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Note

Expression of *lac* and *gal* operons in *Zymomonas mobilis*

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Two *Zymomonas mobilis* strains (ZM63 and ZM6307), containing both lactose and galactose operons, were constructed. β-Galactosidase and galactokinase assays indicated that both operons were expressed in both strains. The transport systems available for lactose uptake by *Zymomonas mobilis* were investigated using ^{14}C-labelled lactose. After the outer membrane, which was considered to be a possible barrier to lactose uptake, was disrupted by treatment with EDTA and Ca^{2+} ions, some increase in lactose uptake was observed in ZM6306 (lac^+) and ZM6307 (lac^+ gal^+), but not in the parent, ZM6. This suggested that the outer membrane of *Zymomonas mobilis* acts as a barrier to lactose uptake to some degree, and also that the lactose permease is operational in *Zymomonas mobilis*.

Zymomonas mobilis is a Gram-negative, obligately-fermentative bacterium which uses the Entner-Doudoroff pathway to convert glucose to ethanol (29). Although this organism can produce ethanol from glucose at a higher specific rate and yield than the traditionally used yeast (25), it has a very narrow substrate range limited to only glucose, fructose and sucrose (29). To develop a commercial ethanol process using *Z. mobilis*, its narrow substrate range must be overcome. Lactose is a potential candidate for extending the substrate range of *Z. mobilis*. It is a disaccharide composed of glucose and galactose and is the major organic constituent of whey (20), a waste material produced in large quantities in the dairy industry. Lactose is cleaved to glucose and galactose by β-galactosidase, but only the glucose moiety can be fermented to ethanol by lac^+ *Z. mobilis* strains, since *Z. mobilis* is unable to utilize galactose to produce ethanol (29). Consequently galactose will accumulate during lactose metabolism and this may be inhibitory (14, 32). To attempt to achieve full utilization of intracellular lactose and to overcome possible galactose inhibition, we have introduced the *E. coli* galactose operon together with the lactose operon into *Z. mobilis*. The poor utilization of lactose by *Z. mobilis* strains containing the lactose operon (14) may be due to very slow uptake of the sugar from the medium. To investigate this, we have carried out ^{14}C-lactose uptake studies on *Z. mobilis*.

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Bacterial Strains and Growth Conditions

Z. mobilis strains were grown in static culture at 30°C in Rich Medium (RM) (13). For enzyme assays, RM medium containing 20 g/*l* glucose was used. ZM6 (27) and ZM6306 (28) were used to construct lac^+ gal^+ strains of *Z. mobilis* and conjugation was performed by 3-way filter mating as described by Goodman and Strzelecki *et al.* (28). *E. coli* strains, JC3272 containing lac^+ plasmid RP1::Tn*951* (9) and JP3438 containing gal^+ plasmid pMU616 (J. Pittard Melbourne University), were grown in Luria broth (LB) (23) at 37°C. M56 minimal medium (6) was used for galactokinase assays and eosin-methylene blue (EMB) agar plates containing galactose (23) were used to screen for gal^+ *E. coli*.

Construction of lac^+ gal^+ Strains of *Z. mobilis*

Gal^+ recombinant plasmids, pOK3 and pOK6, were constructed by insertion of the *gal* operon into two *E. coli/Z. mobilis* shuttle vectors pOK2 (7) and pOK5 (this study), respectively (Fig. 1 and 2). In both cases, the source of the *gal* operon was pMU616, comprising a 5 kb *Pst*I *gal*-encoding fragment of *E. coli* cloned into pACYC184. Mobilization of pOK3 and pOK6 from *E. coli* into *Z. mobilis* was achieved with the aid of the mobilizing plasmid, pRK2013 (11).

Two lac^+ gal^+ strains of *Z. mobilis*, ZM63 and ZM 6307, were constructed. ZM63 was derived from the wild type, ZM6 (27), into which both RP1::Tn*951* (lac^+) and pOK3 has been transferred by conjugation. ZM6307 was constructed by mobilization of pOK6 into ZM6306 (lac^+) which contains RP1::Tn*951* integrated into its genome (28).

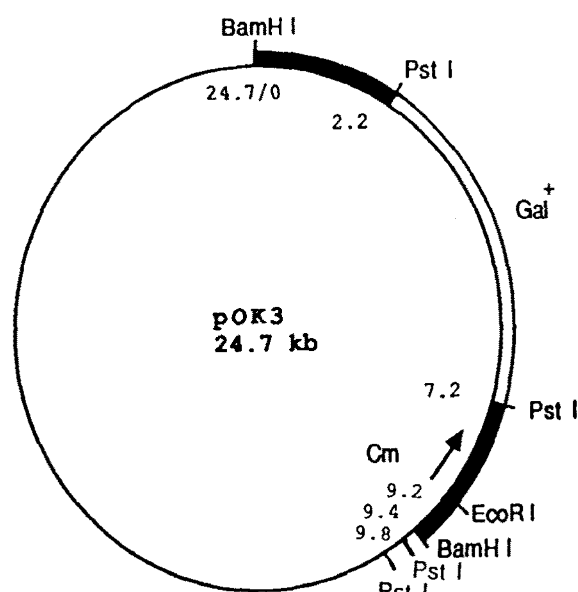

Fig. 1. Physical map of pOK3.
The numbers indicate size in kb. ■ pBR329; — pNSW2; □ *gal*-encoding fragment.

Expression of *lac* and *gal* Operons in *Z. mobilis*

The expression of *lac* and *gal* operons in *Z. mobilis* was studied by assaying for β-galactosidase (*lac*Z) and galactokinase (*gal*K), respectively (Table 1 and 2). Both enzymes were present under uninduced conditions. β-Galactosidase was induced both by lactose and isopropyl-β-thiogalactopyranoside (IPTG) and expressed at similar levels in ZM63 and ZM6307. Enzyme activities were similar to those reported previously (14) except that the levels induced by IPTG were much higher in this study. The level of enzyme activity detected in ZM6 without the *lac* operon was less than 5 U[mg protein]$^{-1}$.

Galactokinase activity was induced 2~3 fold by 0.3% (w/v) galactose. Galactose can also be transported by methyl-β-D-thiogalactoside permease, the product of the *lac*Y gene (1). This might explain the greater induction of galactokinase in ZM63 and ZM6307, which contain both the *lac* and *gal* operons, compared to ZM6 (pOK3), which contains only the *gal* operon. Galactokinase activity in ZM6 and ZM6306 was less than 2 U[mg protein]$^{-1}$.

As postulated Buchholz *et al.* (3), the introduction of the three enzymes of the galactose operon (*gal*K, *gal*T, *gal*E) was considered to be sufficient for metabolism of intracellular galactose by *Z. mobilis*. *gal*P (encoding the galactose permease) and *gal*R (encoding the repressor of the galactose operon) should not be required by *Z. mobilis* for the utilization of galactose derived from lactose, as the galactose would be intracellular, after uptake and hydrolysis of lactose, and a derepressed galactose operon would be desirable.

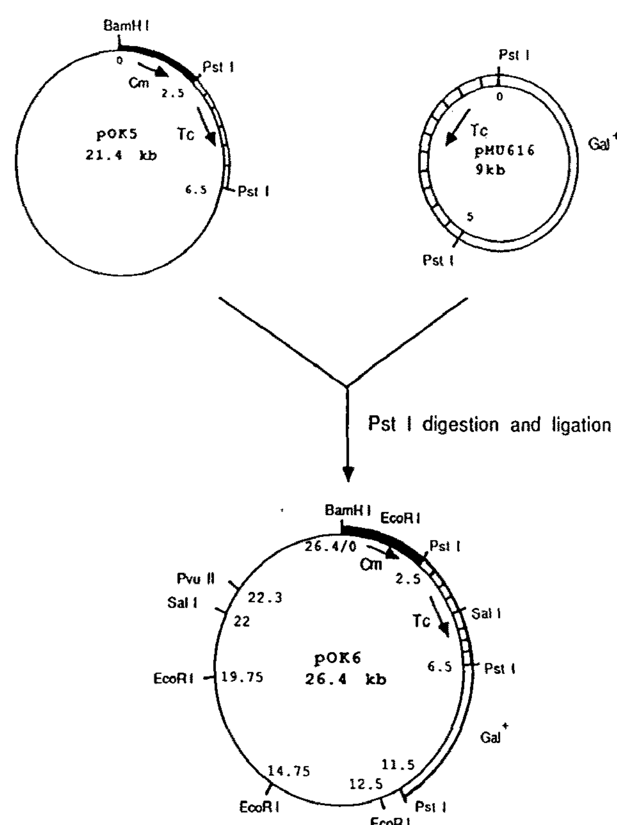

Fig. 2. Construction of pOK6.
The numbers indicate size in kb. ■ pBR325; — pNSW2; ▥ pACYC 184; □ *gal*-encoding fragment.

Table 1. β-Galactosidase activity of ZM63 and ZM6307

Inducer	Specific activity of β-galactosidase (U[mg protein]$^{-1}$)		
	ZM63	ZM6307	JC3272 (RP1::Tn951)
None	200	220	–
Lactose (g/l)			
0.5	300	330	–
5.0	410	360	–
IPTG (10^{-3} M)	770	840	7690

β-Galactosidase was assayed by the method of Miller (23) using chloroform and 0.1% (w/v) sodium dodecyl sulfate to disrupt the cells. One unit of enzyme activity was defined as the amount of enzyme which cleaved 1 nmol of O-nitrophenyl-β-galactopyranoside (ONPG) per minute at 28°C. Protein was measured by the method of Lowry et al. (18).

Although both *lac* and *gal* operons were expressed in *Z. mobilis* strains, the production of ethanol from lactose by these strains was still slow and these strains were unable to grow on lactose. It may be due to inadequate expression of the *lac* genes or the *gal* genes, or both, in *Z. mobilis*. The level of expression could be increased by combining these genes with DNA sequences of *Z. mobilis* containing a functional promoter.

Lactose Uptake by *Z. mobilis*

The outer membrane, which is specific to Gram-nega-

Table 2. Specific activity of galactokinase in ZM63, ZM6307 and ZM6 (pOK3)

Strain	Specific activity of galactokinase (U[mg protein]$^{-1}$)		
	Without inducer	With inducer (0.3% galactose)	Induction ratio (induced: uninduced)
ZM63	23	77	3.3
ZM6307	31	93	3.0
ZM6 (pOK3)	20	33	1.6
JP3438 (pMU616)	740	1280	1.7

Gaslactokinase was assayed essentially as described by McKenney et *al.* (21). Galactokinase activity was expressed as nmoles of galactose phosphorylated per minute per *ml* of cells at an OD_{650} of 1.0. Protein was measured by the method of Lowry *et al.* (18).

tive bacteria, and the cytoplasmic membrane of *Z. mobilis* may be possible barriers to lactose uptake. To investigate the effect of these on lactose uptake by *Z. mobilis* strains, the outer membrane was disrupted and ^{14}C-lactose uptake compared with intact cells.

The outer membrane of *Z. mobilis* was disrupted by EDTA and Ca^{2+} treatment since both EDTA and Ca^{2+} are reported to increase outer membrane permeability (15, 16, 17, 19, 22). Increased permeability was confirmed by an increase in sensitivity of treated cells to the macrolide antibiotic, tylosin, which is known to be ineffective against Gram-negative bacteria mainly due to its inability to penetrate the bacterial outer membrane (24). It was found that *Z. mobilis* was not affected by 50 µg/*ml* tylosin. By contrast, the viability of *Z. mobilis* cells treated by EDTA and Ca^{2+} decreased eight folds in the presence of 50 µg/*ml* tylosin.

Table 3 shows that, while lactose uptake by *Z. mobilis* was low compared to *E. coli*, treatment of cells with EDTA and Ca^{2+} ions increased lactose uptake by *lac*⁺ *Z. mobilis* strains, ZM6306 and ZM6307, by approximately three folds but had little effect on uptake by the parent, ZM6. Nevertheless lactose uptake by ZM6306 and ZM6307 after treatment was still only 8% of that of *E. coli*. Because the lactose was labelled in the glucose moiety it could be argued that this incorporation of label was due to the uptake of labelled glucose released from lactose extracellularly by β-galactosidase leaking from cells treated with EDTA and Ca^{2+}. Since no β-galactosidase activity could be detected in cell-free supernatants of treated cells this possibility seemed unlikely.

Considering the length of time required for ethanol production from lactose by *Z. mobilis* (8), it is likely that the inadequate lactose transport into the cell may also be limiting lactose utilization by *Z. mobilis*. The level of β-galactosidase activity in *Z. mobilis* was approximately 10% of that in *E. coli*. It may be presumed from this result that the expressed level of *lac* permease in *Z. mobilis* is likely to be approximately 10% of that of *E. coli* as well. In fact, the low level of functional

Table 3. Lactose uptake by ZM6, ZM6306 and ZM6307

Strain	Lactose uptake (cpm[mg protein]$^{-1}$)	
	Untreated cells	Treated cells
ZM6	1.1×10^3	1.4×10^3
ZM6306	1.3×10^3	3.5×10^3
ZM6307	1.0×10^3	3.4×10^3
JC3272 (RP1::Tn951)	4.3×10^4	–

The method of DiMarco and Romano (10) was used with some modifications. Log. phase cells (OD_{600}=0.6) were harvested, washed and resuspened in saline phosphate buffer (SPB; pH 7.0) to an OD_{600} of 1.5. [D-*glucose*-1-^{14}C]lactose (1 mM; 1 µCi/µmol) was then added and samples (0.2 *ml*) were removed at 2 min intervals for 10 min, the cells collected by vacuum filtration through nitrocellulose membrane filters and washed twice with 5 *ml* of cold SPB. The filters were dried and the radioactivity counted. Uptake was expressed as increase in cpm[mg protein]$^{-1}$.

lac permease in *Z. mobilis* was reported previously (5, 32, 33). Also, the different membrane composition of *Z. mobilis* compared to *E. coli* (2, 4, 12, 26, 30, 31) may result in reduced efficiency of the *lac* permease in *Z. mobilis*. There was some increase in lactose uptake by *lac*⁺ *Z. mobilis* strains, treated with EDTA and Ca^{2+}, compared to the wild type strain, ZM6. This suggested that the outer membrane of *Z. mobilis* acts in part as a barrier to lactose uptake, and also that the lactose permease is functional in *Z. mobilis*. The problem of lactose transport may be circumvented by isolating lactose permeable mutants or β-galactosidase secretion mutants of ZM63 and ZM6307.

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