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Genetic engineering of soft-rot bacteria for ethanol production from lignocellulose

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SUMMARY

The soft-rot bacteria *Erwinia carotovora* SR38 and *Erwinia chrysanthemi* EC16 have been genetically engineered to efficiently produce ethanol and carbon dioxide as primary fermentation products from cellobiose, glucose and xylose. These organisms have the native ability to secrete a battery of hydrolases and lyases to aid in the solubilization of lignocellulose. Both strains of ethanologenic *Erwinia* fermented cellobiose at twice the rate of the cellobiose-utilizing yeasts (Spindler et al., 1992. Biotechnology Letters 14: 403–407) and may be useful in simultaneous saccharification and fermentation processes.

INTRODUCTION

The solubilization of lignocellulosic biomass by chemical or enzymatic means is a prerequisite for microbial fermentation to ethanol. A cadre of enzymes is needed to depolymerize cellulose, hemicellulose and associated carbohydrates. No organisms are known from nature that rapidly and efficiently produce ethanol from this feedstock. Many plant pathogenic bacteria such as *Erwinia carotovora* and *E. chrysanthemi* have evolved sophisticated systems of hydrolases and lyases to macerate and penetrate plant tissues [11]. However, these organisms produce a mixture of acidic and neutral fermentation products [13] which are of little value. Other organisms, such as *Zymomonas mobilis* and *Saccharomyces cerevisiae*, efficiently ferment glucose to ethanol but produce no enzymatic activities to aid cell wall dissolution.

Many studies have been reported in which cloned genes encoding glycoside hydrolases have been expressed in *Z. mobilis* or *S. cerevisiae* to develop improved organisms for fermentation [3,7]. The genetic engineering of ethanol production in organisms which produce native hydrolases represents an equally attractive alternative approach. Initial studies were only partially successful in redirecting fermentative metabolism in *Erwinia chrysanthemi* [17], *Klebsiella planticola* [18] and *E. coli* [4] by inserting the *Z. mobilis* gene encoding pyruvate decarboxylase (*pdc*). By also including the *Z. mobilis adhB* gene, recombinants of *E. coli* [10] and *K. oxytoca* [12,19] have been constructed which efficiently ferment a variety of sugars to ethanol. This was accomplished by assembling both *Z. mobilis* genes into an artificial operon to produce a portable genetic element for ethanol production (PET operon). In these recombinant organisms, both enzymes (pyruvate decarboxylase and alcohol dehydrogenase) needed to divert pyruvate metabolism to ethanol are present at high levels. The high level of pyruvate decarboxylase and the low apparent K_m of this enzyme for pyruvate effectively divert carbon flow to ethanol even in the presence of native fermentation enzymes like lactate dehydrogenase.

In this study, we have constructed derivatives of the soft-rot bacteria *E. carotovora* and *E. chrysanthemi* which contain the PET operon. These recombinants produced ethanol and secreted hydrolases which may aid in the conversion of lignocellulose to ethanol.

MATERIALS AND METHODS

Organisms and growth conditions

E. carotovora SR38 and *E. chrysanthemi* EC16 [6] were generously provided by Dr. J.F. Preston (University of Florida, Gainesville, FL) [14]. The plasmids pLOI295 [10], pLOI297 [1], and pLOI555 [12] which contain PET operons have been previously described. All organisms were maintained as frozen stocks in 40% glycerol. Unmodified strains were grown in Luria broth lacking sugar [15]. Luria broth or agar containing from 20–100 g/l sugar

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was used for the growth of strains containing PET operons. Ampicillin (50 mg/l), tetracycline (10 mg/l), and chloramphenicol (40 mg/l) were used for selection and maintenance of recombinants. Aldehyde indicator plates were used to confirm high level expression of *Z. mobilis adhB* [10].

Transformation

Strain EC16 was transformed by the calcium chloride method [15]. Strain SR38 was transformed by the method of Hinton et al. [9].

Plasmid stability

Plasmid stability was tested by serially transferring plasmid-bearing strains into fresh medium at 24-h intervals in the absence of antibiotics. Samples were diluted and spread on solid medium lacking antibiotics. Colonies were tested for the retention of the antibiotic markers and ethanologenic trait (large colony phenotype, positive reaction on aldehyde indicator plates). All traits were retained and lost concurrently.

Fermentations

Fermentations were carried out in Luria broth supplemented with chloramphenicol (40 mg/l) and carbohydrate (100 g/l) at pH 6.0, 30 °C, 100 rpm using an inoculum of 1 $OD_{550\,nm}$ of cells (approx. 330 mg cell dry weight per liter) as previously described for *E. coli* [2]. Samples were removed to measure cell mass as $OD_{550\,nm}$ and ethanol concentration [8]. Completion of carbohydrate fermentation was evidenced by a rise in pH due to the catabolism of complex media constituents with the release of ammonia.

Hydrolases

Indicator plates were used to evaluate the production of extracellular enzymes capable of hydrolyzing components of lignocellulose. Strains were tested for their ability to hydrolyze methylumbelliferyl β-D-glucopyranoside, methylumbelliferyl β-D-cellobioside, methylumbelliferyl β-D-mannopyranoside, methylumbelliferyl β-D-galactopyranoside, methylumbelliferyl β-D-xylopyranoside, and methylumbelliferyl α-L-arabinoside (20 mg/l). Activity was evident as bright fluorescent zones under ultraviolet light. Carboxymethyl cellulase activity was measured by the Congo red method [20]. Hydrolyzed zones of *S. cerevisiae* mannan, larch wood arabinogalactan, oat spelt xylan, and potato starch were detected by exposure of plates containing 3 g substrate/l to iodine vapor after incubation for 48 h. Cultures were also screened for proteinases using Luria agar containing 20 g/l of nonfat, powdered milk. Xylan was purchased from the Fluka Chemical Company (Ronkonkoma, NY). All other carbohydrates were purchased from the Sigma Chemical Company (St. Louis, MO).

TABLE 1

Stability of plasmids containing PET operons in *Erwinia* sp.

Strain (plasmid)	Percentage retaining plasmid (generations)	
	24 h	120 h
SR38 (pLOI295)	100 (30)	21 (86)
SR38 (pLOI297)	97 (29)	18 (86)
SR38 (pLOI555)	100 (29)	90 (86)
EC16 (pLOI295)	100 (30)	30 (87)
EC16 (pLOI297)	72 (32)	0 (86)
EC16 (pLOI555)	100 (25)	67 (85)

RESULTS AND DISCUSSION

Strain stability

Transformants were readily obtained in both strains of *Erwinia* with all three plasmids containing the PET op-

TABLE 2

Lignocellulose-degrading activity in *Erwinia* sp.[a]

Organism	Methylumbelliferyl glucosides[b]					Carbohydrate polymers[c,d]			
	Cel	Glu	Xyl	Ara	Man	CMC	Xyl	A/G	Man
SR38 (pLOI555)	–	+	+	–	–	3	18	21	33
EC16 (pLOI555)	–	–	+	–	–	3	32	23	32

[a] Activities are reported after 48 h.
[b] Abbreviations: Cel, celllobioside; Glu, glucoside; Xyl, xyloside; Gal, galactoside; Man, mannoside.
[c] Abbreviations: CMC, carboxymethyl cellulose; Xylan, oat xylan; A/G, larch wood arabinogalactan; Man, *S. cerevisiae* mannan.
[d] Diameters of cleared zones on indicator plates are reported in millimeters.

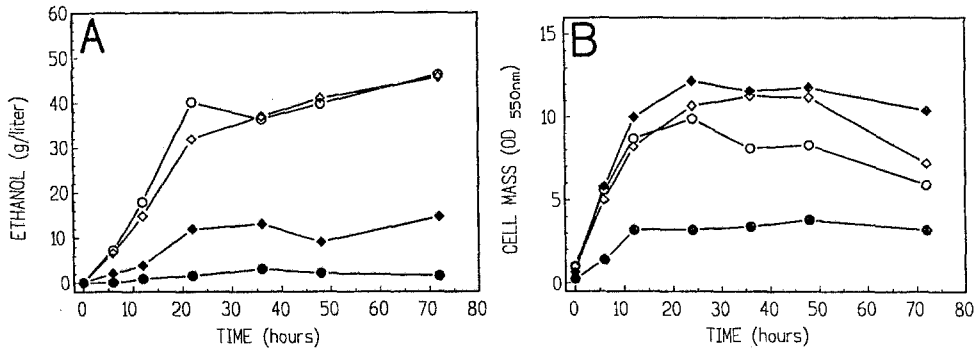

Fig. 1. Fermentation of glucose (100 g/l). A. Ethanol. B. Cell mass. Symbols: ●, SR38; ○, SR38 (pLOI555); ◆, EC16; ◇, EC16 (pLOI555).

eron. All produced large, raised colonies after overnight incubation and all expressed high levels of aldehyde activity on indicator plates. However, these plasmids varied considerably in their stability (Table 1). All plasmids were more stable in SR38 than in EC16. Plasmid pLOI555, which is derived from a cryptic *E. coli* B plasmid [12], was the most stable in both strains. Constructs containing pLOI555 were evaluated in fermentation experiments and for the production of hydrolases.

Hydrolases

The strains used in this study exhibited a wide variety of hydrolase activities (Table 2). The activities present in both recombinants were very similar and degraded all natural polymers tested. Neither produced large zones on carboxymethyl cellulose plates. The apparent absence of arabinosidase and mannosidase activities as evidenced by the lack of hydrolysis of methylumbelliferyl substrates is consistent with the intracellular hydrolysis of dimers and

TABLE 3

Summary of fermentations by ethanologenic *Erwinia* sp.

Organism	Maximum ethanol (g/l)	Ethanol yield (g/g sugar)[a]	Theor. ethanol yield (%)[a]	Time to 90% yield[b] (h)	Max. cell mass ($OD_{550\,nm}$)	Base consumed (mmol/l)
Glucose (100 g/l)						
SR38	3.0	0.03	6	nd	3.8	320
SR38 (pLOI555)	46.2	0.48	94	62	9.9	91
EC16	13.0	0.14	28	nd	11.8	97
EC16 (pLOI555)	45.7	0.48	94	60	11.3	91
Cellobiose (100 g/l)						
SR38 (pLOI555)	51.2	0.54	100	33	13.2	97
EC16 (pLOI555)	49.4	0.52	96	33	14.2	103
Xylose (100 g/l)						
SR38 (pLOI555)	38.3	0.40	78	nd	15.0	103
EC16 (pLOI555)	39.0	0.41	80	nd	14.9	97
Xylose (80 g/l)						
SR38 (pLOI555)	38.1	0.50	98	20	13.7	109
EC16 (pLOI555)	40.7	0.55	108	18	12.0	166

[a] Ethanol values were corrected for dilution by addition of base during fermentation.
[b] Time requred to produce 90% of the theoretical yield of ethanol.

trimers after phosphorylation, analogous to *K. oxytoca* [5,19].

Both strains were also able to degrade starch and pullulan. No proteolytic activity was detected on milk agar after 48 h. However, protein hydrolysis was observed after 5 days of incubation. Proteolysis during prolonged incubation may be due to partial lysis and release of intracellular enzymes rather than secreted proteases.

Ethanol production

As shown in Fig. 1, broth containing 100 g/l glucose was efficiently fermented by both *Erwinia* strains harboring plasmid pLOI555 (Table 3). The amount of ethanol produced from glucose was equivalent to 94% of the theoretical yield (2 mol ethanol per mol of glucose), similar to that obtained with ethanologenic constructs of other bacteria [2,5,12,19]. The maximal rate of ethanol production from glucose occurred between 6 and 24 h approx. 2 g ethanol/l per h. Much lower levels of ethanol were produced by the unmodified parental strains with all sugars tested.

The fermentation of 100 g/l of xylose was slower than that of glucose (Fig. 2A; Table 3). Less than 40 g/l of ethanol was produced after 36 h. The slow fermentation observed with 100 g/l of xylose appears to result from either a sensitivity to high levels of xylose or to the effects of accumulated ethanol on xylose metabolism. In fermentations with 80 g/l of xylose (Fig. 2B), ethanol was produced more rapidly and ethanol yields exceeded the theoretical maximum in 24 h.

Both ethanologic *Erwinia* strains efficiently fermented cellobiose to ethanol (Fig. 2C). Cellobiose is a major product from the enzymatic hydrolysis of cellulose and a competitive inhibitor of cellulase activity [3]. The direct utilization of this dissaccharide has two major advantages for simultaneous saccharification and fermentation. First, the requirement for exogenous β-glucosidase is eliminated. In addition, continuous uptake and metabolism of cellobiose should allow the cellulases to proceed more efficiently. Approx. 50 g/l of ethanol was produced from 100 g/l of cellobiose in less than 48 h with a maximum volumetric productivity of 1.5 g ethanol/l per h.

The rate of cellobiose fermentation by the ethanologenic *Erwinia* was over twice that recently reported for the cellobiose-utilizing yeast, *Brettanomyces custersii* (approx. 0.5 g/l per h) in batch culture [16]. Unlike *B. custersii*, no oxygen or lipid supplements are required for ethanol production by recombinant *Erwinia*. Although expensive complex nutrients were employed in both studies, the level used with *Erwinia* was half that used with *B. custersii*. For *Erwinia* constructs, this medium can be greatly simplified since most are prototrophic [13]. Thus, the combination of nutritional simplicity, high rates of cellobiose utilization,

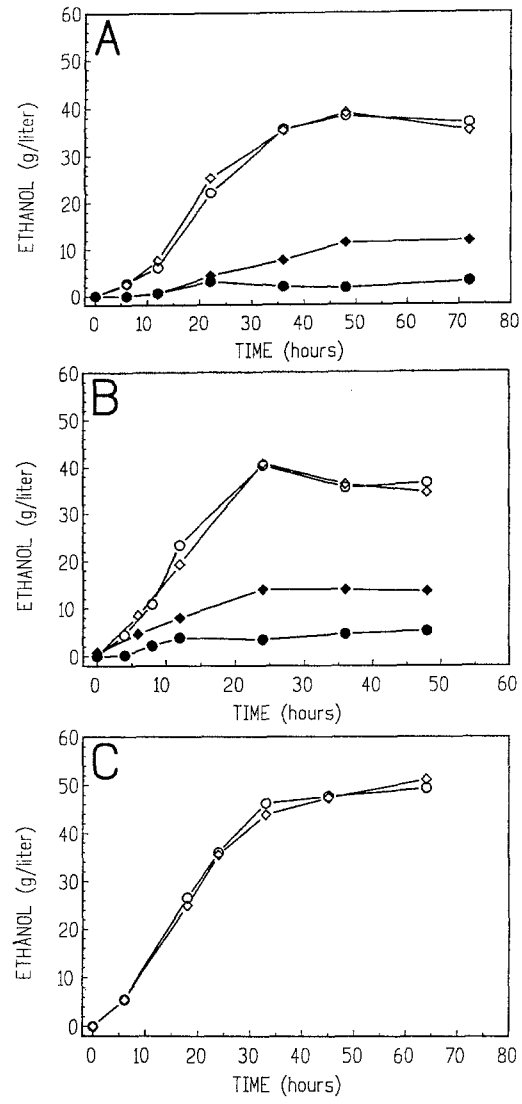

Fig. 2. Fermentation of xylose and cellobiose. A. Xylose (100 g/l). B. Xylose (80 g/l) xylose. C. Cellobiose (100 g/l). Symbols: ●, SR38; ○, SR38 (pLOI555); ◆, EC16; ◇, EC16 (pLOI555).

and the native ability to secrete enzymes which partially degrade lignocellulose make ethanologenic strains of *Erwinia* excellent candidates for further investigations of biomass conversion to ethanol. Although not demonstrated, organisms such as these, but with more active native hydrolases, should reduce the need for external enzymes to convert many polymers into ethanol.

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