

Ethanol fermentation on the move

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The complete genome sequence of the ethanol-producing bacterium *Zymomonas mobilis* provides new opportunities for industrial alcohol fermentation.

Ethanol has been derived from microbial fermentation for thousands of years. It is not only an important product of the alcoholic beverage industry, but also, it is one of the fastest growing fuel sources in the world. In 2004, the United States produced more than 12.5×10^9 liters of ethanol—a 17% increase over the amount generated in 2003¹. Keeping in step with this demand will require the engineering of new strains of fermentative microorganisms that can produce ethanol more efficiently, and more detailed information about the genetic circuits involved. In this issue, Kang and colleagues² report the complete genome sequence of one of these organisms, the ethanologenic bacterium *Zymomonas mobilis*.

The perennial choice for making beverage ethanol is *Saccharomyces cerevisiae*. In contrast, *Z. mobilis* has been shunned because it can spoil fermentations of cider and beer with sulfurous flavors and rotten odors. However, in this rapidly changing industry, *Z. mobilis* might gain popularity. Off-flavors are not a concern in the production of fuel ethanol, so the faster fermentation kinetics and higher product yields of *Z. mobilis* could give it an advantage. Publication of the complete 2.06 Mb *Z. mobilis* genome by a consortium of researchers in Korea should provide a new impetus to efforts to exploit this bacterium for ethanol production.

Since 1970, *Z. mobilis* has been the subject of more than 1,400 research papers. It attracted attention early in the development of ethanol fuel technology because it grows and ferments rapidly, tolerates high levels of ethanol—virtually unique property among bacteria—and has a product yield significantly higher than that of *S. cerevisiae*. Its ethanol production rate is three- to fivefold higher than that of *S. cerevisiae*³, and its ethanol yield approaches 97% of the theoretical maximum⁴, as compared with 90–92% for *S. cerevisiae*⁵.

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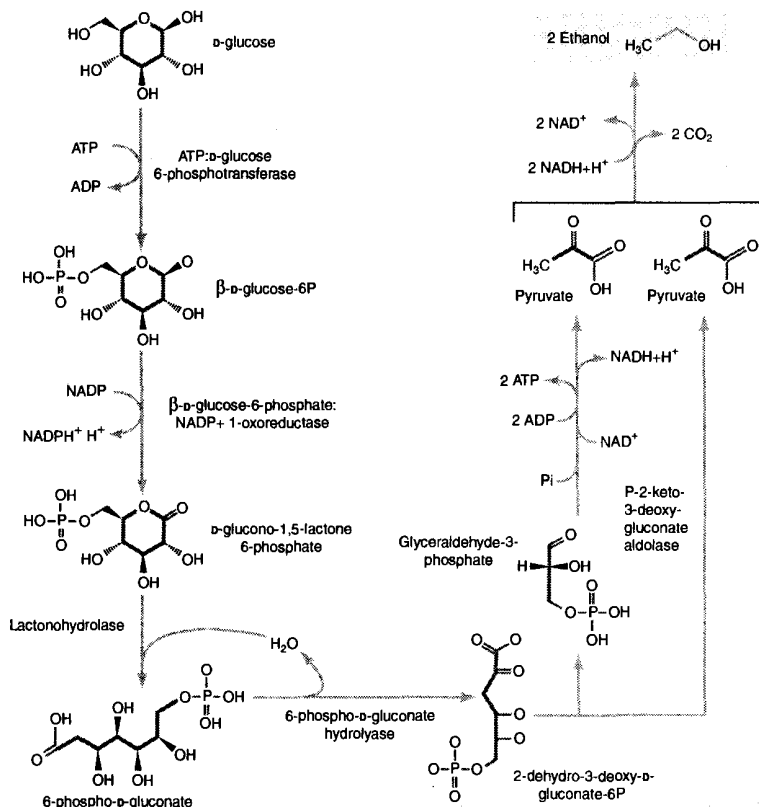


Figure 1 Ethanol fermentation in *Zymomonas mobilis*. The conversion of glucose into two moles of ethanol nets 1 mole of ATP. This pathway is commonly used by aerobic pseudomonads for the metabolism of glucose, but *Z. mobilis* makes use of it uniquely for anaerobic metabolism. The low ATP yield results in a low cell mass and the potential for higher ethanol yields.

Lindner first described *Zymomonas mobilis* (also known as *Z. lindneri*, *Thermobacterium mobile* or *Pseudomonas lindneri*) in 1928. This facultative anaerobe is perhaps most commonly known for the production of Mexican pulque—a white, acidic, viscous alcoholic beverage fermented from agave juice by *Z. mobilis* along with *Lactobacillus plantarum*, *Leuconostoc* sp., and *S. cerevisiae*⁶. (Contrary to popular belief, tequila and mescal, which are also made from agave species, are the results of yeast, not *Z. mobilis*, fermentation.)

Z. mobilis is distinctive in that it uses the Entner-Doudoroff pathway (Fig. 1) for glucose metabolism rather than the more familiar Embden-Meyerhoff-Parnas glycolytic

pathway used by *S. cerevisiae*. Although the Entner-Doudoroff pathway is widely distributed among pseudomonads, it is normally part of aerobic metabolism. Unlike glycolysis, which can theoretically generate two moles of ATP for each mole of glucose fermented to ethanol, the Entner-Doudoroff pathway has a net yield of only one ATP per mole of glucose. This low yield results in low cell mass and allows higher ethanol yields.

As always, a new genome brings surprises. Synteny analysis showed that *Z. mobilis* does not share significant lineage with 76 other published bacterial genomes. What comes the closest, is the obligatory aerobic chemolithotroph, *Novosphingobium hassiacum*^{7,8},

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which has a high capacity for aerobic degradation of polycyclic aromatic hydrocarbons. This relationship raises the question, "How would a novel pathway for glucose fermentation have evolved from a taxon known for versatile aerobic aromatic degradation?"

Perhaps part of the answer can be seen in the Entner-Doudoroff pathway itself—which is derived in part from the NADPH-generating steps of the oxidative pentose phosphate pathway. The key reaction diverting metabolic flux from the non-oxidative phase of the pentose phosphate pathway is the dehydration of 6-phosphogluconate by 6-phosphogluconate dehydratase to form 2-dehydro-3-deoxy-D-gluconate-6-phosphate (Fig. 1). In *Z. mobilis*, the gene that codes for this protein is found in a 6-kb cluster with other genes for glucose metabolism⁹. However, this dehydratase is fairly widely distributed among bacteria, and its occurrence in *Z. mobilis* probably does not tell the whole story.

Perhaps even more important than what is present is what is not present. A total genome sequence can reveal such deficiencies in ways that other approaches cannot. In the case of *Z. mobilis* (and *N. hassiacum*⁸), all genes for the enzymes of the glycolytic pathway are present except for phosphofructokinase, and without this, glycolysis is blocked. Even more confoundingly, *Z. mobilis* is missing most of the enzymes for the pentose phosphate pathway as well. It therefore has few options to metabolize glucose.

Z. mobilis lacks genes for 2-oxoglutarate dehydrogenase and malate dehydrogenase, and consequently has an incomplete tricarboxylic acid cycle. This does not block amino acid synthesis for the most part, because other pathways apparently function in this respect. It does, however, further limit the capacity of the organism to generate ATP through respiration. *Z. mobilis* does have a respiratory system, but it lacks electron acceptor modules, a deficiency that forces the cells to use acetaldehyde (or sulfate) as a terminal electron acceptor. In keeping with this, both pyruvate decarboxylase and alcohol dehydrogenase are highly expressed. Presumably, NADPH from the oxidative pentose phosphate pathway is converted to NADH and oxidized by this route.

Deficiencies in glycolysis and the pentose phosphate pathway greatly constrain the ability of *Z. mobilis* to assimilate other sugars. In fact, it was precisely the objective of adding the capacity for xylose and arabinose metabolism that led researchers at the National Renewable Energy Laboratory to engineer genes for xylulokinase, transketolase, transaldolase, xylose

isomerase and three other enzymes into this organism^{10,11}. Further improvements in substrate utilization can be expected to flow from additional manipulations of the genome.

What are some other ways in which *Z. mobilis* could be engineered to improve its fermentation performance? This bacterium has long been known to require lysine, methionine and several vitamins, and the complete genome has revealed specific reasons for these deficiencies. The only genes missing for lysine and methionine synthesis are *YfdZ* and *MetB*, respectively. By introducing these genes from another source, it might be possible to lower this organism's nutritional requirements. Disruption of the sulfate reduction pathway might be useful in reducing odors for beverage production, but the more likely applications of metabolic engineering are in manufacturing industrial ethanol.

Overexpression of transporters or limiting enzymes, as determined by expression and flux balance analysis, could increase the ethanol production rate. Cell separation and harvest could be improved by inducing genes responsible for flocculation, and alteration of membrane physiology might enhance resistance to inhibitors. *Z. mobilis* is sensitive to the presence of acetic acid, a common contaminant of lignocellulosic hydrolysates. The exact inhibitory mechanism, though probably related to membrane potential, is unknown.

With the completed genome in hand, global expression analysis should reveal ways to improve the performance of *Z. mobilis*, and more approaches to strain improvement will certainly be identified in the near future. As the authors have already demonstrated, *sE* could play a role in resisting ethanol stress, and insights from the *Z. mobilis* genome might also assist in engineering stress or ethanol resistance in other organisms.

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