A work by Kohout *et al.*[6] in this issue has addressed these questions. Motions of the VSD of this hybrid protein were detected by real-time tracing of a fluorescent dye attached to the extracellular side of VSP molecules under the control of transmembrane voltage, a method called voltage clamp fluorometry. As readouts of the enzymatic activity, phosphoinositide-dependent potassium channel currents were electrically measured or a GFP-tagged phosphoinositide sensor protein was imaged using confocal microscopy[7]. Effects of mutations at PBM on enzymatic activities were also examined by measuring their CD spectra and in an *in vitro* enzyme assay. Through combining these approaches, the authors found that coupling between the VSD and the catalytic domain was altered when $PI(4,5)P_2$ levels were depleted. Such an effect of $PI(4,5)P_2$ depletion on coupling was not seen when PBM was mutated. This phosphoinositide regulation of coupling most likely occurs through direct binding to PBM. Surprisingly, the deletion of PBM eliminated enzyme activity measured *in vitro* even with a water-soluble substrate, a feature not shared by the related PTEN. On the basis of these findings, the authors propose that a $PI(4,5)P_2$–PBM interaction is required to maintain active enzyme state, whereas depletion of $PI(4,5)P_2$ inactivates the enzyme. This leads to an intriguing hypothesis under which hyperpolarization disables the interaction of PBM with $PI(4,5)P_2$, keeping the enzyme in an inactive conformation, whereas depolarization-induced motions of VSD regulate the phosphoinositide–PBM interaction to activate the downstream enzyme.

Several details remain to be tested. First, it should be determined whether PBM binds directly to $PI(4,5)P_2$, as has been shown for PTEN. The effects of $PI(4,5)P_2$ depletion on coupling between VSD and enzyme are most likely through release of $PI(4,5)P_2$ from PBM. However, perturbation of phosphoinositide profiles may cause secondary effects. In addition, VSP has a domain (C2) that could potentially interact with membranes. C2 domains from other proteins are known to selectively bind $PI(4,5)P_2$ (ref. 8). Also, the decoupling seen upon depletion of $PI(4,5)P_2$ levels was found only at extremely high membrane potential, so the effects of $PI(4,5)P_2$ on coupling could be conditional.

Studies of Ci-VSP are clearly shifting to address the more biochemical aspects of the protein's functions. It will be important to address how $PI(4,5)P_2$ binding to PBM can activate the enzyme. It is also an open question whether silencing of VSP activities by depletion of $PI(4,5)P_2$ can occur as a negative feedback mechanism to limit hyperactivity associated with persistent VSP activity in any physiological context—for example, in sperm, where VSP is abundantly expressed[1], Answering these questions will also help strengthen our understanding of the molecular mechanisms of PTEN. The PBM motif of PTEN is conserved from slime mold to humans, and mutation in this motif in human PTEN leads to cancer[2]. In PTEN, $PI(4,5)P_2$-dependent regulation of enzymatic activities through PBM would potentially work as a positive feedback mechanism because $PI(4,5)P_2$ is the product of PTEN enzymatic activity. However, whether alteration of $PI(4,5)P_2$ abundance regulates PTEN activity is not well understood in a functional context. Interestingly, deletion of PBM completely eliminates the enzymatic activities of Ci-VSP, whereas the analogous mutant of PTEN retains some activity (particularly toward water-soluble substrates). This raises the simple question of to what extent the PTEN-like region of VSP shares its mechanisms with PTEN.

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References

1. Murata, Y., Iwasaki, H., Sasaki, M., Inaba, K. & Okamura, Y. *Nature* **435**, 1239–1243 (2005).
2. Maehama, T., Taylor, G.S. & Dixon, J.E. *Annu. Rev. Biochem.* **70**, 247–279 (2001).
3. Walker, S.M., Leslie, N.R., Perera, N.M., Batty, I.H. & Downes, C.P. *Biochem. J.* **379**, 301–307 (2004).
4. Campbell, R.B., Liu, F. & Ross, A.H. *J. Biol. Chem.* **278**, 33617–33620 (2003).
5. Villalba-Galea, C.A., Miceli, F., Taglialatela, M. & Bezanilla, F. *J. Gen. Physiol.* **134**, 5–14 (2009).
6. Kohout, S.C. *et al. Nat. Chem. Biol.* **6**, 369–375 (2010).
7. Murata, Y. & Okamura, Y. *J. Physiol. (Lond.)* **583**, 875–889 (2007).
8. Sanchez-Bautista, S., Marin-Vicente, C., Gomez-Fernandez, J.C. & Corbalan-Garcia, S. *J. Mol. Biol.* **362**, 901–914 (2006).

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METABOLISM

Biofuel via biodetoxification

The buildup of toxic intermediates during lignocellulose pretreatment limits the utility of this abundant biomass for biofuel production. A recent study on the degradation pathways of two of the most hazardous toxins, furfural and HMF, now paves the way for mechanism-based enhancements of biodetoxification efficiency.

Hongwei Dong & Jie Bao

High oil prices have driven the global community to increasingly adopt renewable fuels such as ethanol for transportation. As an outcome of this trend, corn is now facing a worldwide shortage. The United States has predicted that corn ethanol is reaching the production ceiling even given efforts to cut back, such as China's 2006 ban on the use of corn for ethanol production. Among other possible feedstock choices, lignocellulose, such as that found in corn stover, rice straw, wheat straw, bagasse or switch grass, is the most abundant source for production of fuel ethanol or other value-added chemicals, and lignocellulose utilization is becoming the primary stream for the bioprocess industry[1]. However, a major bottleneck in using lignocellulose is the generation of toxic compounds during a requisite pretreatment step. A recent paper by Koopman *et al.*[2] now provides potentially key metabolic information for more efficient green processes in which these toxic inhibitors can be removed biologically.

The main difference between starch and lignocellulose is not its chemical composition: corn contains 30%–40% of glucose, and corn stover contains almost the same. The essential difference is in its structure: starch is composed of α-1,4-linked glucose units, whereas cellulose is composed of β-1,4-linked glucose units

to form a crystalline structure. When combined with the hard lignin shell and a hemicellulose glue, lignocellulose is extremely resistant to physical or biological attacks, a feature called biocalcitrance[3]. To overcome this biocalcitrance and access the glucose core, harsh 'pretreatments', such as steam explosion, dilute acid application or ammonia fiber explosion, are required (**Fig. 1**). However, various toxins that inhibit ethanol fermentation strains as well as specific cellulase enzymes are generated from these pretreatments. These toxins include organic acids (acetic acid, formic acid and levulinic acid), furan derivatives (furfural and 5-(hydroxymethyl) furfural (HMF)) and phenol derivatives (vanillin and 4-hydroxybenzaldehyde). The most frequently used 'detoxification' methods—water washing and overliming—generate additional problems[4]. For example, almost 30% of the pretreated lignocellulose solids can be lost during the process, leading to a loss of ethanol of at least the same percentage; the considerable amount of water used leads to high costs of waste water treatment; and finally, the high percentage of water used dilutes the subsequent fermentation, leading to a high energy cost as the ethanol is condensed and distilled. An alternative option for removing toxins that avoids the disadvantages mentioned above is biodetoxification, which relies on microorganisms to degrade the toxins as part of their normal metabolism[5–8]. However, its slow rate of toxin degradation limits its practical application. Furthermore, very limited information about genetic and metabolic pathways by which these toxins are degraded is available, making it difficult to improve the reaction rates[9].

The work by Koopman *et al.*[2] makes major progress toward enhancing the efficiency of this process by elucidating the degradation pathways of two of the most hazardous inhibitors, furfural and HMF. The authors had previously discovered that the bacterium *Cupriavidus basilensis* HMF14 could metabolize furans and could degrade these compounds in particular. To identify the pathways involved, they constructed a large transposon mutant library of 14,000 *C. basilensis* HMF14 colonies to screen for genes relevant to the furfural- and HMF-degrading capacities. They identified 25 transposon mutants unable to grow on furfural and/or HMF and sequenced and analyzed the chromosomal DNA flanking the transposon insertions. Two clusters seemingly essential for the furfural and/or HMF metabolism were discovered. Further analysis to confirm this hypothesis used mutant strains to monitor distinct phenotypes, conversion experiments with cell lysates, enzymatic activity analysis, and observation of accumulated metabolic intermediates of the mutant strains with furfural or HMF as the sole carbon sources. The combined data demonstrated that the enzymes encoded by the *hmfABCDE* cluster constituted the pathway shared by the furfural and HMF metabolism, and an additional *hmfFGH'H* cluster was required for HMF metabolism. In order to further confirm the function of the genes and the putative pathway, the two clusters were also expressed separately in a heterologous host, *Pseudomonas putida* S 12, which normally cannot use furoic acid, furfural, furfuryl alcohol and HMF as carbon sources. With this extensive support, the authors were able to conclude that they had identified the genes essential for furfural and/or HMF metabolism. Although the specific genes required for the conversion of furfuryl alcohol to furfural and furoic acid (and the corresponding conversion of HMF alcohol to HMF and HMF acid) were not found in the two clusters, the authors suggest that nonspecific dehydrogenases abundant in the cell could participate in the conversion, given that the necessary activity—NAD-dependent furfuryl alcohol dehydrogenase—is present. BLAST searches using the sequences of the two clusters demonstrated that the newly revealed furfural and HMF metabolic pathway is present in other Gram-negative bacteria.

This work is significant as the first report, to our knowledge, on the entire metabolic pathway of furfural and HMF degradation with a clear genetic background, and opens new avenues for degradation of lignocellulose-derived inhibitors by *C. basilensis* or other microorganisms with similar functions. The presence of the *hmf* clusters in other bacteria should yield a rich resource for metabolic engineering or microbiology experiments, and the methodology used in this paper should chart a course for the discovery of other degradation pathways for furfural, HMF or other inhibitors such as organic acids and phenol derivatives. To integrate this information into real-world processes, more information is still needed, such as the genetic and metabolic regulation that controls, and could thus accelerate, toxin degradation. Genome sequencing of more microorganisms will be critical for broadening our understanding of both the pathways and the regulation

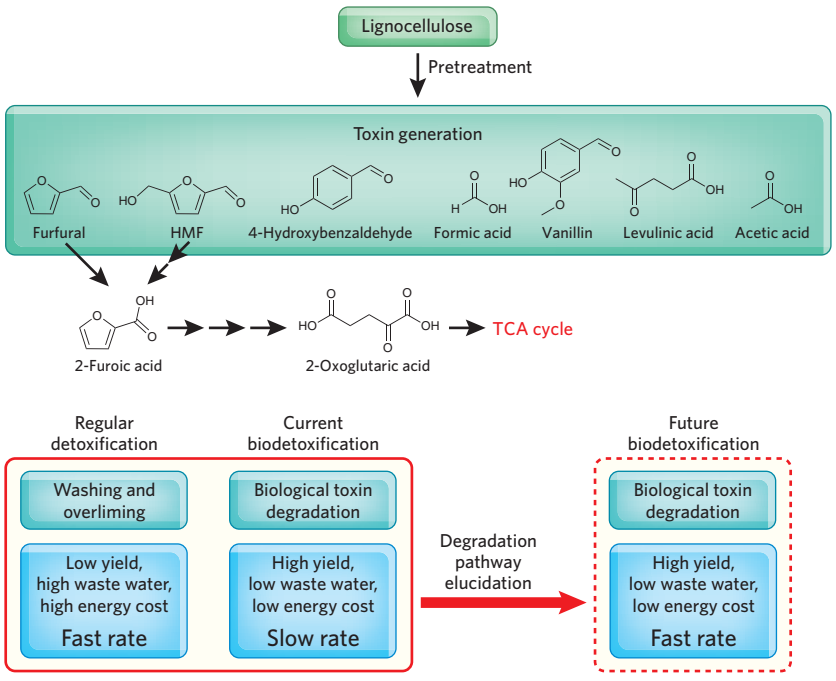

Figure 1 | Detoxification of lignocellulose-derived inhibitor compounds. Toxic inhibitors are generated during lignocellulose pretreatment and removed by washing or overliming in the conventional detoxification methods. Biodetoxification methods provides high yields, uses limited waste water, and has low energy costs, but its slow rate prohibits its practical use. Elucidation of the degradation pathways of two of the most hazardous inhibitors, furfural and HMF, now points to an improved pathway for future processing. TCA, tricarboxylic acid.

of these processes. Though this will be a significant investment, fast inhibitor degradation will lead to significant cost and material reductions compared to current detoxification methods (**Fig. 1**) and, as such, will be important in providing a green, cost-effective option for lignocellulose processing technology, as well as biofuel and biorefinery processes more generally.

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References

1. Lynd, L.R. *et al. Nat. Biotechnol.* **26**, 169–172 (2008).
2. Koopman, F., Wierckx, N., de Winde, J.H. & Ruijssenaars, H.J. *Proc. Natl. Acad. Sci. USA* **107**, 4919–4924 (2010).
3. Himmel, M.E. *et al. Science* **315**, 804–807 (2007).
4. Wyman, C.E. *et al. Bioresour. Technol.* **96**, 1959–1966 (2005).
5. Yu, J. & Stahl, H. *Bioresour. Technol.* **99**, 8042–8048 (2008).
6. Nichols, N.N. *et al. Appl. Biochem. Biotechnol.* **121**, 379–390 (2005).
7. Lopez, M.J. *et al. Appl. Microbiol. Biotechnol.* **64**, 125–131 (2004).
8. Wierckx, N., Koopman, F., Bandounas, L., de Winde, J.H. & Ruijssenaars, H.J. *Microb. Biotechnol.* published online, doi:10.111/j.1751-7915.2009.00158.x (15 December 2009).
9. Nichols, N.N. & Mertens, J.A. *FEMS Microbiol. Lett.* **284**, 52–57 (2008).

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DRUG DISCOVERY

Engineering drug combinations

The level of an individual protein in cells treated with combinations of drugs is best explained by simple linear superposition of the protein levels in response to single drugs. This finding may facilitate rational design of higher order drug combinations.

Scott J Dixon & Brent R Stockwell

As we learn more about disease mechanisms at the molecular level and move toward a more personalized approach to medicine, the need to adjust the composition of therapies for each individual patient continues to grow[1]. Drug combinations can target multiple sites within the same protein, countering the emergence of drug resistance, or multiple nodes within a molecular network, enabling the combinatorial control of biological systems[2,3] (**Fig. 1a**). These considerations make drug combinations leading candidates for personalized therapies. In fact, drug combinations are already in widespread clinical use. Two well-known examples are the three-drug combinations of reverse-transcriptase and protease inhibitors used to treat HIV infection[4] and the four-drug combination comprising DNA-damaging agents, a microtubule disruptor and a corticosteroid (cyclophosphamide, doxorubicin, vincristine and prednisone, together known as CHOP) used to treat non-Hodgkin's lymphoma[5]. Variations on these treatments exist that add even more drugs to the mix. Given this trend, one may ask: what is the most effective drug combination complexity, and how will we know when we get there? In nature, a bacterial endosymbiont growing on the antennae of certain wasp species releases a cocktail of nine different antibiotic compounds that together protect growing wasp larvae from a broad range of fungal and bacterial pathogens[6]. This suggests that we have far to go before achieving the same sophistication in designing drug combinations. Would ten-, fifty- or hundred-drug combinations be more effective than existing three- and four-drug combinations to combat diseases or selectively modulate cell function? How could such combinations be identified? Certainly at this level, both clinical trial-and-error and unbiased screening of all possible combinations of drugs become utterly impractical. We must therefore devise ways to better predict the effects of drug combinations on molecular and cellular networks. In a recent paper, Geva-Zatorsky *et al.*[7] focus on one aspect of this problem, investigating the effects of drug combinations on protein abundances in cells.

Geva-Zatorsky *et al.*[7] investigated what happens to protein levels in cells treated with various drugs. Building on previous work[8,9], they used automated image analysis to examine the expression levels of 15 functionally diverse yellow fluorescent protein (YFP)-tagged proteins in response to 13 different drugs and 19 drug combinations, over the course of 2 days in culture. They observed a surprisingly wide array of protein level changes over time; these changes were unique to each drug–protein pair. Thus, for example, the level of the ribosomal protein RPS3 increased in response to nocodazole but decreased in response to camptothecin; by contrast, the level of the nuclear lamin protein LMNA increased in response to both drugs.

What effect does the combination of two drugs have on specific protein levels? Remarkably, protein levels in cells treated with combinations of two drugs was best described by the weighted sum of the protein level in response to either drug alone (**Fig. 1b**). These weights (from 0 to 1) refer to how much each drug 'counts' toward the final level. The weights were protein specific and varied according to the concentration of drug tested, but they were constant over time and, for the most part, summed to 1. One important caveat is that not all drugs conformed to the linear superposition model. For unknown reasons, the effects of one compound, the phosphatidylinositol-3-OH kinase inhibitor wortmannin, could not be explained by linear superposition. It is not clear whether this is an isolated case or whether a significant fraction of all drugs will produce effects not explicable in terms of the superposition model.

Nevertheless, Geva-Zatorsky *et al.*[7] went on to ask whether it is possible to predict the effects on protein levels of higher order, three- and four-drug combinations using only the observed protein levels in two-drug combinations. In most cases, there was good agreement between the levels predicted from the weighted sums observed for the individual two-drug combinations and the observed levels in the three-drug and four-drug combination experiments (**Fig. 1b**). By implication, all that may be required to predict protein levels in response to any number of drugs is knowledge of each