

EXPRESSION OF A RHODOCOCCAL INDIGO GENE IN *STREPTOCOCCUS THERMOPHILUS*

SUMMARY. A rhodococcal indole oxygenase gene (*ido*) catalyzing the biosynthesis of indigo pigment was cloned into *S. thermophilus* ST128. Expression vectors containing an *S. thermophilus* plasmid replicon (pER8) and promoters (sP1 and ST_{P2201}) were used as the cloning vehicles. Northern blot analysis confirmed the transcriptional expression of the *ido* gene into its mRNA in the ST transformants.

INTRODUCTION

Streptococcus thermophilus (ST) is an important industrial microbe used in the fermentation of yogurt and certain cheese varieties. Because of its generally-recognized-as-safe (GRAS) status, it is an attractive host system for expressing heterologous genes. Earlier efforts had entailed the use of non-ST cloning vectors for the introduction of foreign genes into this organism (Mercenier, 1990; Somkuti *et al.*, 1991 & 1993). We had recently constructed vectors using the replicon and promoter sequences native to ST, and had subsequently showed that they could be used to express heterologous genes such as cholesterol oxidase (*choA*) and tyrosinase (*melC*) in *S. thermophilus* (Somkuti *et al.*, 1995; Solaiman and Somkuti, 1995b). This manuscript describes the successful transfer and transcriptional expression of a rhodococcal indole oxygenase gene, *ido* (Hart *et al.*, 1990), into ST by using similar vectors. Since the ultimate product of *ido* is the indigo pigment, the oxygenase gene is useful as a chromogenic transformation marker (Hart and Woods, 1992) and for the production of a commercially valuable dye (Murdock *et al.*, 1993).

MATERIALS AND METHODS

Bacterial Growth and Plasmids

Escherichia coli DH5 α (BRL Life Technologies, Gaithersburg, MD) and *S. thermophilus* ST128 (laboratory collection) were hosts to the recombinant plasmids. *E. coli* was grown in Luria-Bertani (LB) broth (1% w/v tryptone and 0.5% w/v each of yeast extract and NaCl) containing ampicillin (100 μ g/ml), chloramphenicol (15-35 μ g/ml) and/or erythromycin (200 μ g/ml) as needed. *S. thermophilus* was cultured in a tryptone-yeast extract-lactose broth (Somkuti and Steinberg, 1979) containing erythromycin (10-15 μ g/ml) and/or chloramphenicol (5 μ g/ml). Plasmid pSLH8 containing the rhodococcal *ido* gene (Hart and Woods, 1992) was obtained from ATCC (Rockville, MD). The construction of the cloning vector pER82Pb (Somkuti *et al.*, 1995) and the chimeric plasmid pEU5aML2201a (Solaiman and Somkuti, 1995b) had been described earlier.

Molecular Cloning Procedures

Restriction endonucleases, DNA-modifying enzymes and T4 DNA ligase were obtained from and used according to the protocols of the following vendors: BRL Life Technologies, New England BioLabs (Beverly, MA) and United States Biochemical (Cleveland, OH). Methods for the isolation of plasmids from *E. coli* (Solaiman and Somkuti, 1993) and *S. thermophilus* (Somkuti and Steinberg, 1986) had been described. Further purification of the plasmid DNAs was performed using the Elutip-d columns (Schleicher & Schuell, Keena, NH). The recombinant plasmids were introduced into competent *E. coli* by heat shock protocol as detailed by the supplier, and into *S. thermophilus* by an electrotransformation procedure described previously (Somkuti and Steinberg, 1987). Electrophoresis of DNA was performed in agarose gels using the TBE buffer system (0.089M Tris, 0.089M boric acid, 0.002M NaEDTA).

Northern Blot Analysis

Total RNA of *S. thermophilus* transformants was isolated according to the method of Chomczynski and Sacchi (1987) by using the commercially available TRIzol reagent (BRL Life Technologies). Growth medium (35-45 ml) inoculated with overnight culture of *S. thermophilus* was incubated for 4 hrs at 42°C with 100-rpm rotary shaking. Cells were collected by centrifugation from 25 ml of the culture and resuspended in 2.5 ml of a mutanolysin solution (1.6 U/ μ l; Sigma Chemical Co., St. Louis, MO). Lysozyme (Sigma Chemical Co.) was added to the cell suspension to a final concentration of 10 mg/ml. After an incubation period of 15 min at 37°C, the protoplasts were sedimented by centrifugation. The pellet was resuspended in 1.5 ml of the TRIzol reagent, and subsequent steps for isolating the RNA were performed according to the instruction provided by the supplier. Final RNA pellet was resuspended in 50-100 μ l of H₂O.

The electrophoresis of RNA was performed on a 1.2% agarose/0.37 M

formaldehyde gel in MOPS buffer system (Selden, 1992). Biotinylated lambda/*Hind*III (BRL Life Technologies) and PhiX174/*Hae*III (New England Biolabs) fragments, and the 0.24-9.5 kb RNA ladder (BRL Life Technologies) were used as size markers. The gel electrophoretically resolved RNAs were transferred overnight onto a Nytran membrane (Schleicher & Schuell) in 20xSSC buffer as described (Selden, 1992). Hybridization and chemiluminescent detection were performed using the Rad-Free[®] Probe Labeling and Detection System (Schleicher & Schuell). A 700-bp *Sty*I/*Sph*I fragment of pSLH8 containing the *ido* coding sequence was biotinylated and used as the hybridization probe.

RESULTS

Cloning of *ido* into pER8-based vectors

The construction of the ST expression vector, pER82Pb (4.8 kb; Fig. 1), has been detailed previously (Somkuti *et al.*, 1995). The entire pSLH8 plasmid (Fig. 1) linearized with *Pst*I was spliced into the same restriction site of pER82Pb to generate

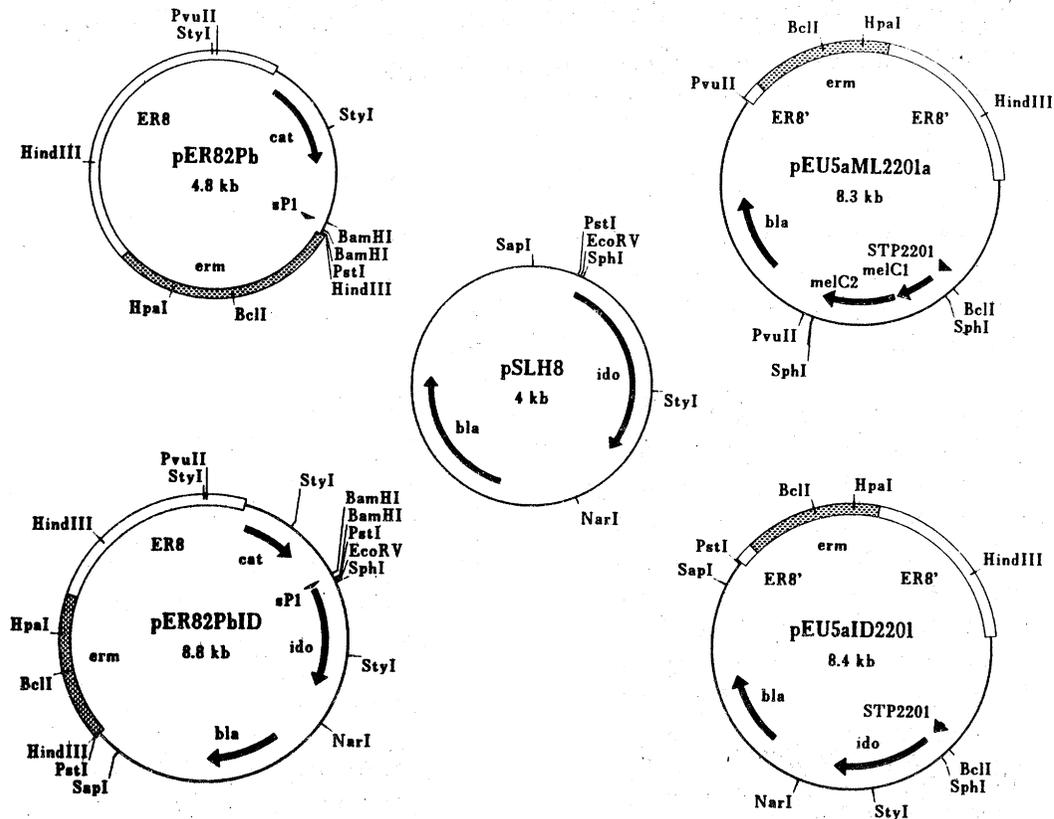


Fig. 1. Restriction Maps of Plasmids. Restriction sites shown were not exhaustive.

pER82PbID (8.8 kb; Fig. 1). In this construct, the *ido* gene is downstream and aligned with the sP1 promoter.

The construction of the *mel*-expressing plasmid, pEU5aML2201a (8.3 kb; Fig. 1), has also been reported (Solaiman and Somkuti, 1995b). In the current study, a 4.5-kb DNA sequence that contains the *S. thermophilus* replicon (ER8) and promoter (ST_{P2201}) and an *erm* selection marker was isolated from pEU5aML2201a by double-digestion with *Pvu*II and *Sph*I. This fragment was directionally ligated with the large, 3.9-kb *Sph*I/*Eco*RV fragment of pSLH8 to generate the pEU5aID2201 recombinant (8.4 kb; Fig. 1). The ST_{P2201} promoter is upstream and aligned with the *ido* gene in pEU5aID2201.

Transcriptional Expression of *ido* in *S. thermophilus*

The Northern hybridization analysis of the total RNA of *S. thermophilus* ST128[pER82PbID] showed that two transcripts (ca. 2.7 and 1.2 kb) complementary to the *ido* probe were present (Fig. 2, lane 1). The RNA nature of the two

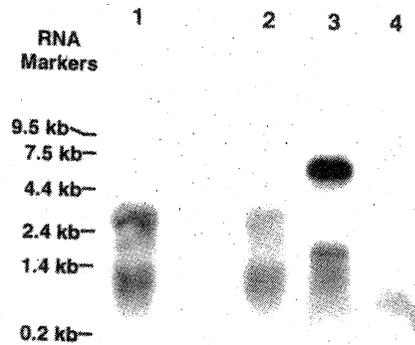


Fig. 2. Northern blot analysis of RNAs from *S. thermophilus* ST128 harboring pER82PbID. The position of the RNA size markers were indicated. *Lane 1:* Total RNA (2 μ l) of ST128[pER82PbID] transformant; *lane 2:* RNA of ST128[pER82PbID] + RNase-free DNase (5 U; Epicentre Technologies, Madison, WI); *lane 3:* Same as *lane 1*, but with 1.12 μ g of *Sph*I-linearized pSLH8 plasmid; *lane 4:* Same as *lane 3*, but with DNase (5 U).

transcripts was confirmed by their susceptibility to RNase action (data not shown) and resistance to DNase digestion (Fig. 2, lane 2). Similar DNase reaction conditions completely digested the pSLH8 plasmid added as internal control (Compare lanes 3 and 4, Fig. 2). These results conclusively showed that pER82PbID directed the transcriptional expression of *ido* in the ST transformant.

In a separate Northern blot experiment, the RNA preparations of ST128[pER82PbID], ST128[pEU5aID2201] and ST128[pER82Pb] were compared (data not shown). As expected, the negative-control sample ST128[pER82Pb] did not produce any *ido*-hybridizing transcripts. Similar to the case with the pER82PbID-transformed cells, however, the ST128[pEU5aID2201] transformant also produced the ca. 2.7 and 1.2 kb mRNAs that hybridized with the *ido* probe. Image analysis of the Northern blot signals standardized to the relative concentrations of the rRNAs (23S and 16S), showed that the two ST transformants produced approximately the same amounts of the 1.2- and 2.7-kb *ido*-hybridizing transcripts.

DISCUSSION

S. thermophilus electrotransformed with pEU5aID2201 and pER82PbID synthesized RNA transcripts of the *ido* gene. Based on the size of the indole oxygenase gene, the ca. 1.2-kb RNA species most likely represents the true transcript of *ido*. The identity of the large mRNA (ca. 2.7-kb) is not known. Based on its size and the maps of the plasmids, however, it very likely represents a read-through transcript that stops at the termination site of the downstream *bla* gene. Although the mRNA transcripts of *ido* were synthesized, indigo pigment production was not apparent in these ST transformants. The possible reasons include an ineffective translation of the transcripts, improper folding of the recombinant polypeptides, or the requirement for accessory metabolic activities as reported for *E. coli* (Hart *et al.*, 1992).

The *ido*-containing chimeric plasmids appeared to be stable in *S. thermophilus*; deletion species was not apparent in the agarose gel electrophoresis of the plasmid preparations. The pEU5aID2201 construct is highly unstable in *E. coli*, where the entire sequence originating from pEU5aML2201a (approximate coordinates of 7250-

bp to 3250-bp clockwise, Fig. 1) was deleted on subsequent culture transfer to fresh medium. The deletion event was accompanied by the concomitant loss of pigmentation of the *E. coli* transformant. In the case with pER82PbID, however, the *E. coli* transformant was not pigmented regardless of the stability of the plasmid. These results agreed with the earlier observations that sP1 was not a strong promoter in *E. coli* (Solaiman and Somkuti, 1995a).

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