

Construction of Lactose-Utilizing *Xanthomonas campestris* and Production of Xanthan Gum from Whey

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Xanthomonas campestris pv. *campestris* possesses a low level of β -galactosidase and therefore is not able to grow and produce significant amounts of xanthan gum in a medium containing lactose as the sole carbon source. In this study, a β -galactosidase expression plasmid was constructed by ligating an *X. campestris* phage ϕ LO promoter with pKM005, a ColE1 replicon containing *Escherichia coli lacZY* genes and the *lpp* ribosome-binding site. It was then inserted into an IncP1 broad-host-range plasmid, pLT, and subsequently transferred by conjugation to *X. campestris* 17, where it was stably maintained. The *lacZ* gene under the control of the phage promoter was expressed at a high level, enabling the cells to grow in a medium containing lactose. Production of xanthan gum in lactose or diluted whey by the engineered strain was evaluated, and it was found to produce as much xanthan gum in these substrates as the cells did in a medium containing glucose.

Xanthomonas campestris pv. *campestris* is an agriculturally and industrially important bacterium. In agriculture, it causes black rot in crucifers, resulting in tremendous economic losses worldwide (22). On the other hand, this bacterium produces xanthan gum, which has many applications in the food, cosmetics, and oil industries (17). Xanthan is a high-molecular-weight exopolysaccharide composed of a cellulosic backbone with trisaccharide side chains attached to alternate glucose residues in the backbone. The side chains are composed of two mannose and one glucuronic acid molecule (9, 15). Owing to its high viscosity, stable properties in extreme chemical and physical environments, and pseudoplastic behavior, this biopolymer has a variety of applications as a stabilizing, viscosifying, emulsifying, thickening, and suspending agent (10, 17).

For the production of xanthan, medium containing glucose, sucrose, or starch as a carbon source is normally used. Due to the low level of β -galactosidase present in *X. campestris*, the bacterium cannot use lactose as an efficient carbon source. Consequently, the bacterium grows poorly and produces little xanthan in a medium containing lactose as the sole carbon source (7).

Whey is a nutrient-rich dairy byproduct which contains 4 to 5% lactose, 0.8 to 1% proteins, minerals, and some small organic molecules (2). It is produced by the dairy industry in such quantities annually that its proper disposal has long been a major problem. The most desirable way of handling this waste is to utilize it as a substrate for production of useful products, such as xanthan. To accomplish this goal, *X. campestris* strains that can efficiently utilize lactose have to be obtained.

In this article, we report the cloning of *Escherichia coli lacZY* genes downstream from the *X. campestris* phage ϕ LO promoter. *X. campestris* cells containing the recombinant plasmid are able to grow and produce xanthan gum with whey as the growth substrate.

(A preliminary report of this work has been presented [J.-F. Fu and Y.-H. Tseng, Abstr. Third Annu. ASM Conf. Biotechnol. 1988, O12, p. 8].)

MATERIALS AND METHODS

Bacteria, plasmids, and phage. The bacterial strains, plasmids, and phage used in this study are listed in Table 1.

Media and cultivation. LB medium (13) was used as a general-purpose medium. XOLN medium plus 0.4% glucose or lactose was used for measurements of cell growth, β -galactosidase activity, and xanthan production. XOLN medium contained (per liter): K_2HPO_4 , 0.7 g; KH_2PO_4 , 0.2 g; $(NH_4)_2SO_4$, 1.0 g; $MgCl_2 \cdot 6H_2O$, 0.1 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $MnCl_2$, 0.001 g; 0.0625% yeast extract; 0.0625% tryptone, pH 7.15. The last two components were added to the medium after autoclaving. *E. coli* and *X. campestris* were cultured at 37 and 28°C, respectively. For liquid cultures, bacteria were grown in 250-ml flasks containing 40 ml of liquid medium with vigorous shaking. Antibiotics were used at concentrations of 50 μ g/ml for ampicillin and 15 μ g/ml for tetracycline.

Preparation of phage ϕ LO and phage DNA. *X. campestris* P20 was inoculated into 40 ml of LB (initial OD_{550} , 0.3), grown to mid-log phase (about 10 h), infected with phage ϕ LO at a multiplicity of 10, and cultured until the cells were lysed. The lysate was centrifuged at $10,000 \times g$ for 10 min at 4°C, and the supernatant was filtered (0.45- μ m pore size; Millipore Corp.). The phage particles in the filtrate were then precipitated with 10% polyethylene glycol (PEG) 6000 at 4°C for 60 min. The solution was centrifuged at $12,000 \times g$ for 10 min, and the phage pellet was suspended in 4 ml of STE buffer (13). The resulting solution was then mixed with an equal volume of chloroform to remove the PEG. The upper aqueous phase was then extracted three times with phenol-chloroform to remove phage coat protein. Phage DNA was then precipitated with ethanol and suspended in 10 mM Tris hydrochloride-1 mM EDTA, pH 8.0.

DNA isolation, cloning, and analysis. Plasmid DNA was prepared by the alkaline method of Birnboim and Doly (1).

Restriction endonucleases and ligase were purchased from Bethesda Research Laboratories, Inc., New England Biolabs, Inc., and Boehringer-Mannheim GmbH-Biochemica and were used as recommended by the suppliers. Electrophoresis was carried out on 0.7% agarose horizontal gels in TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0) (13).

E. coli transformation was conducted by the method of

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TABLE 1. Bacteria, plasmids, and phage

Strain, plasmid, or phage	Phenotype or description	Source or reference
<i>X. campestris</i> pv. <i>campestris</i>		
17	Wild-type strain; isolated in Taiwan	25
11A	Nonpathogenic mutant of XC11; Ap ^r	25
P20	Nonmucoid mutant of XC11A; isolated by NO ₂ ⁻ mutagenesis; Ap ^r	23
<i>E. coli</i> CSH50	<i>ara</i> Δ(<i>lac-pro</i>) <i>strA thi</i>	16
Plasmids		
pLAFR1	Tc ^r Tra ⁻ Mob ⁺	8
pRK2013	Km ^r Tra ⁺ Mob ⁺	6
pKM005	A 9.9-kb derivative of pBR322 carrying <i>E. coli lacZY</i> genes and <i>lpp</i> ribosome-binding site but no promoter (cloning of a promoter will express the β-galactosidase activity.)	14
pKMφ	β-Galactosidase expression derivative of pKM005 carrying a phage φLO promoter-possessing fragment (1.6 kb) inserted upstream of <i>lacZY</i> genes; maintained in <i>E. coli</i> but not in <i>X. campestris</i>	This study
pLT	22.1 kb, derived from the broad-host-range, mobilizable vector pLAFR1 by inserting a 0.5-kb fragment of <i>X. campestris</i> DNA which carried a unique <i>Pst</i> I site	This study
pKMφLT	Recombinant of pKMφ, the β-galactosidase expression plasmid, and the broad-host-range plasmid pLT ligated at the <i>Pst</i> I sites; maintained in both <i>E. coli</i> and <i>X. campestris</i>	This study
Phage φLO	Tadpole-shaped phage of <i>X. campestris</i>	This laboratory

Cohen et al. (3). Transformants were selected on MacConkey agar (Difco) containing ampicillin.

Transfer of plasmid DNA by conjugation. Plasmid pKMφLT was transferred by conjugation from *E. coli* CSH50 into *X. campestris* 17 with the help of pRK2013 (6) as described by Yang et al. (24). Transconjugants were selected on XOLN medium containing 0.4% lactose, tetracycline (15 μg/ml), and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; 40 μg/ml). Blue colonies were selected for further analysis.

Stability test of recombinant plasmid. Transconjugant *X. campestris* 17(pKMφLT) was plated on LB agar containing tetracycline (15 μg/ml). A Tc^r colony was picked and grown in LB broth containing tetracycline for 16 h. The cells were harvested and inoculated into fresh LB without antibiotic, grown till saturation (about four generations), and then diluted into fresh LB medium for further growth. The subculturing was repeated until the cells had undergone at least 10 more divisions. Then the cells were spread out on LB agar without tetracycline, and 200 colonies were patched on LB agar containing X-gal with or without tetracycline.

Enzyme assays and protein determination. β-Galactosidase activity was assayed by the method described by Miller (16). To 0.1 to 0.5 ml of culture, Z buffer (16) was added to a final volume of 1.0 ml. One drop of toluene was added, and the mixture was vortexed vigorously for 10 s. The solution was then shaken at 37°C for 40 min to evaporate the toluene was used as the enzyme source. The enzyme and 0.2 ml of *o*-nitrophenyl-β-D-galactoside (ONPG) (4 mg/ml) were prewarmed separately at 28°C for 5 min, and then the reaction was started by adding the substrate to the enzyme solution. The enzyme reaction was terminated by adding 0.5 ml of 1 M Na₂CO₃ after 5 to 10 min of incubation. The mixture was centrifuged at 12,000 × *g* for 5 min to remove the cells, and the yellow color developed in the supernatant was read at 420 nm with a spectrophotometer. One unit of enzyme was defined as the amount that was required to produce 1 nmol of *o*-nitrophenol per min.

Protein concentration was determined by the method of Lowry et al. (12), with crystalline bovine serum albumin as the standard.

Estimation of xanthan concentration. The amounts of xan-

than were measured by the procedures described previously with some modification (11). The cultures were diluted 2- to 10-fold with distilled water and centrifuged at 12,000 × *g* for 20 min to remove the cells. The exopolysaccharide in the supernatant was precipitated in the presence of 40 mM NaCl with 70% ethanol at -20°C overnight. The solution was then centrifuged at 20,000 × *g* for 30 min. The pellet was washed one time with 70% ethanol and resuspended in distilled water. The amount of xanthan was measured by the anthrone method (11).

RESULTS

Construction and stability of pKMφLT. To construct a lactose-utilizing *X. campestris* strain, a β-galactosidase expression plasmid, pKMφLT, was constructed in *E. coli* and then conjugally transferred into *X. campestris* 17. Phage φLO DNA was cut with *Xba*I and ligated with *Xba*I-digested plasmid pKM005 (14), which carries the *E. coli lacZY* coding region with the *lpp* ribosome-binding site but no promoter. The mixture was used to transform *E. coli* CSH50, and the transformants were grown on MacConkey agar (Difco) containing ampicillin. Transformants giving rise to red colonies, indicating lactose utilization and cloning of a phage promoter, were analyzed for β-galactosidase activity. One of the transformants grew well in XOLN medium containing lactose as the sole carbon source and expressed a high level of β-galactosidase (1,564 U/mg of protein). This transformant contained an 11.5-kilobase (kb) plasmid, which was designated pKMφ. The construction of pKMφ and the restriction map of the insert (1.6 kb) are shown in Fig. 1. Plasmid pKMφ was then inserted into a broad-host-range plasmid, pLT (Table 1). The resulting plasmid, pKMφLT (Fig. 2), was transferred into *X. campestris* 17 via conjugation. Ninety-nine percent of the exconjugant cells formed blue colonies on X-gal plates and were Tc^r after subculturing for more than 14 generations. In other words, plasmid pKMφLT was maintained with high stability in *X. campestris* 17.

Growth of *X. campestris* 17(pKMφLT) in lactose medium. *X. campestris* 17 and *X. campestris* 17(pKMφLT) grew equally well in glucose medium. The generation time for

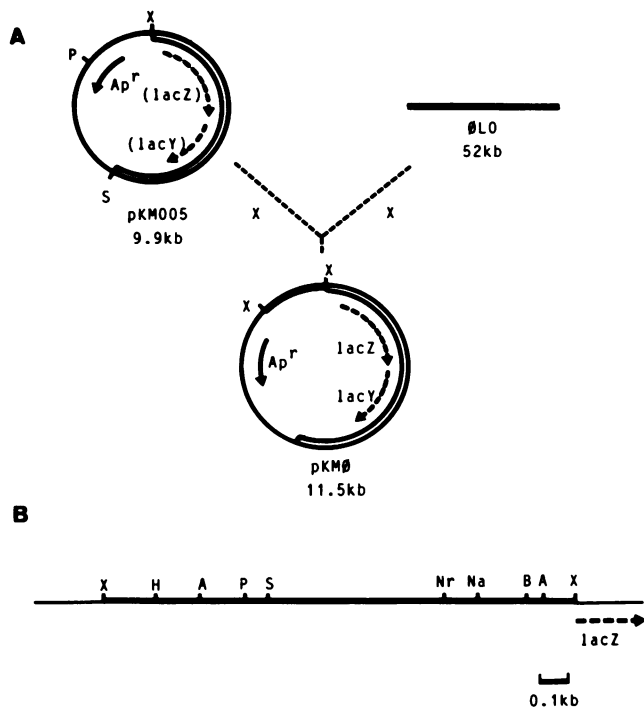


FIG. 1. Construction of β -galactosidase expression plasmid pKM ϕ from pKM005 and phage ϕ LO DNA (A), and the restriction map of the 1.6-kb insert possessing promoter activity (B). Abbreviations for restriction enzymes: (A) P, *Pst*I; S, *Sma*I; X, *Xba*I. (B) A, *Acl*I; B, *Bsp*HI; H, *Hpa*I; Na, *Nae*I; Nr, *Nru*I; P, *Pst*I; S, *Sma*I; X, *Xba*I.

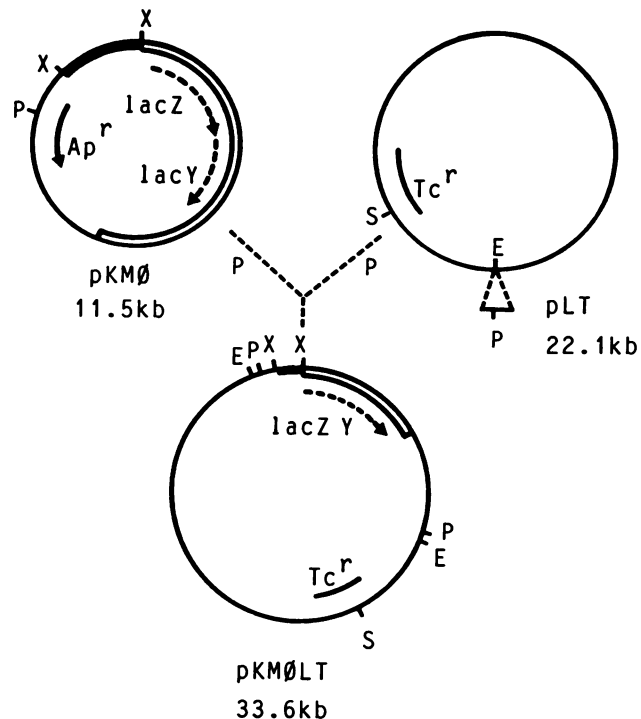


FIG. 2. Insertion of pKM ϕ into the broad-host-range plasmid pLT at the *Pst*I site, forming plasmid pKM ϕ LT. Abbreviations are the same as in Fig. 1, plus E, *Eco*RI.

both strains was approximately 3 h. While *X. campestris* 17 ceased growing after about one generation in lactose medium, *X. campestris* 17(pKM ϕ LT) grew almost as well as in glucose medium (Fig. 3).

Expression of β -galactosidase in *E. coli* CSH50 and in *X. campestris* 17. *E. coli* CSH50 and *X. campestris* 17 showed low β -galactosidase activity (7 and 25 U, respectively) in XOLN medium with 0.4% glucose. On the other hand, *X. campestris* 17(pKM ϕ LT) exhibited 4,290 U. This was approximately 25% of the level found in *E. coli* CSH50(pKM ϕ LT) (16,711 U) but 170-fold higher than the specific activity of the parent strain *X. campestris* 17. Since the *lac* genes were under the control of a phage promoter, neither 0.4% glucose nor 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) in the medium would affect the levels of β -galactosidase. In other words, the *lac* genes were expressed constitutively.

Xanthan production by *X. campestris* 17(pKM ϕ LT) in lactose medium and in whey. *X. campestris* 17 produced 3,530 μ g of xanthan per ml in a medium containing glucose, but only 245 μ g/ml in a medium containing lactose (Table 2). However, the presence of the *lacZY* genes in *X. campestris*(pKM ϕ LT) caused the bacterium to utilize lactose and consequently synthesize as much xanthan as glucose-grown cells did (Table 2). A similar result was obtained when the cells were cultured in a medium containing 10% whey as the carbon and nitrogen source (Table 2).

DISCUSSION

Somkuti and colleagues (7, 21) reported that β -galactosidase levels are low in *X. campestris*. The results presented

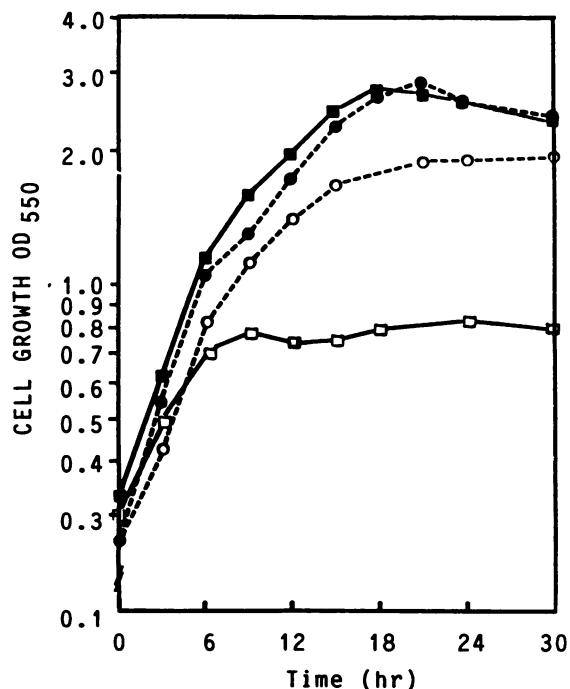


FIG. 3. Growth of *X. campestris*. Cells from an overnight culture were harvested by centrifugation, resuspended, and then inoculated into XOLN medium containing 0.4% glucose or lactose. Symbols: □, strain 17 in lactose; ○, 17(pKM ϕ LT) in lactose; ■, 17 in glucose; ●, 17(pKM ϕ LT) in glucose.

TABLE 2. Xanthan production by *X. campestris*^a

<i>X. campestris</i> strain	Xanthan produced ^a (μg/ml)		
	XOLN + 0.4% glucose	XOLN + 0.4% lactose	10% whey ^b
17	3,530	245	224
17(pKMϕLT)	3,711	3,608	4,241

^a Amounts of xanthan produced are expressed as micrograms per milliliter of culture. The values represent the average of five independent determinations.

^b The whey was diluted and buffered with 0.055 M potassium phosphate, pH 7.15.

here confirmed their results. Because of this low concentration, *X. campestris* 17 was not able to grow for more than one generation in a lactose medium (Fig. 3). Therefore, attempts to use lactose-based substrates, such as whey, for gum production by *X. campestris* 17 would be difficult unless a functional lactose-utilizing system is introduced into and maintained in the bacterium.

Previously, attempts have been made by several groups to construct lactose-utilizing *X. campestris* strains for xanthan production. Schwartz and Bodie (18) were able to select a strain which can utilize lactose for xanthan production, but the strain was not stable. Walsh et al. (21) mobilized Tn951 (4), a transposon containing the *lac* genes, by conjugation from *E. coli* into *X. campestris*. This strain expresses high levels of β-galactosidase and grows well in lactose medium. However, IPTG is required to induce the *lac* genes and antibiotic is required to maintain the plasmid. Xanthan production by this strain was not examined. Recently, Thorne et al. (20) cloned and inserted the *lac* genes from Tn951 into the *X. campestris* chromosome to form a stable recombinant strain. Although the level of β-galactosidase activity was not given, the xanthan produced from whey lactose by this strain was comparable in quality and quantity to the product of wild-type *X. campestris* growing in the presence of glucose.

In this study, a mobilizable β-galactosidase expression plasmid, pKMϕLT (Fig. 2), was constructed. When this plasmid was introduced into *X. campestris* 17, the bacterium was able to produce xanthan from whey in amounts similar to those produced by glucose-grown cells (Table 2). The major parts of this construct are the *lacZY* structural genes, together with an *lpp* ribosome-binding site (14), ligated with a promoter region from *X. campestris* phage ϕLO. The vector parts are composed of a ColE1 replicon, the pKM005 (14) moiety, and an RK2 replicon (19), the pLT moiety. The level of β-galactosidase in *X. campestris* 17(pKMϕLT), expressed constitutively, was 170-fold higher than in the parent strain (4,290 versus 25 U). To our surprise, we found that with such a construct, the expression of β-galactosidase in *E. coli* was higher than it was in *X. campestris* 17 (16,711 versus 4,290 U). This result indicates that this *X. campestris* phage promoter functions well in *E. coli*. It was surprising to find that expression of *lacZY* genes was relatively low in *X. campestris*, possibly because the *lpp* ribosome-binding sequence does not function well in *X. campestris* 17, transcription-translation of this DNA is less efficient in *X. campestris* than in *E. coli*, or β-galactosidase is less stable in *X. campestris* 17 than in *E. coli*. Owing to the differences in copy number, the β-galactosidase activity expressed in *X. campestris*(pKMϕLT) should be higher than the activity expressed in the cells with integrated *lac* genes constructed by Thorne et al. (20). The recombinant plasmid was stable without selective pressure for 14 generations, which is well

over the number of generations required for xanthan production by fermentation.

Other applications of the β-galactosidase expression plasmid pKMϕLT may also be found. In this regard, we have successfully mobilized it into four species of plant bacteria, and the plasmid was stably maintained (unpublished data). By using a monitoring system like that developed by Drahos et al. (5) for detecting engineered *Pseudomonas fluorescens* on X-gal plates, the possible usefulness of the transconjugants is being evaluated for studying the spread of plant diseases caused by these bacteria. Furthermore, due to the mobilization property and the stability of pKMϕLT in different bacteria, the plasmid is potentially useful for the construction of lactose-utilizing industrial bacteria other than *X. campestris* for production of valuable products from whey.

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