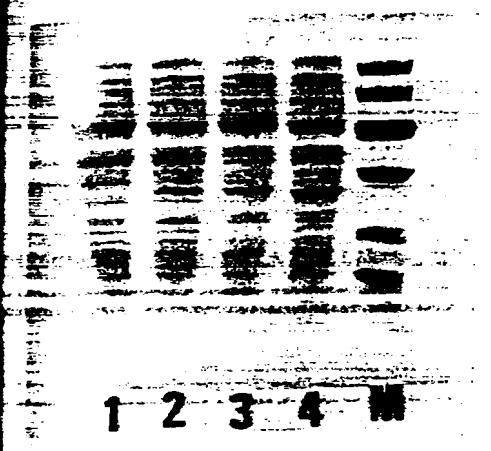


Fig. 3. Gel electrophoresis of bacterial proteins. Samples were electrophoresed on a 12% polyacrylamide gel with SDS. Lanes 1–4, proteins (15 mg) from the crude extract of *E. coli* JM109 carrying the plasmid pTS4 grown at 0.1 MPa without IPTG (lane 1) and with 1 mM IPTG (lane 2), and grown at 30 MPa without IPTG (lane 3) and with IPTG (lane 4). Lane M, LMW (phosphorylase b: 94 kDa, albumin: 67 kDa, ovalbumin: 43 kDa, [illegible]onic anhydrase: 30 kDa, trypsin inhibitor: 20.1 kDa, α-lactalbumin: 14.4 kDa).

Table 2

Comparison of CAT activity encoded by various plasmids in *E. coli* JM109 grown at 0.1 MPa and 30 Mpa.

Plasmid	Gene	Enzyme activity (U/mg)		Ratio
		0.1 MPa	30 MPa	
pTS1	*antitet*-CAT	158	272	1.7
	β-lactamase	14.4	26.0	1.8
pTS2	*tet*-CAT	201	512	2.5
	β-lactamase	30.9	93.8	3.0
pTS3	*amp*-CAT	183	231	1.3
pTS5	*tac*-CAT	438	716	1.6
(−IPTG)	β-lactamase	9.9	14.4	1.5
pTS5	*tac*-CAT	4649	15094	3.2
(+IPTG)	β-lactamase	15.9	43.0	2.7
pBR[illegible]	*antitet*-CAT	645	1481	2.3
	β-lactamase	8.3	21.6	2.6
pACYC184	Tn9-CAT	8576	11564	1.3

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