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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Brefeldin A treatment of a cloned bone marrow (BM) MØ cell line, BM A3.1A7, for 3 hours completely inhibited the generation of OVA-K^b complexes from OVA particles (Fig. 1C). The inhibition was fully reversible (Fig. 1D), and this agent had no effect on the presentation of SIINFKEL added to the extracellular fluids (Fig. 1E). Similar results were obtained with another BM MØ cell line, BM C2.3. These data provide further evidence that peptides from exogenous Ags bind to newly synthesized MHC class I molecules in the central vacuolar compartment.

Antigens in the endocytic compartment of cells are hydrolyzed to oligopeptides by acid-optimal proteases. Agents that raise the pH of the distal acidic vesicles inhibit proteolysis in these compartments (*13*). To determine whether class I–presented peptides from exogenous Ag are generated in the endosomal or lysosomal compartments, we treated a cloned MØ cell line with the weak base chloroquine.

Chloroquine did not affect the presentation of exogenous OVA with MHC class I molecules (Fig. 2A). In contrast, it markedly inhibited the capacity of the same cells to present OVA with MHC class II molecules (Fig. 2C). As expected, treatment with this inhibitor had little effect on the ability of the BM MØ cell line to present, with either class I or class II molecules, synthetic OVA peptide added to the medium (Fig. 2, B and D). The inhibition of the class II pathway of Ag presentation provided a positive control that the chloroquine was effective in blocking catabolism in the acidic vesicles. These data indicate that the generation of peptides from exogenous Ag for MHC class I presentation did not require acid-optimal proteases and imply that the class I– and class II–presented peptides were not generated in the same compartment.

The major nonlysosomal protease that is responsible for degrading the bulk of endogenous cellular proteins is the proteasome (*14*). The catalytic activity of this particle has a neutral pH optimum and is not inhibited by weak bases (*15*, *16*). Proteasomal inhibitors block the proteolytic processing of cytosolic Ag and markedly decrease the supply of peptides to newly synthesized class I molecules (*17*). We therefore tested the effect of peptide aldehyde inhibitors of the proteasome on the presentation of exogenous Ag with MHC class I molecules. The presentation of OVA particles was inhibited when a cloned BM MØ cell line was exposed for 4 hours to MG115 (Fig. 3A).

MG115 inhibits the chymotryptic and peptidylglutamyl peptide–hydrolyzing activity of the proteasome and also inhibits cysteine proteases and calpains. To determine which protease was involved in the presentation of exogenous Ag, we tested two closely related analogs of MG115, LLnL and LLM. All three inhibitors are almost equipotent inhibitors of cathepsin B and calpains but differ markedly in their potency against the proteasome (*17*). Their ability to block the presentation of exogenous Ag with MHC class I (Fig. 3A) correlated with their potency in inhibiting the proteasome.

Several controls indicated that the proteasome inhibitors were selectively interfering with the generation of presented peptides by BM MØs and were not simply toxic. The inhibitory effects of these agents were fully reversible (Fig. 3B). Moreover, they did not block the presentation of an OVA peptide added to the media under conditions where it binds directly to class I molecules on the cell surface (Fig. 3C). Most importantly, these agents also did not inhibit the presentation of the OVA peptide SIINFKEL expressed in the cytosol from a minigene in a vaccinia viral vector (Fig. 3D). Therefore, the peptide aldehydes are blocking the proteolytic generation of peptides in BM MØs without affecting other steps in the class I pathway such as peptide transport, synthesis of class I heavy and light chains, or exocytosis of peptide-MHC complexes.

The requirement for an intact TAP1-TAP2 transporter for Ag presentation demonstrates that peptides derived from phagocytosed Ag gain access to the cytosol. The failure of chloroquine to inhibit presentation indicates that these antigenic peptides are produced in a nonlysosomal compartment. In addition, the inhibition of Ag presentation by LLnL and MG115 but not LLM suggests that the presented peptides are cleaved by the proteasome and hence are generated in the cytosol. Taken together, our results demonstrate a pathway whereby proteins can traffic from the distal vesicular compartments to the cytosol. It is not yet known whether this pathway involves specific transport or some nonspecific transfer as might occur from leaky or ruptured vesicles.

To independently test whether a protein can be transferred from the phagosome to the cytosol, we incubated BM MØ with a ribosomal-inactivating protein conjugated to a phagocytic substrate and measured protein synthesis. Gelonin inactivates ribosomes through a direct enzymatic modification and therefore must enter the cytosol to inhibit protein synthesis. Because this agent

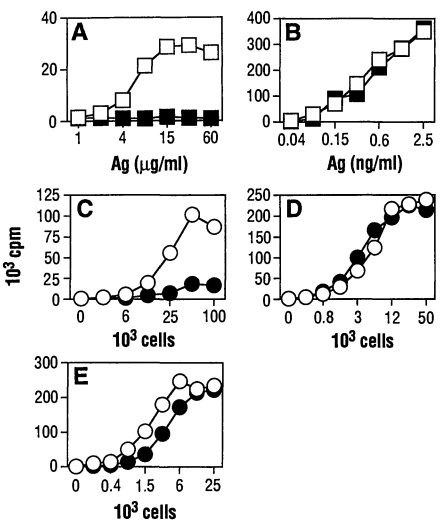

Fig. 1. Presentation of exogenous Ag with MHC class I molecules is dependent on the TAP1-TAP2 transporter and is inhibited by BFA. (**A** and **B**) Peritoneal exudate cells from TAP1 mutant mice (■) and littermate controls (□) (10^5 cells per well) were incubated with the indicated concentration of either OVA-beads (*22*) (A) or SIINFKEL (Ser-Ile-Ile-Asn-Phe-Lys-Glu-Leu) peptide (B) and RF33.70 T-T hybridoma (specific for OVA-K^b) (5×10^4 cells per well) essentially as described (*23*) except that indomethacin (0.25 μM) was added to the culture media. After 18 hours of incubation at 37°C, a sample (100 μl) of the supernatant was removed from duplicate cultures and assayed for interleukin-2 content with HT-2 cells (*24*). (**C** to **E**) BM A3.1A7 cells, a BM MØ cell clone (*19*), were first incubated with either OVA-beads (50 μg/ml) (C and D) or SIINFKEL (7.5 ng/ml) (E) for 2 hours at 37°C. Cultures were then treated with BFA at 5 μg/ml (●) or 0 μg/ml (○) for 3 hours at 37°C. Subsequently, APCs (C and E) were fixed with 1% paraformaldehyde and cocultured with RF33.70 T cells (5×10^4 cells per well). (D) Similar to (C), except that the APCs were not fixed with paraformaldehyde. Before fixation, the viability of APCs as determined by trypan blue was >99% in both treated and untreated cells. Cultures were then treated as described above.

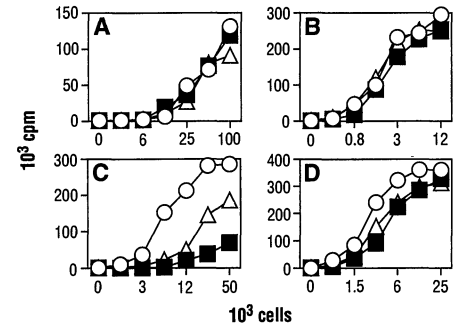

Fig. 2. Processing of exogenous Ag with MHC class I is nonlysosomal. BM A3.1A7 cells were first incubated with either 100 μM (■), 40 μM (△), or 0 μM (○) chloroquine for 1 hour before the addition of either OVA-beads (50 μg/ml) (**A** and **C**) or SIINFKEL (10 ng/ml) (**B**) or $OVA_{258-276}$ peptide (1 μg/ml) (**D**). Cultures were maintained at 37°C in the continuous presence of the inhibitor for 5 hours. APCs were then fixed with paraformaldehyde (1%) and cocultured with either RF33.70 T cells (A and B) or with MF2.2D9 T cells (specific for OVA-IA^b) (C and D). Cultures were then treated as described in Fig. 1.

is membrane impermeable, it is nontoxic to intact mammalian cells (*18*). Gelonin conjugated to beads (gelonin-beads) inhibits protein synthesis partially in the BM A3.1A7, BM B4, and BM C2.3 cell lines (Fig. 4), whereas beads conjugated to a control protein had no effect. In contrast, gelonin-beads did not inhibit protein synthesis in B cell lines (LB27.4 and A20) or in T cell lines (RF33.70 and DO11.10) (Fig. 4). This result confirms that proteins in the phagosome can gain access to the cytosol.

A conclusion of our findings is that there is a final common pathway for MHC class I presentation of exogenous and endogenous Ags. Both are sensitive to proteasomal inhibitors, mutations in TAP1-TAP2, and BFA. The only difference we have found for the presentation of exogenous Ag is that it was not augmented by interferon-γ (*19*). A likely explanation for this difference is that the entry of exogenous Ag into the cytosol may be the rate-limiting step for Ag presentation.

Our findings provide a mechanism to explain how MHC class I–restricted responses are generated against pathogens that reside in the phagosomes and do not enter the cytosol (*8*, *20*). Moreover, this pathway is also likely to explain the phenomenon of "cross-priming," where MØ may phagocytose cell fragments and then represent the Ag in the central lymphoid organs, as hypothesized by Bevan (*4*). Physiologically, this may be of importance for generating CD8 T cell responses to tumors or virally infected somatic cells (*21*).

In contrast, it has been previously shown in MØs that peptides from Ags in phagosomes can be regurgitated into the media and bind to class I molecules (*11*). In this experiment, MØs were fed bacteria that expressed a small peptide sequence of the OVA Ag fused to the COOH-terminus of a bacterial protein. Presumably, lysosomal or bacterial proteases (or both) liberated the antigenic peptide from the fusion protein in sufficient amounts to leak out of the cells. As a result of the binding of this peptide to surface MHC class I molecules, it was not possible to investigate whether there was also a cytosolic pathway of Ag presentation in these cells. We do not detect such a peptide regurgitation (*22*) presumably because of the 20- to 40-fold lower concentration of Ag in our experiments or possibly because the antigenic peptide is cleaved from OVA inefficiently by cathepsins. In any case, it is unlikely that peptide regurgitation from lysosomes is an important pathway for MHC class I presentation under physiological conditions for several reasons. Soluble protein Ags, which are efficiently degraded in lysosomes (providing a source of peptides that could be regurgitated), do not stimulate CD8 T cell responses in vivo. Moreover, immunization with antigenic peptides in vivo (including OVA SIINFKEL) does not generally elicit CD8 responses and therefore peptides, if regurgitated, would be unlikely to account for the generation of CTL responses to particulate Ags (*22*).

Although CTLs play a key role in the immune response against virus and tumors, this form of immunity is generally not primed with nonliving vaccines. However, CTL responses can be primed with proteins conjugated to phagocytic substrates (*22*). An important implication of the present study is that Ag presented through this pathway will be processed in the same manner as the endogenously synthesized Ag and the same epitopes should be presented. Accordingly, this pathway of presentation may be particularly useful for the development of nonliving vaccines against viral infections or tumors.

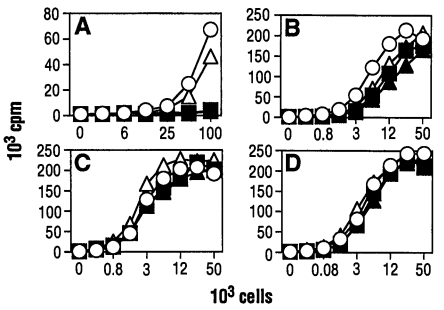

Fig. 3. Presentation of exogenous Ag with MHC class I is blocked by inhibitors of the proteasome. BM A3.1A7 cells were incubated in serum-free OPTIMEM medium (Gibco) supplemented with Nutridoma (1%) (Boehringer Mannheim) with either OVA-beads (50 μg/ml) (**A** and **B**) or SIINFKEL (7.5 ng/ml) (**C**) for 2 hours at 37°C. MG115 (10 μM) (▲), LLnL (50 μM) (■), LLM (50 μM) (△), or no inhibitor (○) were added and the cultures were maintained at 37°C in the presence of inhibitors for an additional 4 hours. (**D**) BM A3.1A7 cells were first incubated for 1 hour with or without inhibitors and then were infected for 3 hours with a recombinant vaccinia virus encoding the SIINFKEL peptide (*25*, *26*) (10 plaque-forming units per cell). After these treatments, APCs were kept alive (B) or were fixed with 1% paraformaldehyde (A, C, and D), and the generation of the OVA-K^b complexes was assayed with RF33.70. Before fixation, the viability of APCs as determined by trypan blue was >99% in all groups. Cultures were otherwise treated as described in Fig. 1. In (A), the closed triangles and the closed squares are superimposed on the baseline.

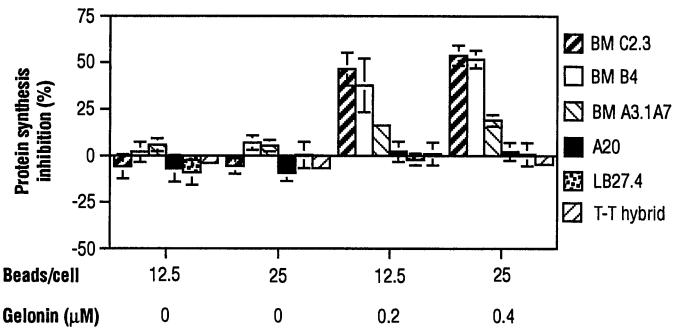

Fig. 4. Exogenous protein in phagosomes gains access to the cytosol. BM C2.3, BM B4, BM A3.1A7 (BM MØ), LB27.4, A20 (B lymphoblastoid cell line), or RF33.70 and DO11.10 (T-T hybrid) (5×10^5 per milliliter) were incubated overnight at 37°C in threonine-free RPMI 1640 (Gibco) supplemented with Nutridoma (1%) with either gelonin-beads, OVA-beads, or without beads. Gelonin was coupled to OVA-beads with a heterobifunctional linker SPDP (Pierce) as described (*27*). Triplicate cell cultures (200 μl) were pulsed for 1 hour at 37°C with 1 μCi of [^{3}H]-threonine. At the end of that time, 100 μl of phosphate-buffered saline, 10% calf serum, and 100 μl of 100% trichloroacetic acid were added. Precipitates were pelleted, resuspended in 50 μl of 88% formic acid, and the incorporation of labeled threonine was determined by scintillation counting. Each bar represents the mean ± SEM percent of the protein synthesis inhibition.

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Requirement of FGF-4 for Postimplantation Mouse Development

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Fibroblast growth factors (FGFs) are thought to influence many processes in vertebrate development because of their diverse sites of expression and wide range of biological activities in in vitro culture systems. As a means of elucidating embryonic functions of FGF-4, gene targeting was used to generate mice harboring a disrupted *Fgf4* gene. Embryos homozygous for the null allele underwent uterine implantation and induced uterine decidualization but did not develop substantially thereafter. As was consistent with their behavior in vivo, *Fgf4* null embryos cultured in vitro displayed severely impaired proliferation of the inner cell mass, whereas growth and differentiation of the inner cell mass were rescued when null embryos were cultured in the presence of FGF-4 protein.

Polypeptide ligands mediate intercellular communication governing cell growth, differentiation, and patterning in the vertebrate embryo. Ligands essential for normal embryogenesis from gastrulation onward include insulin-like growth factors, platelet-derived growth factor, mast cell growth factor, WNT-1, and FGFs (*1–3*). Although several ligands, such as FGFs and transforming growth factors α and β, are expressed in embryos before gastrulation (*4–7*), only maternally expressed leukemia inhibitory factor is known to provide an essential function by mediating uterine implantation of the blastocyst (*8*). By generating mice deficient for FGF-4, we show that embryonically expressed FGF-4 is required for postimplantation development of embryos in vivo and for normal inner cell mass (ICM) proliferation in vitro.

FGFs are a family of nine structurally related ligands (*9*) that can activate a corresponding family of receptor tyrosine kinases (*10*) to elicit a wide range of cellular responses. The 21-kD FGF-4 protein (*11*) is mitogenic toward cultured fibroblasts, melanoblasts, endothelial cells, and embryonic limb mesenchyme (*12, 13*) and induces mesoderm formation in amphibian ectoderm explants (*14*). The *Fgf4* gene is expressed in undifferentiated embryonic stem (ES) cells and their oncogenic equivalent, embryonal carcinoma (EC) cells (*15*), and this expression is governed by an evolutionarily conserved 3′ enhancer element (*16*). In order to achieve a high frequency of *Fgf4* gene disruption in ES cells, we used an "enhancerless" targeting vector (*17*) with a promoterless neomycin-resistance gene downstream from a 5′ homology segment bearing the native *Fgf4* promoter and upstream from a 3′ homology segment lacking the enhancer (Fig. 1A). Homologous recombination of the vector into the *Fgf4* locus deletes exon 1, which encodes the first 109 amino acids of FGF-4, including the NH_2-terminal secretion signal sequence (*18*). When this vector was electroporated into ES cells (*19*), 3 out of 80 G418-resistant ES clones examined showed homologous recombination at the *Fgf4* locus. Two of these clones, 4B and 14E (Fig. 1B), were microinjected into blastocysts to yield chimeric mice that were able to transmit the mutant allele through the germ line (Fig. 1C) (*20*). Unexpectedly, the 14E ES line had lost the Y chromosome and transmitted the mutant allele through female chimeras only (*21*).

Heterozygous males and females were phenotypically normal and fertile. Intercrosses between heterozygotes failed to yield

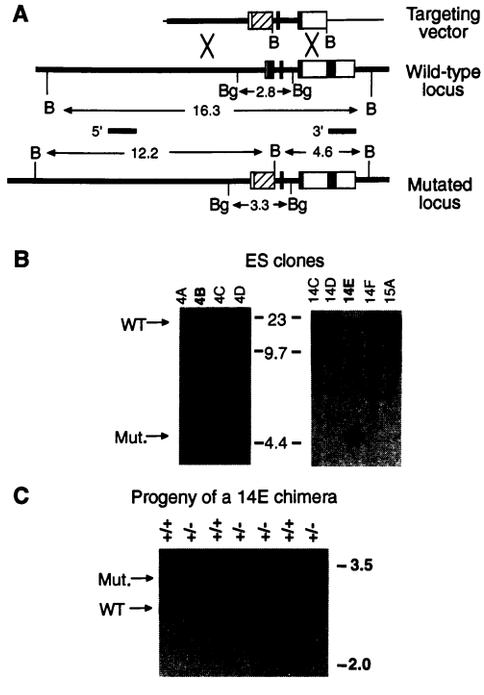

Fig. 1. Strategy of *Fgf4* disruption. (**A**) Targeting vector (*17*), the *Fgf4* locus (*18*), and the mutated *Fgf4* locus. The targeting vector is aligned above the wild-type allele to illustrate the homologous recombination event that generated the disrupted allele, depicted below. The disrupted allele lacks the first 109 codons of *Fgf4*, which encode the NH_2-terminal secretion signal and the first 28 residues of the FGF family core homology region. Shown are the *Fgf4* coding sequence (filled boxes), the 3′ enhancer (*16*) (gray box), other untranslated regions of *Fgf4* exons (open boxes), the Neo^r coding sequence (hatched box), the genomic sequence (thick line), the vector sequence (thin line), the upstream Xba I–Eco RI probe (5′), the downstream Pvu II–Sal I probe (3′), Bam HI (B), and Bgl II (Bg). (**B**) Southern blot analysis of DNA from ES clones. The 3′ probe hybridizes to a diagnostic 4.6-kbp fragment (in addition to the wild-type 16.3-kbp fragment) in Bam HI–digested DNA from targeted clones 4B and 14E. WT, wild-type band; mut., mutant band. Detection of expected 12.2- and 16.3-kbp Bam HI fragments with the 5′ probe confirmed homologous recombination on both sides of the targeting vector (*21*). (**C**) Southern blots of DNA from progeny of 14E-derived chimeras. Bgl II–digested DNAs from litters were probed with a mixture of Neo^r and exon 1 fragments, which detect diagnostic 3.3- and 2.8-kbp fragments, respectively (*20*).

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