Expression of *Streptomyces melC* and *choA* genes by a cloned *Streptococcus thermophilus* promoter ST_{P2201}

Streptococcus thermophilus **(ST) chromosomal DNA (chr DNA) fragments having promoter activity were cloned and selected in *Escherichia coli* using a chloramphenicol acetyltransferase- (*cat*-) based promoter-probe vector pKK520-3. Insertion of a promoterless streptomycete melanin biosynthesis operon (*melC*) downstream from the promoters of the library further identified clone ST_{P2201} as a strong promoter in *E. coli*. Subcloning of a ST_{P2201}-*melC* DNA fragment into the pMEU-series *S. thermophilus* – *E. coli* shuttle vectors yielded pEU5xML2201x plasmids that conferred Mel^+ phenotype to *E. coli*. The pEU5aML2201a was further shown to afford a high level of tyrosinase production (2 units mg^{-1} protein) in *E. coli*, and to produce an apparently inactive *melC* gene product that reacts with anti-tyrosinase antiserum in *S. thermophilus*. Substituting *melC* with a streptomycete cholesterol oxidase gene (*choA*) in the same orientation yielded pEU5aCH2201a that conferred ChoA activity to an *E. coli* transformant at a level of $(1.06 \pm 0.15) \times 10^{-7}$ units mg^{-1} protein. Introduction of this plasmid into *S. thermophilus* by electrotransformation yielded $ChoA^+$ transformant that produced the enzyme at about 25% of the level found in *E. coli*.**

Keywords: lactic acid bacteria; promoter; melanin; tyrosinase; cholesterol oxidase

Introduction

Lactic acid bacteria (LAB) are important microbes used in the fermentation of dairy products. These organisms impart characteristic texture and flavor to cheese and yogurt. Genetic engineering of LAB potentially enables one to control the fermentation process and to modify product attributes. Many molecular biology procedures for the genetic modification of LAB have been developed over the years.

The thermophilic *Streptococcus thermophilus* (ST) is used in the production of yogurt, Swiss-type cheese and certain cooked hard cheese varieties. In attempts to genetically engineer this species, various gene transfer techniques have been tested [15]. The most direct and convenient means for introducing DNA into this bacterium, however, appears to be the electrotransformation procedure [29]. Using electroporation, several cross-species broad-host-range plasmids have been successfully introduced into ST [14,23,29]. In an effort to develop 'food-grade' cloning vehicles, we have constructed a series of shuttle vectors [21] using *Escherichia coli* plasmid pUC19/18 and a *S. thermophilus* cryptic plasmid pER8 [28]. Furthermore, expression vectors based solely on pER8-replicon have also been constructed [27]. In addition to cloning vector development, attempts have been made to isolate native promoter sequences from ST [8,19]. However, detailed characterization of their uses in gene expression was not reported. Based on the nucleotide sequence of a *ca* 300-bp P25 promoter [19], we had synthesized a 65-bp synthetic DNA (sP1) and proceeded to show that it could drive heterologous gene expression in *S. thermophilus* [27] and *E. coli* [22]. In this communication, we report the isolation of a ST_{P2201} promoter from the chromosomal DNA of *S. thermophilus* ST128. We proceed to show that ST_{P2201} is capable of activating promoterless heterologous genes in both *E. coli* and *S. thermophilus*.

The heterologous genes used to demonstrate the promoter activity of ST_{P2201} are the *Streptomyces antibioticus* melanin biosynthesis operon *melC* [11] and the *Streptomyces* sp strain SA-COO cholesterol oxidase gene *choA* [17]. The production of melanin pigments in LAB is of interest because of the potential commercial values of the polymers [2,9] possibly for *in situ* coloration of fermented dairy products [26]. Genetically engineered LAB that express *choA*, on the other hand, would be models for the *in situ* modification of cholesterol in fermented dairy products [24,25].

Materials and methods

Bacteria, culture conditions, and plasmids

E. coli DH5α (BRL Life Technologies, Gaithersburg, MD, USA) and *S. thermophilus* ST108 and ST128 (laboratory collection) were grown in Luria-Bertani (LB) and tryptone-yeast-lactose broth, respectively, as previously described [22,28]. Expression of tyrosinase activity in *E. coli* transformants harboring *melC*-containing plasmids was identified in growth medium supplemented with L-tyrosine (0.3 mg ml^{-1}) and $CuCl_2$ (0.2 mM). Plasmids used or constructed in this study are listed in Table 1.

Plasmid manipulation

Restriction endonucleases and DNA-modifying enzymes were obtained from and used according to the recommended procedures of the following suppliers: BRL Life

Table 1 Plasmids used

Plasmid	Description	Source/reference
pKK520-3	Ap^R; *cat*-based *E. coli* promoter-probe plasmid	[6]
pMEU5a/b	Ap^R; Em^R; *E. coli-S. thermophilus* shuttle vectors with the 1.7-kb *StyI/HinfI* fragment of pER8 cloned into *HincII* site of pUC18; a and b suffixes indicate the two possible orientations of the *erm* gene	[21]
pMEU6a/b	Similar to pMEU5a/b, except the pER8 fragment is orientated in the opposite direction	[21]
pMCU22aPx	pUC18 carrying *S. antibioticus melC* operon with an *S. thermophilus* synthetic promoter sP1 spliced upstream from the *melC*	[22]
pMCU23a	Derivative of pMCU22aPb [22] in which the sP1 promoter had been removed by *BamHI* digestion	This study
pUCO195	Counterpart of pUCO193 [20], in which the 4.1-kb *choA*-containing *KpnI* fragment is oriented in the opposite direction	This study
pKSTs	Cm^R; Ap^R; pKK520-3 derivatives containing putative ST promoters cloned into *BamHI* site	This study
pKST-mels	pKSTs with a 1.4-kb *BamHI/HindIII* fragment of pMCU23a containing the promoterless *S. antibioticus melC*, cloned into *BglII/HindIII* sites downstream from the putative ST promoters	This study
pKST-mel2201	One of the pKST-mels that displays strong Mel^+ phenotype. The *ca* 200-bp putative ST promoter is designated ST_{P2201}	This study
pEU5xML2201a/b	2.56-kb *PstI* fragment of pKST-mel2201 that contains ST_{P2201} and *melC*, cloned into *PstI* site of pMEU5x; a and b suffixes indicate *melC* is aligned and countered to the *bla* gene of pMEU5x, respectively	This study
pEU6xML2201a/b	Similar to pEU5xML2201a/b, except pMEU6x was used as the vector	This study
pEU5a2201a	*melC*-less pEU5aML2201a; the *melC* operon was excised by *SphI* digestion	This study
pEU5aCH2201a/b	2.7-kb *choA*-containing *PstI* fragment from pUCO195 cloned into *SphI* site of pEU5a2201a by blunt-end ligation; suffixes a and b indicate the *choA* gene is aligned and opposite to the ST_{P2201}, respectively	This study

Technologies, New England BioLabs (Beverly, MA, USA), and United States Biochemical (Cleveland, OH, USA). Plasmid DNAs were introduced into *E. coli* by a heat-shock method as detailed by the supplier of the competent cells, and into *S. thermophilus* by electrotransformation [29]. Methods for the isolation of plasmids from *E. coli* [1,21] and *S. thermophilus* [28] were described elsewhere. Agarose gel electrophoresis of plasmid samples was carried out in TBE buffer (0.089 M Tris base, 0.089 M boric acid, 0.002 M Na-EDTA). DNA fragments were isolated as needed from agarose gels by electroelution. When necessary, nucleic acid samples were further purified on Elutip-d columns (Schleicher & Schuell, Keene, NH, USA).

Enzyme assays

Cell-free extracts were prepared from overnight cultures of *E. coli* and *S. thermophilus* transformants by sonication and high-speed centrifugation essentially as described previously [20]. The extracts were subsequently filtered through Centricon-100 filtration units (Amicon Inc, Beverly, MA, USA) and then concentrated on Centricon-10 filters. Fractions retained on the filters were termed Centricon-10 fractions, and were used in enzyme assays. Protein concentrations of samples were estimated by a dye-binding method [4] using Bio-Rad Labs' (Richmond, CA, USA) reagent kit and bovine serum albumin (BSA) as the standard.

Tyrosinase activity was assayed spectrophotometrically using the L-DOPA [3-(3,4-dihydroxyphenyl)-L-alanine] oxidation method as described by Lerch and Ettlinger [13]. Reactions were performed in 1-ml assay mixtures containing 0.1 M Na-phosphate (pH 6), 9.2 mM L-DOPA, and 130–215 μg protein equivalent of Centricon-10 fractions. Oxidation of L-DOPA into dopachrome product was monitored at 475 nm with the cuvette holder of the spectrophotometer maintained at 30° C. The molar extinction coefficient (ϵ) of 3600 was used to calculate enzyme activity. One unit of tyrosinase activity is defined as 1 μmole dopachrome produced per min.

Cholesterol oxidase activity was estimated by measuring the rate of cholesterol depletion at 37° C in a 2-ml reaction mixture containing 0.05 M potassium phosphate (KP; pH 7.4), 0.1% (v/v) Triton X-100, 50 $\mu g\ ml^{-1}$ cholesterol, and 25–50 μg protein equivalent of Centricon-10 fractions. Unreacted cholesterol at the end of a 30-min incubation period was exhaustively extracted with ethylacetate. After evaporating off the solvent, the substrate residue was quantitated colorimetrically using the $FeCl_3$ reagent as previously described [10]. Specific enzyme activity is defined as mole of cholesterol depleted per min per mg protein.

Western hybridization analysis

The presence of *melC* gene product in *S. thermophilus* transformants was determined by a Western hybridization technique described previously [26].

Results

Partial characterization of a subset of pKST promoter library

A pKST-series ST128 promoter partial library was constructed in *E. coli* using pKK520-3 vector [6]. From this partial library, 18 clones containing inserts of $\leq$ 600 bps were pooled and a promoterless *Streptomyces melC* operon was spliced downstream from the ST128 chr DNA inserts to generate pKST-mel recombinants. Plating of *E. coli*

transformants containing the recombinants on tyrosinase indicator plates allowed us to identify melanin-producing clones. Based on restriction analyses of the plasmids from these clones, five classes of tyrosinase-producing pKST-mel constructs were identified. Representative clones from these five classes of melanin-producing transformants contain plasmids with ST128 chr DNA inserts of *ca* 170 bps (pKST-mel2201), 100 bps (pKST-mel1205), 500 bps (pKST-mel3306), 800 bps (pKST-mel1315), and 170 bps (pKST-mel3101). The chr DNA inserts in these constructs were appropriately labeled as ST_{P2201}, ST_{P1205}, ST_{P3306}, etc. When grown in LB liquid medium containing tyrosine and Cu^{2+}, these clones produce melanins with different shades of brown and black. Since culture of pKST-mel2201 exhibited the darkest coloration indicative of high-level *mel*C expression, this construct was selected for further studies. Tyrosinase assays performed on the cell extracts of *E. coli* [pKST-mel2201] yielded a specific enzyme activity value of $(100.5 \pm 7.4) \times 10^{-3}$ u mg^{-1} protein. For comparison, cell extracts of transformants harboring pKST-mel1205 and pKST-mel3306 contain tyrosinase at levels of $(25.5 \pm 6.0) \times 10^{-3}$ and $(26.1 \pm 5.0) \times 10^{-3}$ u mg^{-1} protein, respectively.

STP2201-mediated melC expression in pMEU-series shuttle vectors

Previously-constructed pMEU-series shuttle vectors [21] for *S. thermophilus* and *E. coli* were used to evaluate promoter activity of ST_{P2201}. Accordingly, *Pst*I-digested pKST-mel2201 was ligated with *Pst*I-linearized pMEU5a/b and pMEU6a/b. After introducing the products of the ligation mixtures into *E. coli*, plasmid screenings for the desired pMEUs containing the 2.6-kb ST_{P2201}+ *mel*C cassette yielded only recombinants with the b configuration in which the direction of the *mel*C operon is opposite to the *bla* gene. The only exception was the pEU5aML2201a/b constructs where both the a and b configurations were obtained; the frequency of encountering the former was nevertheless much lower than that of the latter. The pEU5bML2201a construct was subsequently obtained by successively subjecting pEU5bML2201b to *Pst*I digestion and then re-ligating the fragments. As with the pEU5aML2201a/b, plasmid screening of randomly selected transformants identified clones containing pEU5bML2201b at a markedly higher frequency than those harboring pEU5bML2201a. With the pMEU6a/b shuttle vectors, repeated attempts to isolate the pEU6a/bML2201a constructs failed to produce the desired plasmids.

Tyrosinase contents of the cell extracts prepared from *E. coli* transformants harboring pEU5a/bML2201a/b were compared (Table 2). The results showed that the transformant of pEU5aML2201a contained a high level of intracellular tyrosinase activity. Unexpectedly, clones containing its sister plasmid, pEU5aML2201b, contained very low enzyme activity; however, the culture broth exhibited remarkably dark coloration compared to those of the other three transformants. The possibility that cell lysis had occurred with the culture of *E. coli* [pEU5aML2201b], leading to the leakage of tyrosinase, was ruled out because the protein content of the crude cell extracts was compatible to that observed with the pEU5aML2201a sample. It remains to be determined whether secretion of the enzyme or the melanins was responsible for the observed diffused pigmentation. It was also noted that the intracellular tyrosinase contents of *E. coli* harboring either pEU5bML2201a or pEU5bML2201b were comparable and lower than that measured in *E. coli* [pEU5aML2201a].

The functioning of ST_{P2201} in *S. thermophilus* was examined using the pEU5aML2201a construct that was shown to confer high-level intracellular tyrosinase expression in *E. coli*. When tyrosinase assays in the absence or presence of MBTH were performed with the cell extracts of *S. thermophilus* [pEU5aML2201a], enzyme activity was not observed (data not shown). Western blot analysis of the cell extracts, however, revealed that the *mel*C gene product with an apparent molecular weight of *ca* 30 kDa was indeed synthesized (Figure 1). It appeared that ST_{P2201} was in fact capable of expressing the *mel*C operon in *S. thermophilus*; the gene product(s), however, were not enzymatically active in this organism.

Expression of choA by STP2201 promoter

The generality of the promoter activity of ST_{P2201} was further demonstrated using *Streptomyces choA* as a reporter gene. Accordingly, the promoterless *mel*C operon in the pEU5aML2201a plasmid was excised by *Sph*I digestion. In its place, a promoterless *Streptomyces cho*A gene contained in a 2.7-kb *Pst*I DNA fragment of pUCO195 plasmid [20] was inserted in the two possible orientations by blunt-end ligation. Recombinant plasmid with ST_{P2201} located upstream or downstream from the *cho*A gene was labeled pEU5aCH2201a or pEU5aCH2201b, respectively. Assays for ChoA activity in the cell extracts of the transformants showed that only *E. coli* harboring pEU5aCH2201a contained the enzyme (Table 2). Transformants containing the vector pEU5a2201a or the recombinant plasmid pEU5a CH2201b did not produce any measurable ChoA activities under the assay conditions.

The promoter activity of ST_{P2201} in *S. thermophilus* was further confirmed with these *cho*A-containing recombinants. The three plasmids were electrotransformed into ST128 with frequencies in the range of 10^2–10^3 CFU μg^{-1} DNA. Plasmids isolated from the transformants showed that no deletion of the DNAs occurred in this host system (Figure 2). When the Centricon-10 fractions prepared from cell extracts of the transformants were assayed for ChoA activity, only the ST128 [pEU5aCH2201a] sample showed reactivity by completely depleting the substrate in the reaction mixture (Figure 2). The specific activity of this active fraction was estimated in a separate experiment as about one-fourth of that seen with the corresponding *E. coli* sample (Table 2).

Discussion

A partial library of *S. thermophilus* chromosomal DNA fragments with putative promoter activity was constructed in *E. coli* using a *cat*-based promoter-probe vector pKK520-3. By inserting a promoterless *Streptomyces mel*C operon between the cloned ST sequences and the *cat* reporter gene, we demonstrated that the relative promoter strength of the putative promoters could be empirically

Table 2 Specific MelC2 and ChoA enzyme activities of *E. coli* and *S. thermophilus* transformants

Host	Plasmid	Enzyme[a]	Specific activity (u min^{-1} mg $protein^{-1}$) ± SD
E. coli DH5α	pEU5aML2201a	MelC2	1.98 ± 0.42
	pEU5aML2201b	MelC2	0.04 ± 0.01
	pEU5bML2201a	MelC2	0.77 ± 0.09
	pEU5bML2201b	MelC2	0.76 ± 0.09
	pEU5a2201a	ChoA	0[b]
	pEU5aCH2201a	ChoA	$(1.06 \pm 0.15) \times 10^{-7}$
	pEU5aCH2201b	ChoA	0[b]
S. thermophilus ST128	pEU5a2201a	ChoA	ND[c]
	pEU5aCH2201a	ChoA	$(2.78 \pm 0.24) \times 10^{-8}$
	pEU5aCH2201b	ChoA	ND[c]

[a]MelC2, tyrosinase; ChoA, cholesterol oxidase
[b]No ChoA activity detected within experimental errors
[c]Not Determined

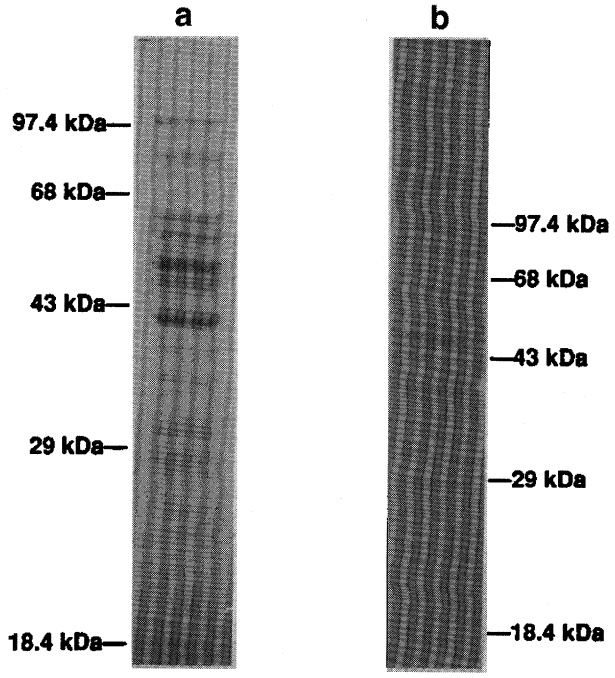

Figure 1 Western-blot analysis of *mel*C expression by *S. thermophilus* [pEU5aML2201a]. Cell-free extracts were prepared as described previously [26]. The total amounts of proteins loaded on polyacrylamide gel (4% stacking/10% analytical) were 70 and 210 μg for lanes shown in panels **a** and **b**, respectively. After electrophoresis, the gel was cut into two halves. One half was stained with Coomassie Brilliant Blue (panel **a**); the other was used to perform Western-blot analysis [26]. Locations of the prestained, high-range protein size markers (BRL Life Technologies) are indicated

assessed based on the extent of dark-brown color development by the transformants. Clones with high levels of tyrosinase expression appeared as colonies surrounded by diffused black pigments on indicator plates and gave pitch-dark coloration to the growth medium in broth cultures. The *mel*C operon thus provides a convenient way for empirically assessing relative strength of cloned promoter sequences. Although the use of *mel*C operon as a transformation marker and promoter probe had been suggested previously [30,31], this study demonstrates for the first time the successful cloning of a promoter sequence using this reporter system. By this method, we were able to identify for further study a putative *S. thermophilus* promoter, ST_{P2201}, that exhibited high gene-expression activity in *E. coli* and was shown to have promoter function in the Gram-positive host, *S. thermophilus*.

The ST_{P2201} promoter was shown to drive the expression of heterologous genes, *mel*C and *cho*A, in Gram-negative *E. coli* and Gram-positive *S. thermophilus* hosts. Using pMEU5a shuttle vector [21] as the cloning vehicle, the levels of ST_{P2201}-driven MelC and ChoA production in *E. coli* transformants were significantly higher than those previously achieved with other plasmid constructs [20]. In fact, they appeared to be comparable to those reported for the tyrosinase and cholesterol oxidase-producing bacteria developed by other investigators [5,9,11,13,16,18]. The pEU5aML2201a- and pEU5aCH2201a-containing *E. coli* could potentially be used as producing strains for the commercially valuable melanins and cholesterol oxidase, respectively.

In the Gram-positive *S. thermophilus*, pEU5aCH2201a was shown to confer $ChoA^+$ phenotype to the host organism, suggesting that the ST_{P2201} promoter is fully functional in this bacterium. The specific enzyme activity detected in the partially fractionated cell extracts was lower than that observed with its *E. coli* counterpart (Table 2). One probable explanation is the difference in plasmid copy number of pEU5aCH2201a in the two host systems. Since the cloning vector pMEU5a relies on the replication functions of the high-copy-number pUC18 and the low-copy-number pER8 [28] for its maintenance in *E. coli* and *S. thermophilus*, respectively [21], the copy-number of pEU5aCH2201a in the former host is expected to be much higher than in the latter. As a result, ChoA would be synthesized in larger amounts in *E. coli* transformant than in *S. thermophilus*. In addition to the copy-number effect, differences in the extent of promoter strength, transcription regulation, mRNA stability, and translation attenuation experienced by

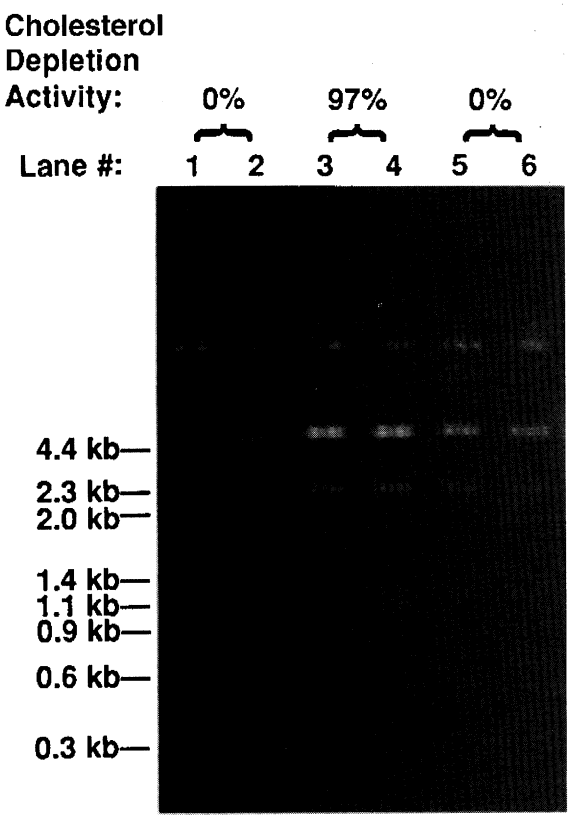

Figure 2 Plasmid stability and cholesterol depletion activity in *S. thermophilus* transformants. Batch cultures (200 ml) of *S. thermophilus* ST128 containing pEU5a2201a, pEU5aCH2201a or pEU5aCH2201b were grown overnight at 37° C with 100 rpm shaking. Prior to preparation of the cell-free extracts, duplicate 5-ml samples were removed from each culture for use in plasmid preparation. The isolated plasmids were digested with *Pvu*II and analyzed on a 1.5% (w/v) agarose gel (SeaKem ME, FMC BioProducts, Rockland, ME, USA); a mixture of lambda-DNA/*Hind*III + PhiX174 RF DNA/*Hae*III fragments were used as size markers (arrows). Cholesterol depletion activity of the cell-free extracts was assayed as follows: Centricon-10 fractions (60–70 μg proteins) from the *S. thermophilus* transformants were added to 2-ml assay mixtures containing 50 mM KP (pH 7.4), 0.1% (v/v) Triton X-100, and 50 μg ml^{-1} cholesterol. Reaction tubes were sealed with parafilm and incubated at 37° C with 100 rpm rotary shaking for 4 days. Residual cholesterol was extracted with ethylacetate and assayed colorimetrically as described under Materials and methods. The results are presented as a percentage of the added cholesterol depleted from the reaction mixtures, with the *S. thermophilus* [pEU5a2201a] sample (lanes 1 and 2) serving as the negative control (ie 0% cholesterol depletion). Lanes 1 and 2, ST128[pEU5a2201a]; 3 and 4, ST128[pEU5aCH2201a]; 5 and 6, ST128[pEU5aCH2201b]

pEU5aCH2201a in the two distinct hosts could not be ruled out.

Although tyrosinase activity was not detected in the cell extracts of *S. thermophilus* containing pEU5aML2201a plasmid, the ST_{P2201}-driven expression of the promoterless *mel*C operon could be inferred from the results of the Western hybridization study (Figure 1). A 30-kDa protein that complexed with the anti-tyrosinase antiserum was detected in the cell extracts of the Mel$^-$ *S. thermophilus* [pEU5a ML2201a] transformant; a similar band was also observed with a positive-control sample of Mel$^+$ *E. coli* [pMCU22-aPa] (data not shown). Furthermore, similar polypeptide was not detected with the negative control sample of *S. thermophilus* [pIL253-3306] in which tyrosinase production was minimal due to the weak ST_{P3306} promoter as shown in *E. coli* (data not shown). This protein band apparently represents the tyrosinase enzyme labeled as the MelC2 gene product by Chen *et al* [7]. In addition to the 30-kDa MelC2 band, the *S. thermophilus* sample also showed a hybridization signal at 46 kDa which was absent in the Mel$^+$ *E. coli* [pMCU22aPa] positive-control sample. The size of this protein species corresponds to the MelC1–MelC2 complex described earlier [7]. It appeared that the *S. thermophilus* transformant contained the binary complex which remained largely undissociated. It is not clear whether the lack of bioavailability of the added copper ion or the absence of other effector molecules was responsible for the failure of the complex to dissociate [7]. Western blots also revealed the presence in both the *E. coli* (data not shown) and *S. thermophilus* cell extracts of a 38-kDa polypeptide that cross-reacted with the anti-tyrosinase antiserum. The identity of this band is not known; the production of an unidentified fusion gene product from the recombinant plasmids is a distinct possibility.

The absence of detectable tyrosinase enzyme activity in the *S. thermophilus* cell extracts was likely due to the presence of some unknown inhibitory factor(s), although the production of an incorrectly folded polypeptide in this heterologous host remained a possibility. In fact, when *S. thermophilus* cell extract was added to reaction mixtures containing the Centricon-10 fraction from Mel$^+$ *E. coli* transformant, color development indicative of tyrosinase activity was markedly inhibited. A study is in progress to determine the possible cause(s) for the failure to detect tyrosinase activity in the *S. thermophilus* transformant.

The cloning of chromosomal promoters of *S. thermophilus* and other lactic acid bacteria had been reported previously [3,12,19,32]; description on their uses for expressing foreign genes was only mentioned in passing. The present study not only describes the cloning of a bifunctional promoter ST_{P2201}, but also characterizes the expression of *mel*C and *cho*A genes under the control of this promoter. Detailed sequence analysis of this useful promoter that is native to a food-grade bacterium is currently underway.

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