

## Expression of *cho* and *melC* operons by a *Streptococcus thermophilus* synthetic promoter in *Escherichia coli*

**Abstract** A 63-base-pair synthetic promoter, sP1, was synthesized on the basis of the nucleotide sequence of a putative *Streptococcus thermophilus* promoter. When inserted upstream from the *Streptomyces cho* operon in a recombinant plasmid, pUCO195P-36, sP1 activated the expression of the *cho* genes in *Escherichia coli*, as shown by the production of cholesterol oxidase by the transformants. The sP1-driven cholesterol oxidase production in pUCO195P-36-transformed cells was estimated to be 40% of that produced by  $P_{lac}$ -mediated *cho* expression in a pUCO193-containing host. The recombinant pUCO195P-36 appeared to be segregationally less stable in *E. coli* DH5 $\alpha$  than in HB101. Its non-expressing counterpart, pUCO195P-1, was stable in both *E. coli* strains. The activity of sP1 was further demonstrated in *E. coli* by the expression of a *Streptomyces melC* operon. When placed upstream from the test operon in the pMCU22aPa construct, sP1 activated the *melC* expression as shown by the production of tyrosinase at  $(3.0 \pm 0.3) \times 10^{-3}$  U/mg and  $(16.0 \pm 1.0) \times 10^{-3}$  U/mg protein equivalent of cell extract in the absence and presence of isopropyl  $\beta$ -D-thiogalactopyranoside, respectively. The presence of a counter-oriented  $P_{lac}$  at the 3' end of the operon in the pMCU22bPa plasmid reduced the sP1-mediated tyrosinase production by about 85%.

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### Introduction

*Streptococcus thermophilus* is an important microorganism used in the manufacture of yogurt and certain cheeses. In our laboratory, genetic engineering of this

bacterium frequently involved the use of *Escherichia coli* as an intermediate host for obtaining large amounts of the recombinant DNA needed for the electroporation of *S. thermophilus* as well as other lactic acid bacteria (Somkuti et al. 1991, 1992). Accordingly, shuttle vectors suitable for carrying DNA inserts into *E. coli* and the lactic acid bacteria were developed (Solaiman and Somkuti 1993; Solaiman et al. 1992). Expression of the cloned genes, however, could not be assured (Solaiman et al. 1992); only when native or some fortuitous promoters were present upstream from the cloned genes did gene expression occur. Consequently, interest has developed in many laboratories in cloning native promoters from the chromosomes of the lactic acid bacteria for use in gene expression undertakings (Bojovic et al. 1991; Koivula et al. 1991; Slos et al. 1991; van der Vossen et al. 1987).

In this paper, we describe a novel approach for obtaining a *S. thermophilus* promoter sequence by *in vitro* synthesis. We chemically synthesized a 63-mer oligonucleotide (designated sP1) based on the sequence of the *S. thermophilus* promoter P25 (Slos et al. 1991) and tested its performance in the intermediate cloning-host *E. coli*. Two *Streptomyces* operons were used as the marker systems to rule out the possibility of a fortuitous gene activation by the sP1 insert. The *cho* operon (Horii et al. 1990) was employed because its expression in *E. coli* and lactic acid bacteria had been extensively studied (Solaiman and Somkuti 1991, 1992; Somkuti et al. 1991, 1992; Solaiman et al. 1992). The *melC* operon (Katz et al. 1983; Leu et al. 1989) was chosen as the other reporter system since the readily detectable melanin pigments provide a potentially useful non-antibiotic marker for the selection of transformants. Our results, showing definitive sP1-mediated gene expression in *E. coli*, demonstrated the validity of chemical synthesis for promoter development. This work also opens up new avenues for preparing active LAB promoters and testing them *a priori* in *E. coli* before finally introducing them into the lactic acid bacteria for optimal gene expression.

## Materials and methods

### Bacterial strains, plasmids, and growth conditions

Competent *E. coli* HB101, DH5 $\alpha$  and DH5 $\alpha$ F'IQ cells were purchased from BRL Life Technologies (Gaithersburg, Md.). Cells were routinely grown in Luria-Bertani (LB) broth (1% w/v tryptone and 0.5% w/v each of yeast extract and NaCl). When needed, antibiotics were included in the growth medium at the following concentrations: ampicillin 100  $\mu$ g/ml, erythromycin 200  $\mu$ g/ml, and kanamycin 10–30  $\mu$ g/ml. Transformants harboring *mel*-containing plasmids were grown in medium supplemented with L-tyrosine (0.3 mg/ml) and CuCl<sub>2</sub> (0.2 mM). 5-Bromo-4-chloro-3-indolyl  $\beta$ -D-galactoside (Xgal) and isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) were used at concentrations of 50  $\mu$ g/ml and 1–2 mM respectively.

The 63-base-pair (63-bp) synthetic promoter sP1 (Fig. 1A) was synthesized by Genset (La Jolla, Calif.). The –35 and –10 regions were based on the putative consensus sequences of the P25 promoter of *S. thermophilus* (Slos et al. 1991). sP1 was initially subcloned into pUC18 to produce pSTP25 (plasmid map not shown) in which the orientation of the insert is opposite to that of the *lacZ* gene. pSTP25 was maintained in *E. coli* DH5 $\alpha$  and served as the source of sP1 for subsequent cloning experiments. The *cho* and *mel* operons originated from the *streptomyces* plasmids pCO-1 (Murooka et al. 1986) and pIJ702 (Katz et al. 1983) respectively. Both plasmids were kindly provided by Y. Murooka (Hiroshima University, Higashi-Hiroshima, Japan).

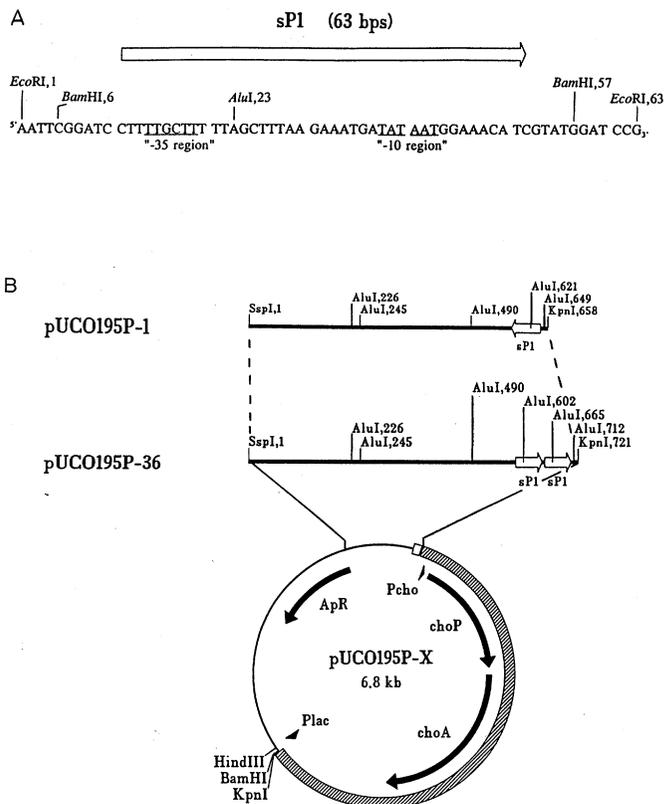
### Molecular cloning procedures

Restriction endonucleases, T4 DNA ligase, calf intestinal alkaline phosphatase (CIAP), Klenow enzyme (*E. coli* PolIk) and T4 DNA polymerase were variously obtained from BRL Life Technologies, New England Biolabs (Beverly, Mass.), or United States Biochemical (Cleveland, Ohio), and were used according to the vendors' specifications.

Transformation of *E. coli* was performed according to suppliers' protocols. Plasmid DNAs were isolated as described (Ausbel et al. 1987; Solaiman and Somkuti 1993). Agarose gel electrophoresis was carried out in TRIS/borate/EDTA buffer system (0.089 M Tris base, 0.089 M boric acid, 0.002 M EDTA). Southern blotting was performed onto Sure-Blot membranes (Oncor, Inc., Gaithersburg, Md) using a semi-dry electrophoretic transfer cell (Bio-Rad Labs, Richmond, Calif.). After immobilizing the nucleic acids in a GS Gene Linker UV chamber (Bio-Rad Labs), the target DNAs were probed with biotinylated sP1 using reagents and protocols from Oncor Inc. The sP1 probe was biotinylated photochemically with *N*-(4-azido-2-nitrophenyl)-*N'*-(3-biotinylamino-propyl)-*N'*-methyl-1,3-propanediamine (photobiotin) as described (Forster et al. 1985).

### Cholesterol oxidase assay

Cell-free extracts were prepared from overnight cultures by sonication and high-speed centrifugation as described (Solaiman and Somkuti 1991). The extracts were concentrated using Centricon-30 filtration units (Amicon Inc., Beverly, Mass.). Protein concentrations were estimated by a dye-binding method (Bradford 1976) using a commercial protein assay kit (Bio-Rad Labs) with bovine serum albumin as standard. Cholesterol oxidase activity was assayed at 37°C by adding about 3.5  $\mu$ g protein equivalent of the extracts to a reaction mixture (30 ml) containing 50 mM potassium phosphate (pH 7.4), 0.1% (v/v) Triton X-100 and 0.19 mM cholesterol (Sigma Chemical Co.). At various times, 2-ml aliquots were removed and the cholesterol contents were determined as described (Solaiman and Somkuti 1991). One unit (U) of cholesterol oxidase activity is defined as one mole of cholesterol oxidized per minute.



**Fig. 1A,B** Structure of sP1 (A) and configurations of pUCO195P-1/-36 (B). Only restriction sites pertinent to this study are shown; numbers following the enzyme denote the position of these sites in nucleotide number. *P<sub>lac</sub>* and *P<sub>cho</sub>*, are promoters of *lacZ'* and *cho* operons respectively

### Tyrosinase assay

Overnight cultures of *E. coli* DH5 $\alpha$ F'IQ transformants were added at 1/20 dilution to 50 ml LB medium supplemented with ampicillin (100  $\mu$ g/ml), kanamycin (30  $\mu$ g/ml), L-tyrosine (0.3 mg/ml) and CuCl<sub>2</sub> (0.2 mM). After growing at 37°C with vigorous shaking for 1 h, the cultures were divided into two halves. IPTG at 1 mM was added to one set of the divided cultures. Incubation with shaking was resumed for an additional 4 h. Cell-free extracts were prepared from these cultures as described above (Solaiman and Somkuti 1991). Tyrosinase activity was estimated at 30°C by adding 100–200  $\mu$ l (approx. 0.4–1.2 mg protein equivalent) of cell extracts to 1 ml assay mixture containing 9.2 mM 3-(3,4-dihydroxyphenyl)-L-alanine in 0.1 M sodium phosphate (pH 6) buffer (Lerch and Ettlenger 1972). The reaction was monitored spectrometrically at 475 nm, and the rates of the reactions were calculated using the molar absorption coefficient of 3600 M<sup>-1</sup>cm<sup>-1</sup> for the dopachrome product. One unit enzyme activity (U) is defined as one micromole of dopachrome formed per minute.

The tyrosinase activity in transformants with low levels of *melC* expression was estimated in a similar assay system, except that 2 mM 3-methyl-2-benzothiazolinone hydrazone was included in the reaction mixture to enhance the colorimetric detection of the dopachrome product (Rodrigues-Lopez et al. 1994).

## Results

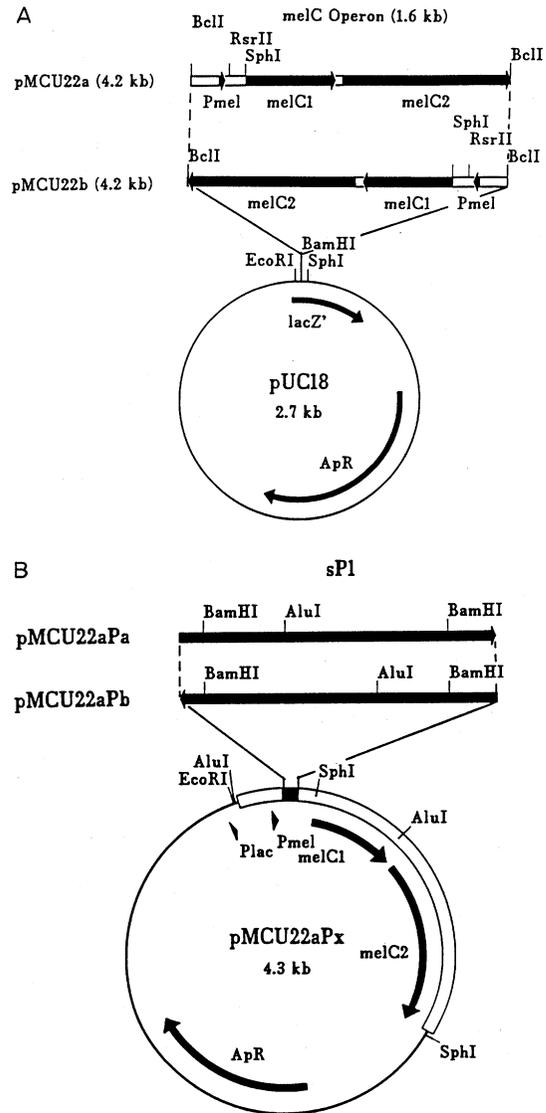
### Construction of plasmids

The construction of pUCO193 in *E. coli* DH5 $\alpha$  has been described in detail (Solaiman and Somkuti 1991). Briefly summarized, a 4.1-kb *Kpn*I-restricted DNA fragment containing the entire *Streptomyces cho* operon (Horii et al. 1990) was subcloned into the unique *Kpn*I site of pUC19 to produce the recombinant. Early studies had established that the expression of the *choA* gene in pUCO193 was directed by the *lac* promoter ( $P_{lac}$ ) located upstream from the cloned operon (Solaiman and Somkuti 1991). We have subsequently isolated pUCO195, the counterpart of pUCO193 in which the 4.1-kb insert is oriented in the opposite direction to that of pUCO193. Since the  $P_{lac}$  in pUCO195 is downstream from, and its direction opposite to, the *cho* operon (Fig. 1B), no expression of *choA* was expected to occur.

Construction of the sP1-containing pUCO195 derivatives is depicted in Fig. 1B. The 63-bp sP1 was excised from pSTP25 by *Eco*RI digestion, gel-purified on a 10% HydroLink Long Ranger gel (AT Biochem, Malvern, Pa.), and ligated with *Eco*RI-linearized/CIAP-treated pUCO195 plasmid. *E. coli* DH5 $\alpha$  transformed with the ligation mixture was selected on LB/ampicillin (100  $\mu$ g/ml) plates. Mini-scale plasmid screening with 36 randomly selected ampicillin-resistant (ApR) clones yielded pUCO195P-1 and pUCO195P-36 (Fig. 1B). We subsequently observed that while pUCO195P-1 was stable in *E. coli* strains HB101 and DH5 $\alpha$ , the pUCO195P-36 construct suffered from segregational instability in the DH5 $\alpha$  strain.

A 1.6-kb DNA fragment containing the *Streptomyces melC* operon was isolated from *Bcl*I-digested pIJ702 (Katz et al. 1983) by gel electrophoresis and electroelution. The fragment was ligated with *Bam*HI-linearized, CIAP-dephosphorylated pUC18 using T4 DNA ligase. Recombinants pMCU22a and pMCU22b (Fig. 2A) were isolated from ApR, non-blue *E. coli* DH5 $\alpha$  transformants selected on LB/ampicillin (100  $\mu$ g/ml)/Xgal(50  $\mu$ g/ml) plates. The orientation of the insert in the recombinants was determined by *Sph*I restriction analysis.

The sP1 promoter sequence was introduced into pMCU22a/b as follows. A molar excess of *Eco*RI-flanked sP1 fragment was mixed with *Rsr*II-linearized, gel-electrophoretically purified pMCU22a/b vector. The mixtures were reacted with *E. coli* PolIk to fill-in the termini of the nucleic acids. After purification on an Elutip-d column (Schleicher & Schüll, Keene, N. H.), a blunt-end-ligation reaction was performed using T4 DNA ligase. *E. coli* DH5 $\alpha$  was used as the plasmid recipient, and transformants were selected on LB/ampicillin (100  $\mu$ g/ml) plates. Clones containing plasmids with the sP1 insert were identified by mini plasmid



**Fig. 2A,B** Maps of the *melC*-operon-containing recombinants. (B) Only the restriction maps of pMCU22aPa and pMCU22aPb are shown. For pMCU22bPa and pMCU22bPb (not shown), the orientation of the pUC18 moiety (drawn as a single line) is reversed relative to that of pMCU22aPa and pMCU22aPb respectively.  $P_{lac}$  and  $P_{mel}$  are the *lac* and *mel* promoter regions respectively

screening (Ausubel et al. 1987) and *Bam*HI restriction analysis. The orientation of the promoter in the recombinants was determined by *Alu*I restriction analysis.

### sP1-mediated *choA* expression

As expected from the results of a previous study (Solaiman and Somkuti 1991), the *E. coli* transformant containing pUCO195 did not express the *choA* gene. When transformants harboring pUCO193 and pUCO195 were cultured in cholesterol-supplemented (50  $\mu$ g/ml) LB/ampicillin (100  $\mu$ g/ml) medium for 4 days at 37 $^{\circ}$  C, we

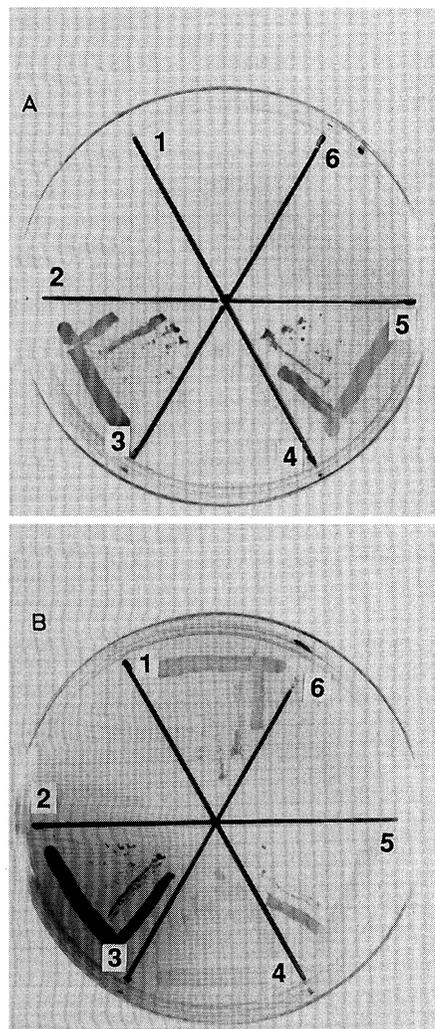
found that only the former was capable of depleting the sterol in the broth as assayed by a colorimetric method (Johnson and Somkuti 1990; Solaiman and Somkuti 1991). The silent *cho* operon in pUCO195 rendered this plasmid suitable for testing the transcription activity of the sP1 in *E. coli*.

When transformants harboring pUCO195P-1 and pUCO195P-36 were similarly screened for cholesterol-depletion activity, we found that the P-36 clone showed cholesterol oxidase activity. Detailed restriction analyses, aided by Southern-blot hybridization with biotinylated sP1 probe (data not shown), of the two plasmids established that the orientation of the sP1 promoter insert was respectively opposed to and aligned with the *cho* operon in the P-1 and P-36 constructs (Fig. 1B). The results also indicated the presence of an additional copy of tandemly arranged sP1 in the pUCO195P-36 plasmid. On the basis of the configuration of these recombinants, we concluded that pUCO195P-36 was capable of conferring cholesterol oxidase activity on its host because the aligned sP1 promoters upstream from the *cho* gene cluster had activated the *cho* operon.

We next estimated the relative cholesterol oxidase activities of the *E. coli* DH5 $\alpha$  transformants containing pUCO193 and pUCO195P-36. The results showed that the specific cholesterol oxidase activity in the extracts of the transformant of pUCO195P-36 was  $0.5 \times 10^{-8}$  unit/mg protein. For comparison, the corresponding value obtained with the pUCO193-containing organism was  $1.3 \times 10^{-8}$  unit/mg protein.

#### sP1-mediated *melC* expression

The ability of sP1 to activate yet another operon was demonstrated, to rule out the possibility that the *choA* expression seen with the pUCO195P-36 construct was simply a fortuitous event. When *E. coli* DH5 $\alpha$ F'IQ transformed with pMCU22a or pMCU22b (Fig. 2A) were streaked on indicator plates without or with IPTG, only the pMCU22a-containing bacteria in the presence of the inducer exhibited the Mel<sup>+</sup> phenotype (Fig. 3). Apparently the *streptomyces* promoter  $P_{mel}$  was not functional in *E. coli* as expected (Sugiyama et al. 1990; Tseng et al. 1990); and the IPTG-responsive *mel* expression was mediated by the *lacZ* promoter ( $P_{lac}$ ). By inserting the sP1 promoter in front of the transcription initiation site of the *melC* operon (Bernan et al. 1985; Leu et al. 1989) in the pMCU22aPa and pMCU22bPa constructs (Fig. 2B), *mel* expression in the absence of IPTG was observed with the corresponding transformants (Fig. 3A; sectors 3 and 5). As expected, melanin production from the pMCU22aPa plasmid was also responsive to IPTG induction (Fig. 3B; sector 3). Tyrosinase synthesis from the pMCU22bPa construct, on the other hand, was not affected by the inducer (Fig. 3B; sector 5). Recombinants pMCU22aPb and pMCU22bPb, in which the upstream sP1 was oriented against the *melC* operon, did not confer the Mel<sup>+</sup> phe-



**Fig. 3A,B** Melanin production by *melC*-containing *E. coli* DH5 $\alpha$ F'IQ transformants. Overnight cell cultures were streaked on Luria-Bertani broth/agar (1% w/v)/ampicillin (100  $\mu$ g/ml)/kanamycin (30  $\mu$ g/ml)/L-tyrosine (0.3 mg/ml)/CuCl<sub>2</sub> (0.2 mM) plates. **A** No isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG); **B** 1 mM IPTG. Sectors: 1 pMCU22a, 2 pMCU22b, 3 pMCU22aPa, 4 pMCU22aPb, 5 pMCU22bPa, 6 pMCU22bPb

notype on their respective hosts in the absence of IPTG (Fig. 3A; sectors 4 and 6). The inducer did activate *melC* expression with the pMCU22aPb construct as expected (Fig. 3B; sector 4). These results showed that sP1 inserted upstream from the *streptomyces melC* operon could indeed activate the expression of the genes in the *E. coli* host.

The relative levels of tyrosinase production by transformants harboring pMCU22a, pMCU22aPa, and pMCU22bPa were compared (Table 1). The results showed that sP1 alone in the pMCU22bPa construct directed the production of 2.3 times as much tyrosinase as did pMCU22a under IPTG induction conditions. Interestingly, sP1 appeared to act synergistically with the  $P_{lac}$  and/or  $P_{mel}$  promoter in the pMCU22aPa plasmid to confer even higher levels of tyrosinase production.

**Table 1** Tyrosinase activities in transformants containing pMCU22xPx. Specific activities were expressed as  $\mu\text{mol}$  dopachrome formed  $\text{min}^{-1}$  ( $\text{mg}$  protein equivalent of cell extracts) $^{-1}$ ; no 3-methyl-2-benzothiazolinone hydrazone (MBTH) was added in the reactions. The relative activities were determined as changes of  $A_{490} \text{ min}^{-1}$  ( $\text{mg}$  protein equivalent of cell extracts) $^{-1}$ , with 2 mM MBTH present in the reaction mixture. All values were normalized to that obtained with the pMCU22a + isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) sample (ND) not determined because the tyrosinase contents were below the detection limit of the assay system)

Resident plasmid	Specific tyrosinase activity ( $\mu\text{mol min}^{-1} \text{ mg}^{-1}$ )	Relative tyrosinase activity ( $\Delta A_{490} \text{ min}^{-1} \text{ mg}^{-1}$ )
pMCU22aPa	$0.003 \pm 0.0003$	15.0
pMCU22aPa + IPTG	$0.016 \pm 0.001$	— <sup>a</sup>
pMCU22a	ND	0.4
pMCU22a + IPTG	ND	1.0
pMCU22bPa	ND	2.3
pMCU22bPa + IPTG	ND	2.3

<sup>a</sup> Not included in the MBTH-mediated assay

## Discussion

We have conclusively demonstrated that the synthetic oligonucleotide sP1 with a sequence based on a strong *S. thermophilus* promoter, is operational in *E. coli*. This occurred in spite of the vast dissimilarity that exists between the “-35 region” of sP1 and the consensus sequence of *E. coli* mRNA promoters (Lisser and Margalit 1993); the “-10 regions” of the two sequences are similar.

The synergetic interaction of sP1 with the  $P_{lac}$  and/or  $P_{mel}$  promoter, as seen with the pMCU22aPa plasmid, is noteworthy. The effect was apparently sequence-specific, since the insertion of a reversed sP1 sequence in the pMCU22aPb construct did not lead to the same levels of IPTG-responsive *melC* expression (Fig. 3). Although the level of tyrosinase production by pMCU22aPa-transformed *E. coli* under induction conditions was only moderate in comparison to other producing strains (Katz et al. 1983; Hintermann et al. 1985; della-Cioppa et al. 1990; Betancourt et al. 1992), further study to optimize fermentation conditions should lead to high levels of melanin activity.

The functioning of a synthetic *S. thermophilus* promoter in *E. coli*, as shown in this study, demonstrates that rational design of a DNA sequence for gene activation/expression can be achieved. We have subsequently shown that the synthetic sequence is capable of directing expression of a promoterless *cho* operon in *S. thermophilus* (Somkuti and Solaiman, manuscript in preparation). These results further support earlier studies (van der Vossen et al. 1987; Bojovic et al. 1991) that it is possible to use *E. coli* as an intermediate host in identifying the promoters of *S. thermophilus* and other lactic acid bacteria; the expression of heterologous genes can be examined or even fine-tuned first in this

versatile organism before introduction into the ultimate lactic acid bacteria host. Furthermore, in vitro design or modification of existing promoter sequences to achieve either increased or controlled (regulated) gene expression is a distinct possibility.

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