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18. Four different preparations of the evolved ribozyme, purified by two phenol extractions, chloroform-isoamyl alcohol extraction, high-resolution denaturing polyacrylamide gel electrophoresis (PAGE), and reversed-phase chromatography (DuPont NENsorb), all exhibited comparable amounts of activity. This activity was lost after incubation with ribonuclease A but was retained after phenol extraction or incubation with proteinase K.
19. The k_{cat} was obtained at a saturating concentration of ribozyme with ribozyme in excess over substrate. Reaction conditions were either 50 nM substrate and 0.5, 1.0, or 2.0 μM ribozyme, or 60 nM substrate and 0.3 or 0.6 μM ribozyme, incubated in the presence of 10 mM $MgCl_2$ and 30 mM *N*-[2-hydroxymethyl]-piperazine-*N*′-[3-propanesulfonic acid] (pH 7.5) at 37°C for 2, 6, or 18 hours. The rate of product formation was linear over the reaction ($r > 0.99$). Product accounted for less than 10% of the starting material, even at the highest ribozyme concentration and latest time point, which allowed us to neglect loss of starting material due to cleavage at the phosphodiester linkage following GGCCCTC. A plot of reaction velocity versus ribozyme concentration was linear ($r = 0.999$), the slope providing an estimate for k_{cat} of $1 \pm 0.3 \times 10^{-6}$ min^{-1}.
20. The uncatalyzed reaction was evaluated by incubation of 50 nM (7.7×10^5 cpm) [5′-^{32}P]-labeled substrate under the standard reaction conditions for 86 hours and analysis of the products with a PhosphorImager (Molecular Dynamics). No product was detected, even though we could detect a minimum of 144 cpm of authentic 5′-labeled product against a background of the same amount of unincubated substrate. The second-order rate constant, k_{OH}, for hydrolysis of *N*-methylacetamide at 25°C was 3.0×10^{-4} M^{-1} min^{-1} [T. Yamana, Y. Mizukami, A. Tsuji, Y. Yasuda, K. Masuda, *Chem. Pharm. Bull.* **20**, 881 (1970)]. At pH 7.5 and 25°C, the pseudo–first-order rate constant was 9.4×10^{-11} min^{-1}. Hydrolysis of the unusually reactive Phe-Gly peptide linkage of Phe-Phe-Phe-Gly, attached by the NH_2-terminal Phe to a polyacrylamide resin, occurred at a rate of 2×10^{-7} min^{-1} at pH 7.0 and 25°C [D. Kahne and W. C. Still, *J. Am. Chem. Soc.* **110**, 7529 (1988)]. Hydrolysis of either Phe-Phe linkage could not be detected under these conditions, occurring at a rate $< 2 \times 10^{-9}$ min^{-1}.
21. Amino acid–containing substrates were prepared by chemical aminoacylation of [5′-^{32}P]$DNA1_{NH_2}$ in aqueous solution with carbonyldiimidazole as a condensing agent [K. W. Ehler and L. E. Orgel, *Biochim. Biophys. Acta* **434**, 233 (1976)]. Reaction conditions were as follows: 1 μM [5′-^{32}P]$DNA1_{NH_2}$, 0.1 M amino acid, and 0.2 M carbonyldiimidazole, freshly prepared in 0.5 M imidazole buffer (pH 8.0) and incubated at 4°C for 1 hour. Compounds corresponding to addition of one or more amino acids were separated by electrophoresis in a 20% polyacrylamide–8 M urea gel [G. Zieboll and L. E. Orgel, *J. Mol. Evol.* **38**, 561 (1994)], eluted from the gel, and purified by reversed-phase chromatography and a second round of denaturing PAGE and reversed-phase chromatography.
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27. We thank R. Lerner, D. Herschlag, and the members of our laboratory for helpful suggestions; U. Pieles (Ciba-Geigy) for synthesis and initial purification of the DNA-flanked amide substrates; and J. Tsang for providing evolved ribozyme G27-48. Supported by the NASA Specialized Center for Research and Training (NSCORT) in Exobiology (grant NAGW-2881).

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Metabolic Engineering of a Pentose Metabolism Pathway in Ethanologenic *Zymomonas mobilis*

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The ethanol-producing bacterium *Zymomonas mobilis* was metabolically engineered to broaden its range of fermentable substrates to include the pentose sugar xylose. Two operons encoding xylose assimilation and pentose phosphate pathway enzymes were constructed and transformed into *Z. mobilis* in order to generate a strain that grew on xylose and efficiently fermented it to ethanol. Thus, anaerobic fermentation of a pentose sugar to ethanol was achieved through a combination of the pentose phosphate and Entner-Doudoroff pathways. Furthermore, this strain efficiently fermented both glucose and xylose, which is essential for economical conversion of lignocellulosic biomass to ethanol.

The rapid and efficient fermentation of the pentose sugars found in lignocellulosic feedstocks is an absolute requirement for the economical conversion of biomass to ethanol. Lignocellulosic feedstocks, such as agricultural and forestry residues, are composed principally of cellulose, hemicellulose, and lignin. While there are microorganisms that efficiently ferment the glucose derived from cellulose, the conversion of the pentose sugars prevalent in hemicellulose to ethanol has been difficult because a suitable biocatalyst has not been available (*1*). Consequently, the discovery and development of microorganisms capable of converting pentose sugars, particularly xylose, to ethanol has been the focus of much research during the past 20 years. Recently, *Escherichia coli* and *Klebsiella oxytoca* have been successfully engineered for this purpose by the addition of genes for ethanol production from *Zymomonas mobilis*. In spite of the development of microorganisms with improved fermentation performance (*2*–*6*), high ethanol yields and concentrations from lignocellulosic feedstocks and short fermentation times have yet to be achieved.

Zymomonas mobilis is a bacterium that has been used as a natural fermentative agent in alcoholic beverage production. This organism demonstrates many of the traits sought in an ideal biocatalyst for fuel ethanol production, such as high ethanol yield and tolerance, high fermentation selectivity and specific productivity, the ability to ferment sugars at low pH, and considerable tolerance to the inhibitors found in lignocellulosic hydrolysates. In addition, the distillers dried grain from a *Zymomonas* fermentation is generally recognized as safe (GRAS) for use as an animal feed. Comparative performance trials have suggested that *Zymomonas* may become an important fuel ethanol–producing microorganism because it achieves 5 to 10% higher yield and up to fivefold higher volumetric productivity when compared with traditional yeast fermentations (*7*). Despite these potential advantages, fermentation processes using *Zymomonas* are still considered an immature technology compared with the industrial practice of yeast fermentation and have yet to be commercialized for fuel ethanol production from starch-based feedstocks.

Zymomonas mobilis has demonstrated ethanol yields of up to 97% of theoretical yield and ethanol concentrations of up to 12% (w/v) in glucose fermentations (*8*). These notably high yields have been attributed to reduced biomass formation during fermentation: whereas yeast produces 2 mol of adenosine triphosphate (ATP) per mole of glucose through the Embden-Meyerhoff-Parnas pathway, *Zymomonas* ferments glucose through the Entner-Doudoroff pathway (Fig. 1) and produces only 1 mol of ATP per mole of glucose (*9*, *10*). *Zymomonas*' facilitated diffusion sugar transport system (*11*) coupled with its highly expressed pyruvate decarboxylase and alcohol dehydrogenase genes (*12*) enable rapid and efficient conversion of glucose to ethanol. However, *Z. mobilis* can only ferment glucose, sucrose, and fructose and lacks the pentose metabolism pathways necessary to ferment the xylose commonly found in hemicellulose. Consequently, metabolic engineering of xylose fermentation in *Z. mobilis* is an essential step toward its development as a biocatalyst for fuel ethanol production from lignocellulosic feedstocks.

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Previous attempts to introduce a xylose catabolic pathway into *Zymomonas* have met with limited success (*13*, *14*). Although the xylose isomerase (*xylA*) and xylulokinase (*xylB*) genes from either *Xanthomonas campestris* or *Klebsiella pneumoniae* were functionally expressed in *Z. mobilis*, the strains were incapable of growth on xylose as the sole carbon source. The presence of low 6-phosphogluconate dehydrogenase and transketolase activities in *Z. mobilis* has been shown, but transaldolase activity was undetected (*14*), which indicates that *Z. mobilis* lacks a complete pentose phosphate pathway. Consequently, the introduction and expression of the genes encoding xylose isomerase, xylulokinase, transaldolase, and transketolase were necessary for the completion of a functional metabolic pathway that would convert xylose to central intermediates of the Entner-Doudoroff pathway and enable *Zymomonas* to ferment xylose to ethanol.

For construction of a xylose-fermenting strain, the *E. coli xylA* and *xylB* genes were cloned precisely under the control of a strong, constitutive *Z. mobilis* glyceraldehyde-3-phosphate dehydrogenase (GAP) promotor (*15*) by polymerase chain reaction (PCR)–mediated overlap extension (*16*). The resulting xylose assimilation operon (Fig. 2) was transformed into *Z. mobilis* CP4. Although both genes were functionally expressed, the transformants were unable to grow on xylose as a sole carbon source because transaldolase and sufficient transketolase activities were absent. Growth on glucose was inhibited by the addition of xylose to the medium, which suggests an intracellular accumulation of nonmetabolizable intermediates as had been observed previously with a similar strain (*14*).

The next step was to complete a pentose phosphate pathway that would allow conversion of xylulose-5-phosphate to the fructose-6-phosphate and glyceraldehyde-3-phosphate intermediates of the central Entner-Doudoroff pathway and subsequently to ethanol. Because no functional bacterial transaldolase genes are known, an open reading frame postulated to encode a transaldo-

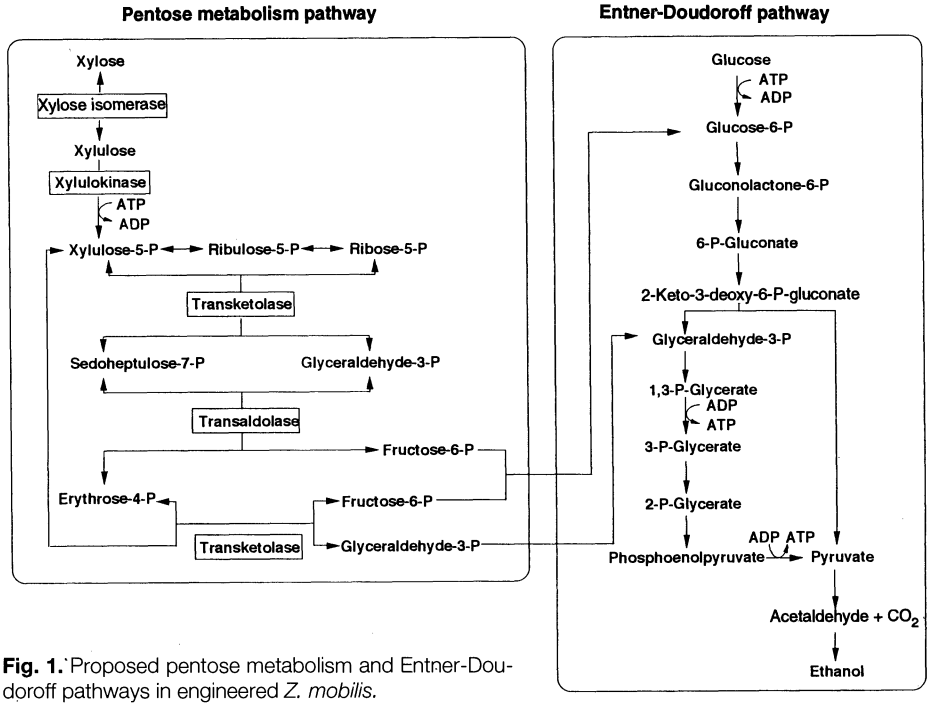
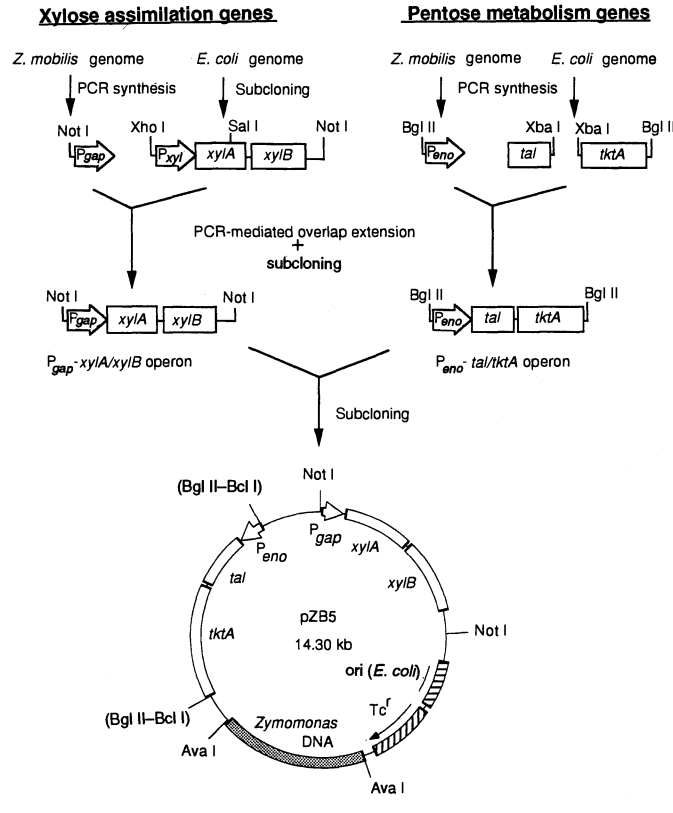


Fig. 1. Proposed pentose metabolism and Entner-Doudoroff pathways in engineered *Z. mobilis*.

Fig. 2. Construction of the P_{gap}-*xylA/xylB*, P_{eno}-*tal/tktA* operons and plasmid pZB5. The 7-kb Hpa I–Eco RI restriction fragment containing the *E. coli xylA* and *xylB* genes was obtained from plasmid pLC1-3 (*24*) and was subcloned into a pBlueScript plasmid (Strategene). The 308 base pairs (bp) of 5′-flanking DNA comprising the GAP promoter and the first 893 bp of *xylA* were separately synthesized with a common linking oligonucleotide primer. The individual DNA fragments were combined in a second PCR in which the complementary ends at the 3′ end of the GAP promoter and the 5′ end of the *xylA* gene were annealed. The addition of the 5′-GAP and 3′-*xylA* primers allowed the synthesis of a 1213-bp DNA fragment comprising a precise fusion of the GAP promoter to the *xylA* gene. This DNA fragment was used to replace a 2.5-kb Xho I–Sal I restriction fragment containing the native *xylA* promoter and 5′ end of the *xylA* gene. The *xylA* and *xylB* genes cloned under the control of the GAP promoter were contained on a 4.1-kb Not I fragment. The 1174-bp DNA fragment comprising the transaldolase gene homolog (*tal*) (*17*) was synthesized from *E. coli* genomic DNA and cloned under the control of the ENO promoter. The 2073-bp transketolase gene (*tktA*) was subsequently subcloned downstream of the transaldolase gene. A shuttle vector was constructed by ligation of pACYC184 (New England BioLabs, Beverly, Massachusetts) to the 2.7-kb plasmid from *Z. mobilis* ATCC 10988 and designated pZB186. This vector was modified to generate pZB188 by addition of a Not I linker to the filled-in Eco RI site. The ~3-kb Bgl II restriction fragment containing the P_{eno}-*tal/tktA* genes was ligated to pZB188 and designated as pZBET. The 4.1-kb Not I fragment containing P_{gap}-*xylA/xylB* was ligated to Not I–linearized pZBET. The plasmid containing the xylose assimilation operon in clockwise orientation and the pentose phosphate pathway operon in counterclockwise orientation was designated pZB5.



lase homolog at 0 to 2.4 min on the *E. coli* chromosome (*17*) was synthesized by PCR. This open reading frame was then subcloned under the control of the *Z. mobilis* enolase (ENO) promoter (*18*) by PCR-mediated overlap extension. The transketolase gene (*tkt*A) (*19*), which includes its ribosome binding site and transcriptional terminator, was synthesized from *E. coli* W3110 genomic DNA, and its expression was confirmed by complementation of an *E. coli* BJ502 transketolase-deficient mutant. This gene was then subcloned immediately downstream of the transaldolase homolog translation termination codon to form an operon encoding the nonoxidative portion of the pentose phosphate pathway (Fig. 2). Both transaldolase and transketolase genes were highly expressed in *E. coli* under control of both *lac* and *eno* promoters, thus confirming their accurate synthesis by PCR and that the transaldolase homolog encoded a functional transaldolase.

The two operons comprising the four xylose assimilation and pentose phosphate pathway genes were simultaneously transferred into *Z. mobilis* CP4 on a chimeric shuttle vector constructed from a 2.7-kb *Z. mobilis* native plasmid and pACYC184 (Fig. 2). Enzymatic analyses of *Z. mobilis* CP4 (pZB5) grown in a glucose-based medium demonstrated the presence of xylose isomerase (0.11 U/mg), xylulokinase (1.5 U/mg), transaldolase (0.88 U/mg), and transketolase (0.16 U/mg) activities (*14*, *20*). These enzymatic activities were largely undetectable in the control strain that contained the shuttle vector alone and, except for the higher transaldolase activity, were similar to the enzyme activities previously reported for *Z. mobilis* transformed with the *Klebsiella xylAB* and *E. coli tkt* genes (*14*).

The recombinant *Z. mobilis* CP4 (pZB5) was capable of growth on xylose as the sole carbon source and efficient ethanol production (Fig. 3A). Cell growth on xylose occurred at a rate of 0.057 per hour with ethanol as the principal fermentation product. Ethanol was produced at a yield of 0.44 g per gram (1.43 mol per mole) of xylose consumed, corresponding to 86% of theoretical yield. In contrast, the control strain that contained the shuttle vector alone did not grow on xylose or produce ethanol from xylose. The recombinant and control strains achieved 94% and 97% of theoretical ethanol yield from glucose within 16 hours, respectively (Fig. 3B). The control strain grew to a higher cell density and slightly faster (0.19 per hour) than the recombinant strain (0.15 per hour) on glucose. This result probably reflects the metabolic burden imposed on *Z. mobilis* by the high-level expression of the four additional genes. Furthermore, in the presence of a mixture of glucose and xylose, the recombinant strain fermented both sugars to ethanol at 95% of theoretical yield within 30 hours (Fig. 3C), thus providing a foundation for advanced process designs that require cofermentation of mixed-sugar feedstocks. Although no diauxic effect was apparent, glucose was preferentially utilized at a faster rate than xylose. Xylose transport is apparently mediated by the indigenous glucose-facilitated transport system and is likely to be inhibited competitively at high glucose concentrations (*11*).

Efficient fermentation of a pentose sugar to ethanol was thus obtained through a combination of the pentose phosphate and the Entner-Doudoroff pathways. *Zymomonas mobilis* is the only organism known to use the Entner-Doudoroff pathway anaerobically (*9*, *10*). With the introduction of the xylose assimilation pathway and the nonoxidative portion of the pentose phosphate pathway into this organism, xylose is presumably converted to xylulose-5-phosphate (Fig. 1) and then further metabolized to glyceraldehyde-3-phosphate and fructose-6-phosphate, which effectively couples pentose metabolism to the glycolytic Entner-Doudoroff pathway. In the overall fermentation reaction, 3 mol of xylose are converted to 5 mol of ethanol. Neglecting the NAD(P)H balance, the stoichiometry can be shown by the equation

$$3\text{xylose} + 3\text{ADP} + 3\text{P}_i \rightarrow 5\text{ethanol} + 5\text{CO}_2 + 3\text{ATP} + 3\text{H}_2\text{O}$$

The theoretical ethanol yield based on this stoichiometry is 0.51 g of ethanol per gram of xylose or 1.67 mol of ethanol per mole of xylose. In this new pathway, the net ATP yield from 3 mol of xylose is 2 mol less than that postulated for conventional xylose fermentation through a combination of pentose phosphate and Embden-Meyerhoff-Parnas pathways. Because less substrate is used for biomass formation, ethanol production via xylose fermentation in the engineered *Z. mobilis* is more efficient than in any other known microorganism.

Metabolic engineering has been defined as the purposeful modification of intermediary metabolism through the use of recombinant DNA techniques (*21*). One practical application of this approach is to broaden the substrate range of the host organism so that more economical raw materials can be used in industrial processes. Whereas many studies have shown the feasibility of metabolic engineering, few have achieved the yields, productivity, or final product concentrations required for practical processes (*22*).

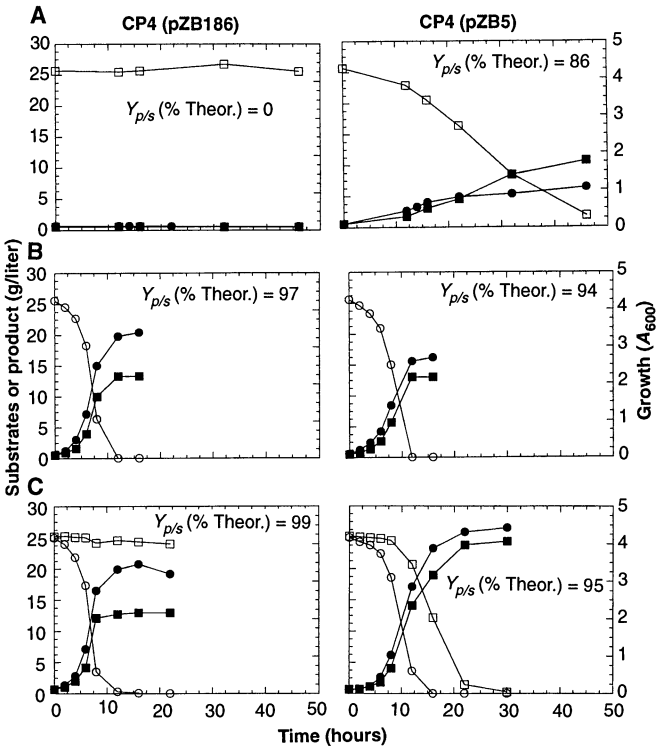


Fig. 3. Comparative fermentation performance of *Z. mobilis* CP4 (pZB186) and *Z. mobilis* CP4 (pZB5) in xylose, glucose, or mixed glucose-xylose media. (**A**) CP4 (pZB186) (left) and CP4 (pZB5) (right) in xylose medium (25 g/liter). (**B**) CP4 (pZB186) (left) and CP4 (pZB5) (right) in glucose medium (25 g/liter). (**C**) CP4 (pZB186) (left) and CP4 (pZB5) (right) in glucose (25 g/liter) and xylose (25 g/liter) medium. Cell growth was monitored by absorbance at 600 nm (A_{600}) (●) with a Spectronic 601 (Milton Roy) spectrophotometer. Glucose (○), xylose (□), and ethanol (■) were analyzed with a Hewlett-Packard 1090L HPLC equipped with an HP 1047A refractive index detector and a Bio-Rad HPX-87H organic acid analysis column operating at 65°C with a 0.01 N sulfuric acid mobile phase flow rate of 0.6 ml/min. The fermentation medium contained yeast extract (10 g/liter) (Difco), KH_2PO_4 (2 g/liter), and the appropriate carbohydrates. CP4 (pZB5) was grown anaerobically at 30°C until late log-phase in fermentation medium containing either glucose (25 g/liter) or xylose (25 g/liter) and tetracycline (10 μg/ml) and then inoculated to an initial A_{600} of 0.1 into 100 ml of the same fermentation medium (except media containing both sugars, which was inoculated with xylose-grown cells). CP4 (pZB186) inocula were prepared in fermentation medium containing glucose. The cultures were grown at 30°C without agitation. Ethanol yield ($Y_{p/s}$) is based on consumed sugar. Theoretical yield is 0.51 g of ethanol per gram of glucose or xylose.

This limitation has been attributed to a control architecture thought to prevent the radical alterations of metabolic flux distributions that are required for overproduction of certain metabolites at maximum yields (*23*). Here, metabolic engineering of primary glycolytic metabolism in *Z. mobilis* was achieved by the expansion of its fermentable substrate range to include the pentose sugar, xylose. This xylose-fermenting *Z. mobilis* is capable of achieving high ethanol yield, productivity, and concentration. Efforts are now underway to optimize strain performance in commercial feedstocks.

Ethanol is an important industrial chemical, and a national effort directed toward its development as an alternative transportation fuel will be successful if it can be produced from renewable feedstocks at economical cost. To this end, *E. coli* has been metabolically engineered to increase the ethanol yields from hexose and pentose sugars by introduction of the pyruvate decarboxylase and alcohol dehydrogenase genes from *Z. mobilis* (*2*). The results presented here demonstrate the feasibility of the complementary approach of introducing the xylose assimilation and pentose phosphate pathway genes from *E. coli* into *Z. mobilis*. This industrial microorganism is already recognized for its ability to produce ethanol at high yield and productivity from glucose-based feedstocks and is naturally tolerant of the high ethanol concentrations that would be encountered in a commercial biomass to ethanol process.

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A Phagosome-to-Cytosol Pathway for Exogenous Antigens Presented on MHC Class I Molecules

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Peptides from endogenous proteins are presented by major histocompatibility complex class I molecules, but antigens (Ags) in the extracellular fluids are generally not. However, pathogens or particulate Ags that are internalized into phagosomes of macrophages (MØs) stimulate CD8 T cells. The presentation of these Ags is resistant to chloroquine but is blocked by inhibitors of the proteasome, a mutation in the TAP1-TAP2 transporter, and brefeldin A. Moreover, phagocytosis of a ribosomal-inactivating protein inhibited MØ protein synthesis. These results demonstrate that MØs transfer Ags from phagosomes into the cytosol and that endogenous and exogenous Ags use a final common pathway for class I presentation.

A key event in the generation of an immune response is the display of antigenic fragments by major histocompatibility complex (MHC) molecules at the cell surface. In this process, peptides from proteins synthesized in the cell are generated in the cytosol and are transported through the TAP1-TAP2 peptide transporter into the endoplasmic reticulum (ER), where they bind to newly synthesized MHC class I molecules (*1*, *2*). Mutant cells that lack a functional TAP1 gene are therefore unable to present most peptides from endogenously synthesized Ags to CD8 T cells (*2*, *3*).

In most cells, exogenous Ags are not presented with MHC class I molecules; however, sometimes exogenous Ags can prime a cytotoxic T lymphocyte (CTL) response (*4*–*7*) and pathogens in phagosomes can generate a $CD8^+$ immune response (*8*). To determine whether the route taken by peptides from proteins in the phagosome overlaps with that used by peptides from endogenously synthesized proteins, we used antigen-presenting cells (APCs) from TAP1 mutant mice (*9*). These MØs, which lack the TAP1 gene, did not present the particulate exogenous ovalbumin (OVA) with MHC class I molecules (Fig. 1A). Similarly, these cells did not present soluble OVA loaded into the cytosol by electroporation (*9*, *10*). In contrast, MØs from wild-type mice presented the exogenous OVA particles with MHC class I molecules (Fig. 1A).

It has been suggested that MØs present exogenous Ag by regurgitating peptides from the phagosome into the extracellular fluids, where the peptides bind surface MHC class I molecules (*11*). However, MØs from TAP1 mutant and wild-type mice presented the OVA peptide SIINFKEL added to the culture medium equally well (Fig. 1B). Under these conditions the exogenous peptide binds directly to surface class I heterodimers. Thus, peptides released into the medium should have been presented equivalently by mutant and wild-type cells. Therefore, the requirement for the TAP1-TAP2 transporter indicates that peptides from exogenous Ag must be transported from the cytosol into the central vacuolar compartment.

In the ER, peptides from endogenous Ag bind to newly synthesized MHC class I molecules, and these complexes are transported to the cell surface. Brefeldin A (BFA) inhibits exocytosis of proteins from the ER and Golgi complex and thus prevents newly assembled peptide–MHC class I complexes from reaching the cell surface (*12*). To determine whether peptides from exogenous Ag travel similarly, we investigated whether BFA affected the presentation of phagocytozed Ag with MHC class I molecules.

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