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Metabolic Engineering of *Klebsiella oxytoca* M5A1 for Ethanol Production from Xylose and Glucose†

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The efficient diversion of pyruvate from normal fermentative pathways to ethanol production in *Klebsiella oxytoca* M5A1 requires the expression of *Zymomonas mobilis* genes encoding both pyruvate decarboxylase and alcohol dehydrogenase. Final ethanol concentrations obtained with the best recombinant, strain M5A1 (pLOI555), were in excess of 40 g/liter with an efficiency of 0.48 g of ethanol (xylose) and 0.50 g of ethanol (glucose) per g of sugar, as compared with a theoretical maximum of 0.51 g of ethanol per g of sugar. The maximal volumetric productivity per hour for both sugars was 2.0 g/liter. This volumetric productivity with xylose is almost twice that previously obtained with ethanologenic *Escherichia coli*. Succinate was also produced as a minor product during fermentation.

Pentose sugars are abundant in nature as a major component of lignocellulosic biomass. These sugars represent a potential resource for the production of fuel ethanol (23). However, no naturally occurring microorganisms have been found which rapidly and efficiently ferment pentoses to high levels of ethanol (13, 26). One approach to solving this problem has been to transfer genes encoding the ethanol pathway in *Zymomonas mobilis* (*pdc* and *adhB*) into *Escherichia coli* and to express appropriate levels of pyruvate decarboxylase (PDC) and alcohol dehydrogenase II (ADHII) (1, 10–12, 18). This metabolic engineering of *E. coli* has produced recombinants capable of rapid fermentation to ethanol of all sugars which are constituents of biomass (20, 21).

In principle, the transfer of *Z. mobilis* genes to other organisms could be used to produce recombinants with additional useful traits for ethanol production. Recombinants of *Erwinia chrysanthemi* (28) and *Klebsiella planticola* (8, 29) have been constructed by using the *Z. mobilis pdc* gene alone. However, pyruvate metabolism was incompletely diverted to ethanol as a product of fermentation. Under selected conditions, ethanol yields per gram of sugar utilized were quite good, although the final levels of ethanol achieved were low. Low levels of ethanol were also produced by *E. coli* recombinants expressing only the *pdc* gene (3). Problems associated with these *pdc* recombinants included plasmid instability, decreased ethanol tolerance (which appeared to limit final product concentrations), decreased growth rate under fermentative conditions, decreased cell yield, incomplete conversion of substrate, and accumulation of acidic fermentation products.

Two factors were identified as contributing to the poor performance of the original *K. planticola* recombinant containing pZM15(*pdc*) (29). The low expression of PDC from pZM15 was proposed as being insufficient to divert pyruvate metabolism. Higher ethanol yields were subsequently obtained with constructs which expressed higher levels of PDC (8). The accumulation of organic acids (formate and acetate) was proposed to contribute to increased ethanol toxicity. Formate and acetate production was blocked by the selection of *K. planticola* pyruvate formate-lyase mutants (8). Although the elimination of this competing fermentation pathway improved ethanol production, the performance of these mutants remained below that observed previously with *E. coli* recombinants expressing both the *Z. mobilis pdc* and *adhB* genes (21).

Recombinants which contain the *pdc* gene alone are dependent on endogenous levels of native ADHs to couple the reduction of acetaldehyde to the oxidation of NADH. Since ethanol is only one of several abundant fermentation products normally produced by these enteric bacteria (19), it seemed possible that a deficiency in ADH (and accumulation of NADH) could contribute to the adverse effects of *pdc* on growth and ethanol tolerance. In this study, we have investigated the expression of both enzymes from the *Z. mobilis* ethanol pathway (*pdc* and *adhB* products) on ethanol production by a related enteric organism, *Klebsiella oxytoca* M5A1.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Strains and plasmids used in this study are listed in Table 1. Taxonomic methods for *Klebsiella* species identification were used as recommended in *Bergey's Manual of Systematic Bacteriology* (22).

Strains were subcultured on Luria agar plates with no added sugar (15) unless they harbored plasmids encoding *Z. mobilis* genes. Recombinants containing *adhB* and *pdc* require a fermentable carbohydrate for survival and were maintained on plates containing 2% glucose or xylose (21). Antibiotic concentrations were as follows: ampicillin, 50 μg/ml; chloramphenicol, 40 μg/ml; and tetracycline, 12.5 μg/ml. Expression of *Z. mobilis* ADHII in recombinants was screened by using aldehyde indicator plates (7).

Genetic procedures and recombinant techniques. Standard procedures were used for plasmid preparations, restriction enzyme digestions, ligations, transformations, and agarose gel electrophoresis (25). *E. coli* TC4 (6) was used as the host for all plasmid constructions.

Plasmid constructions. Since M5A1 is relatively resistant to penicillin and its derivatives, we have constructed *E. coli* shuttle vectors carrying the *cat* (Cm^r) or *tet* (Tc^r) genes. A *tet* gene was added to pLOI276 containing *Z. mobilis pdc* by

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
K. oxytoca M5A1	Wild type	P. W. Wilson
E. coli B	Wild type	ATCC 11301
E. coli TC4	*recA lacZ*	Our laboratory (6)
pcos2EMBL	Tc^r	Our laboratory (24)
pLOI276	Ap^r *pdc*$^+$	Our laboratory (6)
pLOI297	Tc^r *pdc*$^+$ *adhB*$^+$	Our laboratory (1)
pLOI510	Cm^r *pfl*$'$ *pdc*$^+$ *adhB*$^+$	Our laboratory (21)
pLOI555	*pdc*$^+$ *adhB*$^+$ Cm^r	This study
pLOI560	Ap^r Tc^r *pdc*$^+$	This study

inserting a 2.6-kbp *Eco*RI fragment from pcos2EMBL (24) into the *Sal*I site of pLOI276. Cohesive ends were removed by treatment with the Klenow fragment of *E. coli* DNA polymerase before ligation. The resulting construct was confirmed by restriction analysis and designated pLOI560.

Preliminary studies indicated that *E. coli* B (ATCC 11303) harbored cryptic low-copy-number plasmids. A new and useful vector was constructed in vivo by randomly integrating the *Z. mobilis pdc* and *adhB* along with *cat* into these plasmids. This was done by isolating a 4.6-kbp promoterless *Pst*I fragment containing *pdc*, *adhB*, and *cat* from pLOI510 (21). No replication functions are present on this fragment. After being circularized by self-ligation, this fragment was transformed into *E. coli* B with selection for Cm^r on Luria agar plates containing 2% glucose. Transformants were tested on aldehyde indicator plates, and dark-red clones were selected for high-level expression of the *adhB* gene. Plasmid preparations from these strains were tested for their ability to transfer antibiotic resistance and *Z. mobilis* genes into *E. coli* TC4 by transformation. All recombinants were sensitive to ampicillin, indicating a lack of the pUC18 fragment containing *bla* and the *colE1* replicon. One of these, pLOI555 (8.4 kbp), produced the most intensely red colonies on aldehyde indicator plates, conferred excellent ethanol production ability to *E. coli*, and appeared to be present in low copy number on the basis of yields from small-scale plasmid isolations. This plasmid was used to transform *K. oxytoca* M5A1 with selection for Cm^r.

Plasmid stability in M5A1. Cells harboring pLOI555, pLOI297, or pLOI560 were serially transferred in Luria broth containing 10% glucose without antibiotics for more than 60 generations at 30°C. Appropriate dilutions of cultures were plated on Luria agar containing 2% glucose without antibiotics. Colonies were tested on aldehyde indicator plates for retention of the ethanol production genes from *Z. mobilis* and for resistance to appropriate antibiotics.

Fermentation experiments. Fermentations were carried out in Luria broth containing 10% (wt/vol) glucose or xylose at 30°C and pH 6.0 with 100-rpm agitation as previously described (21). Inocula were grown overnight at 30°C from isolated colonies in unshaken flasks. Fermentations were inoculated to an initial optical density at 550 nm of 1.0 (330 mg [dry weight] of cells per liter). A Bausch & Lomb Spectronic 70 spectrophotometer was used to monitor growth.

Ethanol concentrations were determined by gas-liquid chromatography (1). Conversion efficiencies were corrected for volume changes caused by the addition of base and assumed that all sugar had been metabolized. The maximum theoretical yield of ethanol from xylose and glucose was calculated to be 0.51 g of ethanol per g of sugar, with the balance as carbon dioxide. Volumetric productivities were estimated from the most active periods and represent maximum values. All fermentation data in the tables and figures are averages from two or more batch fermentations.

Isolation of cytoplasmic protein fraction. Cells were grown for 24 h under the conditions of the fermentation experiments, chilled to 0°C, harvested by centrifugation (7,000 × *g* for 10 min), washed twice with one-third volume of 5 mM sodium phosphate buffer (pH 6.5) containing 10 mM 2-mercaptoethanol, and stored frozen at −20°C. Cell pellets were resuspended in an equal volume of buffer and broken by two passages through a French pressure cell at 20,000 lb/in^2. Membranes were removed by centrifugation at 4°C for 90 min at 100,000 × *g*. The supernatant containing soluble cytoplasmic proteins was stored at −70°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cytoplasmic proteins. DNase I-treated protein extracts were separated with a Mini-Protein II electrophoresis unit (Bio-Rad Laboratories, Richmond, Calif.) by using 10% acrylamide (14, 21). Protein was estimated by using the Bradford reagent (2). Approximately 10 μg was loaded into each lane. Molecular weight markers (Low Molecular Weight Calibration Kit; Pharmacia, Piscataway, N.J.) were included in a separate lane (phosphorylase *b*, 94,000; albumin, 67,000; ovalbumin 43,000; carbonic anhydrase 30,000; trypsin inhibitor, 20,100; and α-lactalbumin 14,400). Proteins were stained with Coomassie blue R-250.

Assay of PDC activity. PDC activity in heat-treated French press extracts (see above) was measured as described previously (6). Heat treatment was used to inactivate competing native enzymes which may complicate measurements of PDC activity in recombinants.

Analysis of volatile and nonvolatile acids. Samples were removed for organic acid analyses after 96 h of fermentation and analyzed for acetic, lactic, and succinic acids as previously described (21). Butanediol was determined essentially as described for ethanol, except that column and injection temperatures were increased to 190°C.

RESULTS

Taxonomic position of strain M5A1. The *Klebsiella* strain used in this study was previously designated as *Klebsiella pneumoniae* M5A1 (16). This strain, a dinitrogen-fixing organism, was originally identified as *Aerobacter aerogenes* but renamed on the basis of antigenic properties. Since new taxonomic criteria for *Klebsiella pneumoniae* were defined in *Bergey's* (4, 22), the species to which this strain belonged was investigated further. Strain M5A1 grew at 10°C in glucose minimal medium but failed to produce gas from lactose at 44.5°C. This strain was indole positive and utilized both *m*-hydroxybenzoate and gentisate as sole sources of carbon for growth at both 30 and 37°C. On the basis of these tests, M5A1 was designated *K. oxytoca*.

Plasmid stability in M5A1. Excessive instability of *Z. mobilis* genes in pBR322-based vectors was reported previously for *K. planticola* (29) and would not be acceptable for industrial processes. As shown in Table 2, the two pUC18-based vectors, pLOI560 and pLOI297, which also contain *colE1* replicons were unstable in M5A1. Only a small fraction of the population retained these plasmids after cultivation for 60 generations in the absence of antibiotic selection. In contrast, recombinants harboring pLOI555 were very stable, with 98% of the population retaining both the antibiotic resistance gene and the genes from *Z. mobilis*. Although only *adhB* expression is detected by aldehyde indicator plates, these recombinants also retained the large-colony

TABLE 2. Plasmid stability and *pdc* expression in recombinant strains of *K. oxytoca* M5A1

Plasmid	PDC activity after 24 h (U/mg of protein)	% retaining traits[a] (no. of generations)
pLOI555	3.6	100 (38.5), 98 (68.5)
pLOI297	28.0	52 (38.6), 10 (68.6)
pLOI560	27.0	97 (32.9), 0 (62.0)

[a] Cells were sampled twice during cultivation. The aldehyde trait and antibiotic resistance were lost concurrently: Cm^r for pLOI555; Tc^r for pLOI297; and Tc^r for pLOI560.

phenotype (10), indicative of expression of both *pdc* and *adhB*.

Expression of *Z. mobilis* genes in M5A1. Three plasmid constructs containing the *Z. mobilis* gene encoding PDC were transformed into M5A1 (Table 2). The levels of PDC were eightfold higher with the two pUC-based constructs (pLOI297 and pLOI560) than with pLOI555. Assuming a maximum specific activity for pure PDC of 100 U, this enzyme makes up more than 25% of the cytoplasmic protein in M5A1(pLOI297) and M5A1(pLOI560) and 3.6% in M5A1 (pLOI555).

The expression of *Z. mobilis* genes in M5A1 was further confirmed by SDS-PAGE (Fig. 1). Bands which contain PDC and ADHII were easily identified by comparison with the native strain (Fig. 1). The band containing PDC is much larger in pUC-based recombinants than in M5A1(pLOI555), consistent with measurements of enzymatic activity. Although ADHII is less abundant than PDC, the relative expression of this *Z. mobilis* gene is also higher in M5A1 (pLOI297) than in M5A1(pLOI555). No band corresponding to ADHII is evident in M5A1(pLOI560), which contains only the *Z. mobilis pdc* gene.

The copy number of pLOI555 was estimated to be less than 1/10 that of the two other constructs on the basis of yields in small-scale plasmid preparations. Although this estimate is only approximate, it is clear that the high levels of PDC present in the pUC-based constructs are due in part to higher copy number.

Ethanol production from xylose and glucose. Figure 2A and C and Table 3 compare the effects of *Z. mobilis pdc* and *pdc* plus *adhB* on ethanol production from glucose and xylose to ethanol production by the native strain M5A1. Recombinants containing the *pdc* gene alone produced more than twice the parental level of ethanol. Recombinants containing both *pdc* and *adhB* produced ethanol more rapidly and with higher efficiency than did M5A1(pLOI560) containing *pdc* alone. M5A1(pLOI555) was clearly the best construct for ethanol production, with maximum volumetric productivities of 2.1 g/liter/h for both glucose and xylose. With either sugar, this recombinant produced approximately 37 g of ethanol per liter after 30 h. Fermentation of these sugars was essentially completed after 48 h, with 45 g of ethanol per liter.

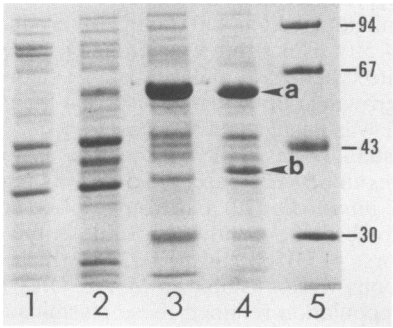

FIG. 1. SDS-PAGE gel comparing soluble proteins from recombinant strains of *K. oxytoca* M5A1. Molecular weights and marker proteins are shown in lane 5 and indicated on the right (in thousands). Arrows mark the bands which contain *Z. mobilis* PDC (a) and ADHII (b) proteins. Lanes: 1, control without plasmid; 2, pLOI555; 3, pLOI560; 4, pLOI297.

Under pH-controlled growth conditions, the addition of *pdc* alone reduced the maximal cell density and rate of growth by more than 50% compared with those of the native strain (Fig. 2B and D; Table 3). Growth was also limited in M5A1(pLOI297) containing a complete *Z. mobilis* ethanol pathway. As with ethanol production, the growth of M5A1 (pLOI555) was clearly superior. Growth of this recombinant was almost equivalent to that of the parental strain. However, unlike the parental strain, cell density progressively declined after reaching a maximum at 15 h. This decline may reflect a reduction in refractility as ethanol accumulated, since lysis was not evident. Without the addition of base to control pH (data not shown), recombinants containing both *pdc* and *adhB* grew to more than twice the density of the parent organism as a result of a reduced rate of acid production (higher proportion of neutral fermentation products), as observed previously with *E. coli* (10).

Organic acid production. Despite the efficient production of ethanol by some recombinants of strain M5A1, acidic fermentation products were also formed by all recombinants, as evidenced by the consumption of base to maintain pH (Table 3). All recombinants produced higher levels of fermentation products from xylose than did the parent (Table 4). Acetate was the dominant acidic fermentation product in the parental strain, whereas the dicarboxylic acid succinate was more abundant in the recombinant containing *pdc* alone (pLOI560) and in recombinants containing the complete *Z. mobilis* pathway. Base in excess of that needed to neutralize the measured acidic products was consumed by the parent organism (190 mmol of KOH per liter), and this additional base may serve to neutralize formate, which was not measured. In contrast, recombinants harboring pLOI560, pLOI297, and pLOI555 produced more acid than could be neutralized by the base consumed (70, 90, and 110 mmol of KOH per liter, respectively). Analogous recombinants of *E. coli* catabolize complex nutrients and produce ammonia (20, 21). A similar catabolism of complex nutrients by M5A1 recombinants may contribute natural buffers to maintain pH and reduce base consumption.

DISCUSSION

Our best recombinant strain, M5A1(pLOI555), contained both *Z. mobilis pdc* and *adhB* genes and fermented xylose to ethanol more effectively than did the best previously reported strains of *E. coli* (21). The maximal volumetric productivities (2.1 g of ethanol per liter per h) are almost double those of *E. coli* recombinants, and similarly high efficiencies and final ethanol concentrations are maintained. Unlike *E. coli* (20), M5A1(pLOI555) ferments xylose and glucose at equivalent rates. Plasmid pLOI555 was stably maintained in M5A1 in the absence of antibiotic selection. Since the range of substrates for M5A1 is equivalent to that of *E. coli*, M5A1 recombinants offer a distinct and unexpected advantage for ethanol production.

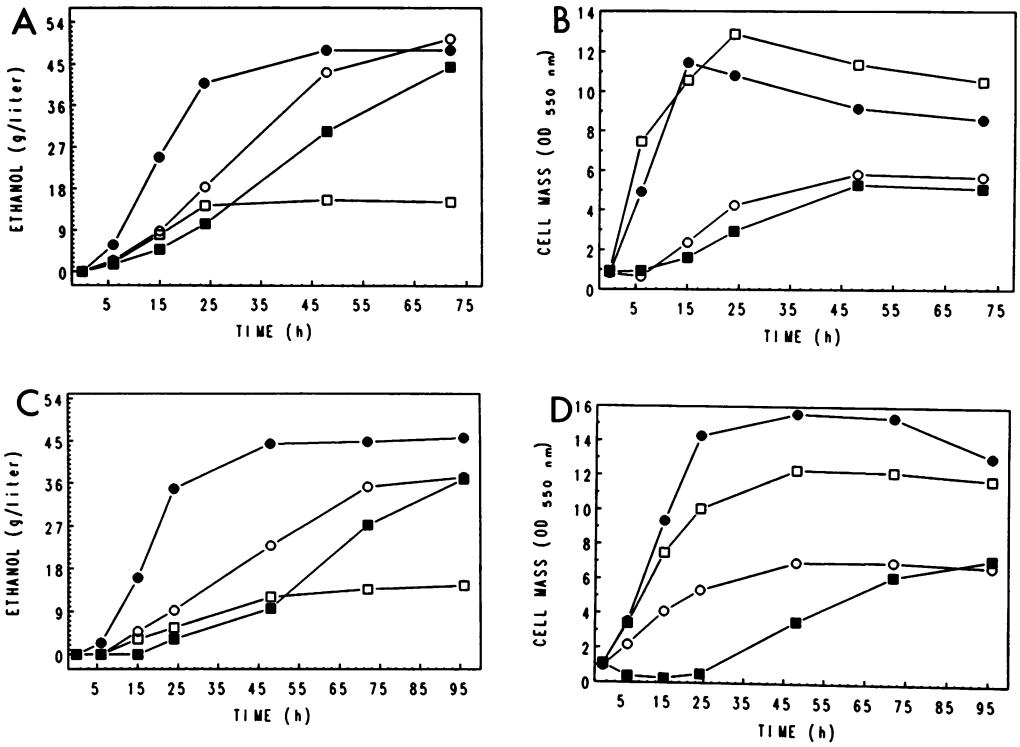

FIG. 2. Ethanol production (A and C) and growth (B and D) of *K. oxytoca* M5A1 recombinant strains during batch fermentation. Panels A and B show fermentation of 10% glucose. Panels C and D show fermentation of 10% xylose. Symbols: □, control without plasmid; ●, pLOI555; ○, pLOI297; ■, pLOI560. Abbreviation: OD_{550}, optical density at 550 nm.

Our studies have confirmed previous reports (3, 8, 28, 29) of poor ethanol production by enteric recombinants which contain only the *Z. mobilis pdc* gene. This poor performance is due in large part to the accumulation of acetaldehyde, indicating a requirement for additional ADH activity. The level of expression of the *Z. mobilis pdc* and *adhB* genes is also an important consideration. Excessive levels of the *Z. mobilis* enzymes, such as observed with M5A1(pLOI297), appear to retard growth and slow fermentation.

The instability of high-copy-number plasmids with the *colE1* replicon in *K. planticola* (29) was also confirmed for M5A1. Neither the replicon nor the promoter expressing the *Z. mobilis* genes is known for pLOI555. However, this plasmid was maintained well and appears to provide near-optimal levels of PDC and ADH activities for ethanol production.

Expression of *pdc* in M5A1(pLOI560) resulted in an increase in the production of succinate. Succinate levels were not reported in analogous constructs of *E. chrysanthemi* (28) or *K. planticola* (29). No increase in succinate was observed in *E. coli* ED8767 (pZM11*pdc*) during anaerobic growth without pH control (3). Simultaneous expression of

TABLE 3. Ethanol production from glucose and xylose by recombinant strains of *K. oxytoca* M5A1[a]

Plasmid genes[b]	Base (mmol/g of sugar)[c]	Time[d] (h)	Ethanol yield		Theoretical yield (%)	VP (g/liter/h)[e]	30-h ethanol amt (g/liter)	Cell yield (g/g of sugar)
			g/liter	g/g of sugar				
Glucose								
No plasmid	1.0	48	15	0.16	31	1.1	15	0.044
pLOI560 *pdc*	0.8	72	44	0.46	90	1.1	15	0.018
pLOI297 *pdc adhB*	0.7	72	50	0.52	102	1.3	24	0.020
pLOI555 *pdc adhB*	1.0	48	48	0.50	98	2.1	43	0.040
Xylose								
No plasmid	1.9	96	14	0.16	31	0.5	7	0.044
pLOI560 *pdc*	0.7	96	37	0.38	75	1.2	5	0.025
pLOI297 *pdc adhB*	0.9	96	37	0.39	76	1.0	12	0.024
pLOI555 *pdc adhB*	1.1	48	46	0.48	94	2.0	37	0.054

[a] Calculations are based on total sugar added initially.
[b] Genes indicated are from *Z. mobilis*.
[c] Amount of base consumed to maintain a pH of 6.0 during fermentation.
[d] Time of maximum ethanol concentration.
[e] VP, Maximum volumetric productivity during batch fermentation.

TABLE 4. Acidic and neutral fermentation products from 10% xylose (666 mM) after 96 h

Plasmid	Concn of organic acid (mM)			Concn of neutral products (mM)		% Carbon recovery[a]
	Acetic	Lactic	Succinic	Butanediol	Ethanol	
None	61	19	10	85	292	34
pLOI560	44	10	46	8	771	56
pLOI297	38	15	41	Tr[b]	771	55
pLOI555	33	7	59	Tr	958	67

[a] Percentage of carbon recovered as fermentation products. Residual xylose and carbon dioxide were not determined.
[b] Tr, trace.

adhB did not prevent the increase in succinate in the three recombinants of M5A1 expressing *pdc*. The level of succinate was similar in all three recombinants despite the expression of only one-eighth the level of PDC in M5A1(pLOI555). Both *pdc* and *adhB* are assembled into an artificial operon (11), and similar differences are expected in the level of *Z. mobilis* ADHII. The lack of a large difference in succinate production between these M5A1 recombinants which expressed different levels of PDC is consistent with a lack of direct competition between the enzyme which commits glycolytic intermediates to succinate (phosphoenolpyruvate carboxylase) and PDC.

Four fermentation pathways function in *Klebsiella* strains (19): the pyruvate formate-lyase pathway, which produces formate (hydrogen and carbon dioxide) and acetate plus ethanol in equimolar amounts; the lactic acid pathway; the succinate pathway; and the butanediol pathway. All but the last of these also functions in *E. coli* (5). In both organisms, it has been possible to divert more than 90% of carbon flow from sugar catabolism away from the native fermentative pathways to ethanol. Partial success has been achieved with the *pdc* gene alone in *E. chrysanthemi* (28) and *K. planticola* (29). In these cases, the low K_m of PDC for pyruvate allows this enzyme to compete effectively even in the presence of native pathways. Genetic approaches to metabolic engineering of other pathways have also been successful. The pathways for antibiotic biosynthesis have been merged in actinomycetes to produce novel compounds (9). Pathways encoding the synthesis of storage polymers (27) and carotenoids (17) have been functionally transplanted from one organism to another and have been integrated into the host metabolism. These results from different laboratories are representative of the interchangeable nature of metabolic pathways in microorganisms and the potential for metabolic engineering. As with the increased production of succinate, the integration of foreign pathways may have unexpected consequences and are not always predictable. However, nature has provided a marvelous diversity of metabolic activities in contemporary organisms. With a foundation of knowledge in microbial physiology and biochemistry and the tools of genetics, rational metabolic engineering of new recombinant organisms should provide an important route for the development of fine chemicals, chemical feedstocks, and fuels from contemporary biomass.

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