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Growth of *Zymomonas* on lactose: gene cloning in combination with mutagenesis

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SUMMARY

Wild-type strains of *Zymomonas mobilis* have a limited substrate range of glucose, fructose and sucrose. In order to expand this substrate range, transconjugants of *Z. mobilis* containing Lac^+ plasmids have been constructed. Although β-galactosidase is expressed in such strains, they lack the ability to grow on lactose. We now report the development of *Z. mobilis* strains capable of growth on lactose. This was achieved in two stages. First, a broad host range plasmid was constructed (pRUT102) which contained the lactose operon under the control of a *Z. mobilis* promoter plus genes for galactose utilization. *Z. mobilis* CP4.45 containing pRUT102 was then subjected to mutagenesis combined with continued selection pressure for growth on lactose. One strain, *Z. mobilis* SB6, produced a turbid culture that yielded 0.25% ethanol from 5% lactose (plus 2% yeast extract) in 15 days.

INTRODUCTION

The bacterium *Zymomonas mobilis* is an aerotolerant gram-negative anaerobe which produces ethanol as a major fermentation product [6,9,23,32]. *Z. mobilis* is capable of ethanol production at more than double the reported rates for yeasts, and recent advances in bioengineering have demonstrated the potential superiority of this organism over yeasts in other aspects of industrial ethanol production [15,17,18,20,27,28]. However, commercial production of ethanol by *Z. mobilis* has been limited in part by its native substrate range being restricted to glucose, fructose and sucrose. It should be possible to increase the substrate range of *Zymomonas* to include other industrially attractive substrates, such as lactose (found in the waste product milk whey) or cellulose, via the transfer of appropriate hydrolase genes.

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Until recently, there have been no reports of efficient growth of *Zymomonas* on substrates other than glucose, fructose and sucrose, although limited growth has been reported on raffinose [22,34] and sorbitol [22]. We recently reported the use of 'evolutionary selection techniques' to expand the range of carbohydrates utilized by *Zymomonas* to include rapid fermentation of mannitol [4]. However, despite the efforts of many researchers, growth of *Z. mobilis* on other sugars has not been as successful. Initial genetic experiments to gain lactose-utilizing strains of *Zymomonas* have proven negative. These experiments included transfer of the broad host range Lac^+ plasmid pGC91.14 [8] to *Z. mobilis*, yet resulted in cells which, though they express β-galactosidase, cannot grow on lactose [5,11,14,31].

Bottlenecks in this general problem can include gene expression, lactose uptake, galactose toxicity and production of products other than ethanol, the latter being encountered during growth of *Zymomonas* on mannitol [4]. No clear rationale has emerged to solve all of these problem areas. However, it should be possible to transfer the appropriate genetic material to *Z. mobilis*, which in combination with selective pressure for growth, will yield strains capable of efficiently utilizing additional substrates. This approach is illustrated in the preliminary selection of *Z. mobilis* strains with enhanced enzymatic capability which are capable of limited growth on lactose.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Z. mobilis* CP4 and CP4.45 have been described [3,32]. *Escherichia coli* strains and plasmids are listed in Table 1.

Media and reagents. *Z. mobilis* strains were grown in static culture at 28°C in GYx medium consisting of D-glucose (2%), yeast extract (2%) and KH_2PO_4 (0.1%), or in LYx medium consisting of lactose (4%) and yeast extract (2%). GalYx medium consists of D-galactose (2%) and yeast extract (2%). Minimal medium was prepared as described by Goodman et al. [13]. *E. coli* strains were grown in LB medium consisting of tryptone (1%), sodium chloride (1%), and yeast extract (0.5%), or on MacConkey agar base (BBL Microbiology Systems, Cockeysville, MD) supplemented with either lactose (2%) or D-galactose (2%). TMg buffer (10 mM Tris base, 33 mM $MgCl_2$, pH 8.0) (S. Buchholz, 1987, Ph.D. Dissertation, Rutgers University) was used to wash and dilute *Zymomonas* cells. Yeast extract was purchased from Oxoid U.S.A. (Columbia, MD). Restriction endonucleases were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Other chemicals and reagents were purchased from Fisher Scientific (Springfield, NJ) or from Sigma Chemical Co. (St. Louis, MO).

In vitro DNA manipulation and plasmid transfer. Extraction of plasmid DNA from *Z. mobilis* was performed essentially as described by Stokes et al. [30]. Plasmid DNA from *E. coli* was extracted in the same manner except that 0.3% Triton X-100 was used in place of 2% sodium dodecyl sulfate during cell lysis. Plasmid DNA was characterized electrophoretically in 0.7% agarose gels using TAE buffer (40 mM Tris-HCl, 40 mM acetate, 2 mM EDTA, pH 7.8) [19]. Restriction endonuclease digestion and ligation of plasmid DNA was essentially as described by Perbal [25]. Conjugal transfer of plasmids to *Z. mobilis* and selection of transconjugants was as previously described [3]. Transformation of *E. coli* with plasmid DNA was via the calcium chloride/rubidium chloride procedure [19].

Mutation. Mutation of *Z. mobilis* strains was by NTG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) serial dilution. Serial dilutions (1:10) were made in GYx (supplemented with antibiotics as required) with an NTG-saturated solution of 50% ethanol. Each tube was inoculated with 50 μl (approximately 1×10^7 cells/50 μl) of a *Z. mobilis* culture and incubated overnight at 28°C. Cultures with visible turbidity at the highest concentration of NTG were selected for continued development. The cells were washed three times with TMg buffer and plated for mutant selection.

Chemostat fermentations. Chemostats (New Brunswick Scientific model C-30, working volume 425 ml) were set at 28°C. Cultures were aerated by impeller only. Medium composition and agitation are described for each specific fermentation.

Table 1

Plasmids and *E. coli* strains used in the development of lactose-positive strains of *Z. mobilis*

Plasmid	*E. coli* strain	Relevant characteristics	Source or Ref.
–	HB101	*lacY1, galK2*, Rec$^-$	21
–	DH5-α	*lacZ* (M15), Rec$^-$	33
–	WA802	*lac3, galK2, galT22*, Rec$^-$	*E. coli* Genetic Stock Center, Yale University
pGC91.14	HB101	Am^r, Km^r, Tc^r, Lac$^+$	8
pAA-E-gal$^+$	WA802	Am^r, Tc^r, Gal$^+$ (*galE, galK, galT*)	1
pKT210	HB101	Cm^r, Sm^r	2
pBR322-Km^r	DH5-α	Am^r, Tc^r, Km^r	J. Philbrick, personal communication
pCMD1	DH5-α	Cm^r, promoter probe (Lac)	10
pCMD128	DH5-α	Cm^r, Lac$^+$	10
pRUT100	WA802	Cm^r, Gal$^+$	this study
pRUT101	DH5-α	Cm^r, Km^r, Lac$^+$	this study
pRUT102	WA802	Km^r, Gal$^+$, Lac$^+$	this study

Analytical methods. Optical density (OD) was determined at 600 nm using a Gilford Instrument Stasar II spectrophotometer. Ethanol concentrations were determined via gas chromatography (Gow-Mac series 750 using a flame ionization detector and a Porapack-Q column). β-Galactosidase ('Miller units') was assayed by the method of Miller [21] using 20 min incubations.

RESULTS

Mutation of Z. mobilis CP4

Mutation of *Z. mobilis* CP4 was used as a control for all experiments which included mutation of *Z. mobilis* CP4 containing transferred plasmids. In all cases, mutation alone of *Z. mobilis* CP4 (lacking transferred plasmids) failed to yield cells capable of growth on lactose or galactose.

Gene cloning for growth of Z. mobilis on lactose

Z. mobilis cells containing pGC91.14 appear blue (β-galactosidase-positive) on GYx plates containing Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and IPTG (isopropyl-β-D-thiogalactopyranoside). Similarly, transfer of pGC91.14 to *Z. mobilis* CP4 results in β-galactosidase production without the ability to grow on media where lactose is the sole carbohydrate source. Induction of the lactose operon of pGC91.14 with IPTG yields 73 and 980 Miller [21] units of β-galactosidase activity in *Z. mobilis* CP4 and *E. coli* HB101, respectively.

Several factors were postulated as barriers to growth of *Z. mobilis* containing pGC91.14 on lactose. These included insufficient β-galactosidase production (73 units with pGC91.14), galactose toxicity and inadequate transport of lactose. It was possible that the level of β-galactosidase in *Zymomonas* may have been too low for growth. Thus, increased expression of the *lacZ* gene was attempted by placing it under the control of a *Zymomonas* promoter. *Zymomonas* promoters were isolated using a broad host range promoter probe vector, pCMD1, constructed for this purpose [10]. Plasmid pCMD1 contained the entire lactose operon (*lacZ, lacY, lacA*) but lacked a promoter and operator. β-Galactosidase activity was utilized to monitor relative strength of promoters inserted into the unique *Hin*dIII site. Isolation of *Zymomonas* promoters using pCMD1 yielded plasmids with a variety of β-galactosidase activities [10]. One construction, pCMD128, gave 125 Miller units of β-galactosidase activity (versus 73 units for pGC91.14) and 16 units of permease activity in *Z. mobilis* CP4 [10]. Even so, *Z. mobilis* CP4 containing pCMD128 failed to grow on solid media containing lactose as the sole carbo-

hydrate source, although equivalent activities were quite adequate for growth of *E. coli* on lactose [10].

Resuspension of glucose-grown *Z. mobilis* CP4 containing pCMD128 in 20% lactose (plus 2% yeast extract and 0.1% KH_2HPO_4) yielded 1.23% (w/v) ethanol in 12 days. This ethanol production is equivalent to 24% efficiency of fermentation of the glucose moiety of the lactose. Mutation of *Z. mobilis* CP4 containing pCMD128 with NTG yielded 11 small colonies on LYx agar (0.2–1.2 mm diameter). *Z. mobilis* Lac$^+$2 produced faint growth on LYx agar after only 1 week whereas the remaining ten mutants took several weeks to grow on LYx agar. No colonies were formed from NTG-mutation of *Z. mobilis* CP4 containing pCMD1. Only *Z. mobilis* Lac$^+$2 gave visible limited turbidity in LYx broth, i.e., flocculent growth was seen after vortexing, increasing from an OD_{600} of 0.06 to 0.41 in 16 days. A corresponding culture in 2% yeast extract showed no increase in optical density, emphasizing that growth of the Lac$^+$ strains was not due to growth on yeast extract. *Z. mobilis* Lac$^+$2 yielded 104 units of β-galactosidase activity, but there was no detectable ethanol (<0.05%). Growth of strains Lac$^+$2, 6, 8 and 9 on lactose minimal medium was evident after prolonged (8 weeks) incubation.

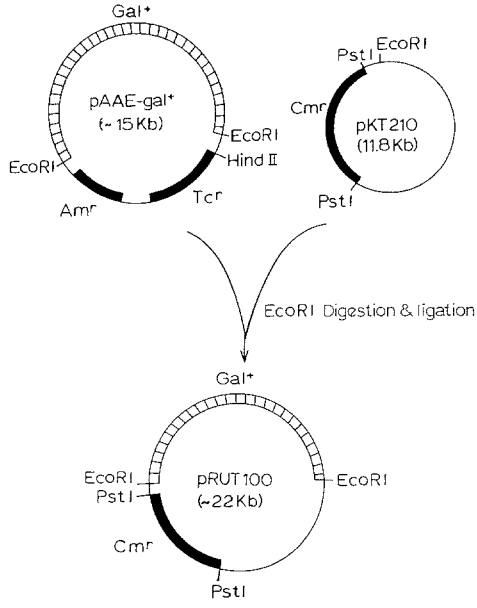

Fig. 1. Insertion of the galactose operon from pAA-E-gal$^+$into pKT210 to form pRUT100. Abbreviations: Gal$^+$, galactose operon (*galE, galK, galT*); Am^r, ampicillin resistance gene; Cm^r, chloramphenicol resistance gene; *Eco*RI, *Hin*dIII and *Pst*I are restriction endonuclease sites.

Galactose utilization

Z. mobilis CP4 grown on glucose (2%) and on glucose plus galactose (2% and 10%, respectively) results in nearly identical growth rates of the two cultures; i.e., galactose does not appear toxic, though we did not illustrate that extracellular galactose entered the cells. Therefore, it is still possible that the intracellular release of galactose following uptake of lactose could restrict the growth of *Z. mobilis* on lactose.

It is possible to insert the entire galactose operon into *E. coli* using plasmid pAA-E-gal$^+$ (*galE, galK, galT* inserted into the *Eco*RI site of pBR322) [1]. However, as pBR322 and its derivatives are not easily transferred to or maintained in *Zymomonas,* the galactose operon was subcloned into the unique *Eco*RI site of the broad host range plasmid pKT210 to yield plasmid pRUT100 (Fig. 1). Plasmid pRUT100 was maintained under selective pressure either by growth on M9 minimal medium containing galactose [21] or on rich media (LB or MacConkey galactose agar) containing chloramphenicol (50 μg/ml). The new plasmid conferred stable utilization of galactose in *E. coli* WA802, a trait that was unstable with pAA-E-gal$^+$.

Transfer of pRUT100 to *Z. mobilis* resulted in cells which maintained the galactose operon but could not grow on galactose, an expected result in that galactose permease was lacking. Plasmid pRUT100 was then isolated from *Z. mobilis* and retransformed into *E. coli* WA802, without apparent phenotypic change of the plasmid. Mutation of *Z. mobilis* CP4 containing pRUT100 by NTG serial dilution yielded microcolonies (<0.15 mm diameter) on galactose-yeast extract agar after 2–3 weeks of incubation. This was a presumptive indication of the use of galactose by *Z. mobilis*. Several of the largest colonies were harvested and resuspended in TMg buffer, and subsequently used for selective cultivation on galactose in a chemostat.

Chemostat selection on galactose

The 'galactose-chemostat' contained D-galactose (2%) and yeast extract (1%), and the reaction vessel contained additional D-glucose (0.2%) and chloramphenicol (150 μg/ml) to support initial cell growth and plasmid maintenance of the inoculum. Cells were cultured for 7 days prior to initiation of flow (dilution rate of 0.007 vol./h; 3 ml/h) from the reservoir. The 'galactose-chemostat' was operated in a single continuous trial for 69 days, at 100 rpm agitation and 28°C. After 3–4 weeks, flocculent growth was evident attached to the baffles. Suspension of a portion of the attached flocs was achieved via momentarily increasing the agitation to 500 rpm, followed by sampling for novel strains and resumption of agitation at 100 rpm. No sustained increase in turbidity was achieved. A portion of each sample was also assayed for culture purity.

As D-galactose contains a minor glucose impurity (1–2%; Sigma Chemical Co.), and 2% galactose was used in the chemostat (total glucose concentration of up to 0.04%), the negative control (2% yeast extract) agar plates were supplemented with 0.05% glucose. Chemostat isolates yielded colonies of 0.10–0.20 mm diameter on GalYx plates after 3 weeks of aerobic incubation, indicating that very limited growth on galactose was possible. The isolates yielded colonies of <0.10 mm diameter on the negative control plates, and wild-type *Z. mobilis* CP4 also formed colonies of <0.10 mm diameter on GalYx. A culture, isolated from the 'galactose-chemostat' and forming colonies up to 0.20 mm diameter on GalYx agar, was designated Gal$^+$-*Z. mobilis*. It was resistant to 300 μg/ml chloramphenicol in GYx agar, indicating maintenance of some plasmid functions. Although the galactose operon activities were not assayed in this *Z. mobilis* strain, the increased colony size on GalYx agar infers function. Plasmid pRUT100 was stably maintained as evidenced by increased chloramphenicol resistance in *Zymomonas* and also retransformation into *E. coli* WA802.

Simultaneous incorporation of lactose and galactose utilization genes

Simultaneous incorporation of both lactose and galactose utilization genes (*galEKT*) into *Z. mobilis* was first attempted by conjugation of either Gal$^+$-*Z. mobilis* or *Z. mobilis* CP4 containing pRUT100 (Inc. Q) with *E. coli* HB101 containing pGC91.14 (Inc. P1). Plasmids pRUT100 and pCMD128 are both contained in incompatibility group Q and therefore could not be maintained simultaneously in *Z. mobilis*. Repeated trials of the conjugations failed to yield *Zymomonas* colonies which were resistant to both chloramphenicol (pRUT100) and tetracycline (pGC91.14), or that were capable of growth on LYx (lactose). A single plasmid was therefore constructed which contained both the lactose and the galactose operons.

In the first phase, the kanamycin resistance gene (on a *Pst*I cassette) of pBR322-Km^r (courtesy of J. Philbrick) was subcloned into pCMD128. The new plasmid, pRUT101 (Fig. 2), was isolated in *E. coli* DH5-α by selection on LB agar containing kanamycin (50 μg/ml) and screening for blue colonies (Xgal hydrolysis). Conjugation of pRUT101 from *E. coli*

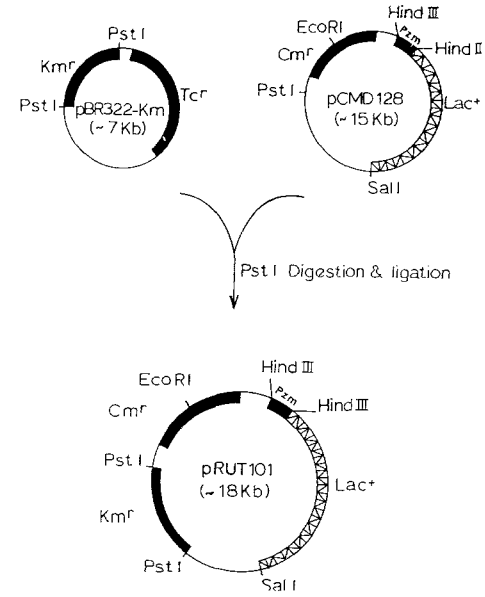

Fig. 2. Construction of pRUT101 by insertion of the kanamycin resistance gene from pBR233-Km^r into pCMD128. Abbreviations: Lac$^+$, lactose operon (*lacZ, lacY*, partial *lacA*; Pzm, promoter functions from *Z. mobilis* CP4; Cm^r, chloramphenicol resistance gene; Km^r, kanamycin resistance gene; Tc^r, tetracycline resistance gene; *Eco*RI, *Hin*dIII, *Pst*I and *Sal*I are restriction endonuclease sites.

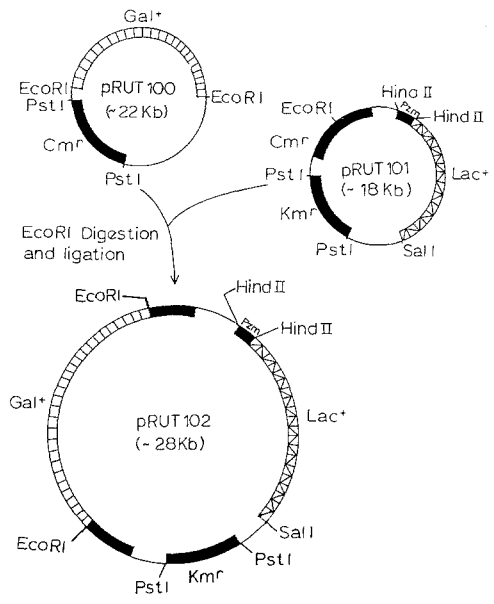

Fig. 3. Construction of the Gal$^+$–Lac$^+$ broad host range plasmid, pRUT102, by *Eco*RI digestion and ligation of pRUT100 and pRUT101.

DH5-α to *Z. mobilis* CP4.45 results in *Zymomonas* cells which express β-galactosidase but are unable to visibly grow on lactose, as is the case for *Z. mobilis* CP4 containing the original pCMD128.

The second phase involved subcloning of the galactose operon from pRUT100 into the unique *Eco*RI site in the chloramphenicol gene of pRUT101 to form pRUT102 (Fig. 3). The new plasmid pRUT102 contains a kanamycin resistance gene, the galactose operon (*galEKT*) and a lactose operon under the control of a *Zymomonas* promoter. As all kanamycin-resistant transformants should form lactose-positive colonies, the ligation mix was transformed into *E. coli* WA802 and screened for galactose-positive colonies on MacConkey galactose agar containing kanamycin (50 μg/ml). Of 16 galactose-positive, kanamycin-resistant colonies examined, only five were found to be positive for lactose upon isolation and subsequent transformation into *E. coli* DH5-α. One of the latter five isolates, designated pRUT102, was randomly selected and used for further experiments.

Conjugation of pRUT102 from either *E. coli* WA802 or DH5-α to *Z. mobilis* CP4.45 resulted in *Zymomonas* cells which expressed β-galactosidase (148 Miller units versus 504 units in *E. coli* DH5-α) and increased kanamycin resistance, upon selection on GYx plates containing kanamycin (200 μg/ml) and streptomycin (100 μg/ml). No conjugants were found on LYx plates containing streptomycin (100 μg/ml) even after 3 weeks of incubation. Very light growth of *Z. mobilis* CP4.45 containing pRUT102 was evident from subcultures incubated for 1 week on LYx plates. This growth is comparable to the growth on LYx agar of *Z. mobilis* Lac$^+$2 (the most rapidly growing NTG-induced mutant of *Z. mobilis* CP4 containing pCMD128). It is important to note that cultures of *Z. mobilis* CP4 containing pCMD128 and CP4.45 containing pRUT101, which had not been treated with NTG, failed to grow on LYx agar. Mutation of *Z. mobilis* CP4.45 containing pRUT102 with NTG (via serial dilution) resulted in small colonies on LYx plates within 1 week, some of which produced visible turbidity in LYx broth within 10 days. Ten such strains were designated *Z. mobilis* SB1–SB10. In preliminary results, three strains, *Z. mobilis* SB5, SB6 and SB10, attained optical densities of 0.2–0.4 and respectively produced 0.09%, 0.25%, and 0.08% (w/v) ethanol from 5% lactose (plus 2% yeast extract) in 15 days. Correspondingly, *Z. mobilis* CP4.45 containing pRUT102 which had not been treated with NTG produced 0.06% ethanol from 5% lactose in 15 days. β-Galactosidase activities of the 15-day cultures were 169 units (SB5), 154 units (SB6) and 142 units (SB10); lactose permease activities were not determined. These three cultures failed to grow on lactose minimal agar within 4 weeks, although SB5 grew on lactose minimal agar supplemented with 0.05% casamino acids.

DISCUSSION

Growth of *Zymomonas* on lactose would serve as a model for genetic engineering in *Z. mobilis*, as well as being useful in the conversion of agricultural waste (whey) to ethanol. It has become relatively common to isolate transconjugants of *Z. mobilis* containing the broad host range Lac$^+$ plasmid

pGC91.14, although β-galactosidase is expressed without the corresponding ability to grow on lactose [5,7,11,14,31]. The inability to grow on lactose can be considered in part a problem of β-galactosidase expression from pGC91.14, as low enzyme activity was detected in *Zymomonas* compared to *E. coli* HB101 containing this plasmid (73 versus 980 Miller units, respectively). These enzyme activities are in genéral agreement with those previously reported using similar strains [5], although higher β-galactosidase activities have been reported for pGC91.14 in *Z. mobilis* ZM6 and its derivatives [7,31].

We therefore constructed a broad host range promoter probe plasmid, pCMD1, to simultaneously isolate *Zymomonas* promoters and gain (increased) expression of β-galactosidase [10]. One plasmid construction, pCMD128, expressed 125 Miller units of β-galactosidase activity, and 16 units of permease activity in *Z. mobilis* CP4. These enzyme levels should have been more than adequate for growth of *Zymomonas* on lactose, as introduction of other constructions (*Zymomonas* promoters in pCMD1) into *E. coli* yielded as little as 34 Miller units of β-galactosidase activity and 7.5 units of permease activity, yet conferred to *E. coli* the ability to grow on lactose [10]. As strains of *E. coli* which produced less than 70 Miller units of β-galactosidase were capable of growth on lactose, while *Zymomonas* cells producing nearly double that amount of β-galactosidase were incapable of growth on this disaccharide, presumably the β-galactosidase levels obtained were sufficient for growth of *Zymomonas* on lactose.

Limitations to growth of *Zymomonas* on lactose, other than β-galactosidase expression, can include lactose transport, galactose inhibition (either as intracellular concentration or formation of a toxic metabolite – although *Zymomonas* is not sensitive to extracellular galactose) or an overall limitation on the energy yield of lactose metabolism for *Zymomonas*. In this latter vein, one ATP molecule is expended for each lactose molecule transported across the membrane via (lactose permease) proton symport [16,29]. Since *Z. mobilis* can metabolize only the glucose portion of lactose and also generates only 1 mol of ATP per mol of glucose fermented, there is no net gain of energy for cell metabolism and growth.

This problem may be circumvented in three ways. Lactose uptake-positive mutants of *Z. mobilis* could be isolated. Secondly, genes for galactose utilization could be incorporated into a plasmid containing the lactose operon. In the latter case, metabolism of galactose would eliminate potential galactose inhibition and result in the theoretical formation of 2 mol of ATP per mol of lactose utilized. Alternatively, β-galactosidase secretion mutants could be isolated, which would result in extracellular hydrolysis of lactose and subsequent growth of *Zymomonas* on the liberated glucose moieties. Apparent leaky (lactose uptake-positive or β-galactosidase secretion) mutants were previously obtained by mutating *Z. mobilis* CP4 containing pCMD128, although we note that high-level expression of some exportable β-galactosidase fusion proteins is lethal in *E. coli* [24,26]. These mutants, *Z. mobilis* 'Lac$^+$1–Lac$^+$11, formed small colonies on LYx agar and one (Lac$^+$2) grew to yield turbidity in LYx broth, yet without detectable ethanol production. These feebly lactose-positive mutants, including *Z. mobilis* Lac$^+$2 which produced 104 units of β-galactosidase activity, were considered to be 'leaky' in either lactose uptake or β-galactosidase excretion. We decided to combine these approaches by introduction of galactose plus lactose utilization genes, followed by selection of leaky mutants.

We consider the three enzymes of the galactose operon (*galE, galK, galT*) to be sufficient for metabolism of intracellular galactose by *Zymomonas*. *galP* (galactose permease) and *galR* (repressor of the galactose operon) are not required in the above lactose utilization scheme for *Zymomonas*, as the galactose would be intracellular after uptake and hydrolysis of lactose and unrepressed expression of the galactose operon was desired [12]. If mutants that secreted β-galactosidase were obtained, *galP* would be required for galactose uptake if metabolism of galactose was to occur, but no inhibition or energy-yield problems should arise from the presence of extracellular galactose during growth on the liberated glucose moieties. However, upon intro-

duction of the galactose operon into *Z. mobilis*, we were unable to simultaneously introduce a second plasmid (pGC91.14) containing the lactose operon. We then constructed a single broad host range plasmid containing both the galactose and lactose operons, which could then be inserted into *Z. mobilis*.

The final plasmid (pRUT102; Fig. 3) should yield a Km^r, Gal^+, Lac^+ phenotype. Of the galactose-positive, kanamycin-resistant clones examined in *E. coli* WA802, only one third were found to be β-galactosidase-positive upon subsequent transformation into *E. coli* DH5-α. Although it is possible that some cells of the initial recipient strain could have been Km^r or Gal^+ spontaneous mutants, none of the negative control plates showed any growth of *E. coli* WA802. Several other possibilities were ruled out in that upon agarose gel electrophoresis all of the clones examined contained plasmids the same size (28 kb) and yielding identical endonuclease restriction digest patterns. We concluded that either pRUT102 was unstable in the *E. coli* genetic backgrounds or construction of pRUT102 resulted in loss of promoter activity. The former is evident as isolation of pRUT102 and subsequent transformation into *E. coli* DH5-α results in approximately 30% of the transformants being negative for β-galactosidase, indicating some form of plasmid–host instability. Regardless, transfer of pRUT102 to *Z. mobilis* CP4.45 results in β-galactosidase (148 units)-positive cells exhibiting increased kanamycin resistance.

The *Zymomonas* transconjugants containing pRUT102 are capable of only very feeble growth on LYx agar and virtually no growth in LYx liquid media. This growth was similar to that observed with the mutated strain *Z. mobilis* Lac^+2, which lacked the galactose operon, thereby providing some indirect evidence that the galactose operon provides a selective advantage to *Zymomonas* cells grown on lactose. NTG mutation of *Z. mobilis* CP4.45 containing pRUT102 yielded colonies on LYx agar within 1 week, although no growth was evident on lactose minimal agar within 4 weeks. These mutants gave visible turbidity plus detectable ethanol in 5% LYx broth. The best lactose-positive mutant of *Z. mobilis* CP4.45 containing pRUT102 was *Z. mobilis* SB6, which is capable of producing visible turbidity (OD_{600} = 0.33) and 0.25% (w/v) ethanol from 5% lactose in 15 days; i.e., positive but extremely slow growth. The growth rate of *Z. mobilis* SB6 in 5% lactose was a meager 0.02 doublings per h by optical density (approximately one doubling every 2.35 days), similar to that observed with strain Lac^+2. Although other researchers have reported greater rates of ethanol production from lactose in the presence of glucose [7], our strains produced ethanol from lactose alone and additionally yielded an increase in cell mass (as evident by turbidity and growth on plates) when grown on lactose. Furthermore, during our efforts to obtain *Z. mobilis* cells capable of growth on lactose, we succeeded in gaining some slight growth on galactose.

Further improvements in the growth rate of *Z. mobilis* SB6 are currently under examination via selection in a chemostat including additional mutagenesis of the strain. Isolation of the lactose-positive strains of *Z. mobilis*, such as SB6, indicates that it is possible to genetically manipulate *Zymomonas* to utilize alternative substrates, but selection of (leaky) mutants is further required for improved growth rates. Preliminary data suggest that growth of *Z. mobilis* on lactose is not due to cell lysis and concomitant liberation of β-galactosidase, as there was no significant increase in extracellular soluble protein after 7 days with resuspended cells. We have gained a true lactose-utilizing strain. Efforts are now directed to unravelling its mechanism of metabolizing this disaccharide, besides gaining more effective mutants.

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