After Horizontal Gene Transfers, Metabolic Pathways May Need Further Optimization

Effective use of a horizontally transferred pathway can require co-evolutionary changes in the host or pathway

Joshua K. Michener and Christopher J. Marx

One surprising discovery of the genome sequencing era is the sheer ubiquity of horizontal gene transfers (HGT), particularly among bacteria. Genetic material encoding single enzymes and entire metabolic pathways has moved between distantly related species, with important consequences for microbial evolution, physiology, and ecology. HGT facilitated the rise of antibiotic resistance, the emergence of new bacterial species, and the adaptation of microbes to new ecological niches. By drawing on the metabolic potential of this "flexible genome," novel microorganisms are capable of adopting a diverse array of phenotypes.

However, the frequency of HGT should not obscure the challenges involved for recipient cells trying to accommodate their new genes. After all, we observe the results of only those rare successful transfers that led to improved cells, rather than the vastly more common cases of unfit recombinant cells that failed to survive.

HGT Succeeds Only Rarely

To transfer a gene or genes successfully from one cell to another, a DNA molecule must first move into its new host by means of transduction, conjugation, or natural competence. Next, the transferred DNA must elude any recipient-cell defense mechanisms that target incoming genes, including CRISPR/Cas or restriction/modification systems, and then stably replicate within the recipient cell. Depending on the source of the DNA, the transferred gene may need to acquire appropriate transcription and translation signals if it is to become active in its new environment. Any necessary posttranslational interactions, including localization or chaperone-assisted folding, must be maintained in or adapted to the new host. Even if all of these challenges are overcome, a gene may still fail to function effectively.

Because cells contain complex, interconnected metabolic and regulatory networks, perturbing such systems by adding new genes can prove harmful, compromising both the horizontally transferred pathway and the native pathways of the new host cell. Many of the genes that we believe were successfully transferred are predicted to lie at the periphery of these networks, minimizing the chances for deleterious interactions. However, even genes that interact with few partners can be highly disruptive, and transfers of core functions may endow recipients with strong selective benefits that overcome transient fitness costs. In these cases, effective use of newly acquired pathways may depend on post-transfer refining mutations to minimize deleterious interactions and their costs to the recipient cells.

Experimental Evolution and Metabolic Engineering Highlight the Challenges of HGT

Studying transient post-transfer refinements in natural isolates can be challenging, since natural selection quickly eliminates any cells with de-

SUMMARY

- **Pathways acquired through horizontal gene transfer (HGT) may impose new stresses on recipient cells.**
- **HGT-related stresses can limit the utility of newly acquired genes.**
- **Effective use of horizontally transferred genes may require refining mutations to both the recipient and the transferred genes.**
- **Synthetic biologists deliberately transfer genes between species and seek to determine how such genes can be optimized to function properly within recipient cells.**

AUTHOR PROFILE

Michener: from Home Algebra to Horizontal Gene Transfers and Kombucha Tea Preps

Josh Michener had not planned to study biology. "I had an empty slot in my schedule for my senior year of high school, and took a genetics class," he says. "I had a great teacher, really enjoyed the class, but still was leaning towards majoring in computer science." As a college freshman, "I signed up for another genetics class. One thing led to another, and I eventually added a major in biology."

Today, Michener, a postdoctoral fellow at the Massachusetts Institute of Technology (MIT), studies how microbes evolve after gaining new functions either through horizontal gene transfer or genetic engineering. His research has moved from engineered to natural systems, "looking at how nature solves similar problems when they occur during horizontal gene transfer," he says.

Michener, 30, grew up in Chapel Hill, N.C., where his parents work at the Duke University Medical Center. His father is Chair of the Department of Community and Family Medicine, while his mother is an assistant consulting professor with the Division of Community Health. An older sister teaches high school science in Charlottesville, Va.

His parents nurtured his love of math and science, even to the point of teaching him algebra at home. He made the U.S. Physics Olympiad team when he was a high school junior. He spent his final two years of high school at the North Carolina School for Science and Math, a public boarding school in Durham, an experience he calls one of the most influential of his young life. Later, he earned his Bachelor of Science degree in 2006 from MIT, and his Ph.D. in Bioengineering in 2012 from the California Institute of Technology.

What really "kick started" his career was the opportunity to conduct research as an undergraduate with Drew Endy, a synthetic biologist and bioengineer, now at Stanford University, but earlier at MIT, Michener says. "As both a biologist and an engineer, the idea of deliberately making biology easier to engineer was extremely attractive. I sent him an e-mail, cold, asking if I could work in his lab. He agreed, and I ended up spending much of the rest of my time in college working in the Endy Lab." A research fellowship took Michener to Sweden in 2011 to work with Jens Nielsen at Chalmers University of Technology. "Working with Jens, we tried to look at my strains more holistically, to see all the different ways in which my engineered pathway was interacting with the host microbe."

Michener's wife is a physical therapy assistant at Massachusetts General Hospital, who plans to return to school for her doctorate in physical therapy. Michener, an enthusiastic cyclist, also enjoys cooking, sometimes incorporating fermentation steps into his culinary efforts—for example, by making sauerkraut and kombucha tea. "Friends have tried to convince me to brew my own beer, but that's probably not going to happen any time soon," he says.

Marlene Cimons

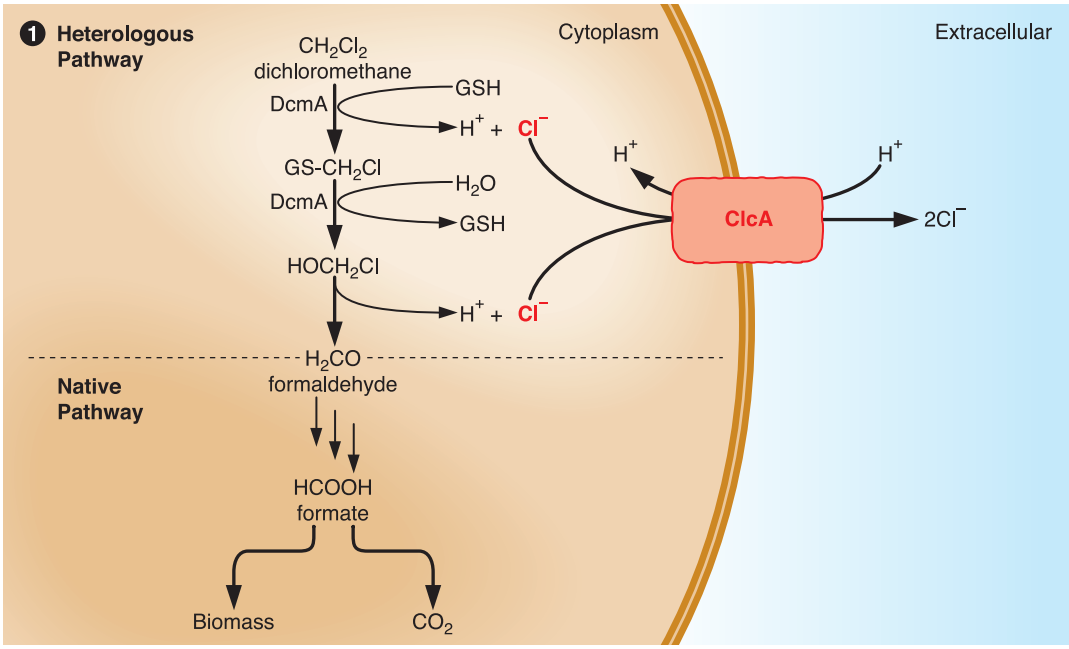
Marlene Cimons lives and writes in Bethesda, Md.

creased fitness. However, synthetic biologists and metabolic engineers are effectively conducting HGT by moving genes between distantly related organisms. Some of these engineering projects have yielded microbes that can produce pharmaceuticals, specialty chemicals, and biofuels, and these efforts offer an opportunity to appreciate the challenges of HGT.

Unfortunately, for every successful metabolic engineering project, many more unreported attempts fail, when enzymes and pathways that function well in their original host cells are ineffective in the recipient strain. In some cases—for instance, when genes encoding membrane-bound eukaryotic enzymes are transferred into bacteria—these failures are not surprising. In many others, however, the reasons for failure are neither expected nor explained, forcing investigators to change their experimental approaches. Consequently, to rapidly engineer metabolic pathways, we need to confront these barriers to HGT and learn how to overcome them.

At the same time that we develop improved engineering strategies, we also wish to understand natural evolutionary processes. Since engineers use different strategies than nature does, a pure engineering approach offers limited insights into natural processes. An alternative approach combines aspects of synthetic biology and experimental evolution. We can deliberately recreate HGT in the laboratory by transferring pathways into hosts and then selecting for use of the new ability. If a newly acquired pathway functions poorly, mutations that increase pathway effectiveness will provide a selective advantage to their host. Working backwards from these mutations allows us to identify both the challenges that previously limited the effectiveness of the pathway and the biochemical mechanisms for overcoming those challenges.

FIGURE 1



A heterologous pathway for dichloromethane (DCM) catabolism in *Methylobacterium extorquens* converts DCM into a native metabolic intermediate, formaldehyde. Hydrochloric acid is produced as a byproduct of DCM dechlorination, and accumulation of chloride ions limits growth on DCM. However, overexpression of a chloride/proton antiporter (ClcA) increases chloride export and dramatically improves growth on DCM.

These evolutionary solutions also begin to develop a toolbox of design strategies for engineering applications.

Fitness Costs May Accompany Antibiotic Resistance Gene Transfers

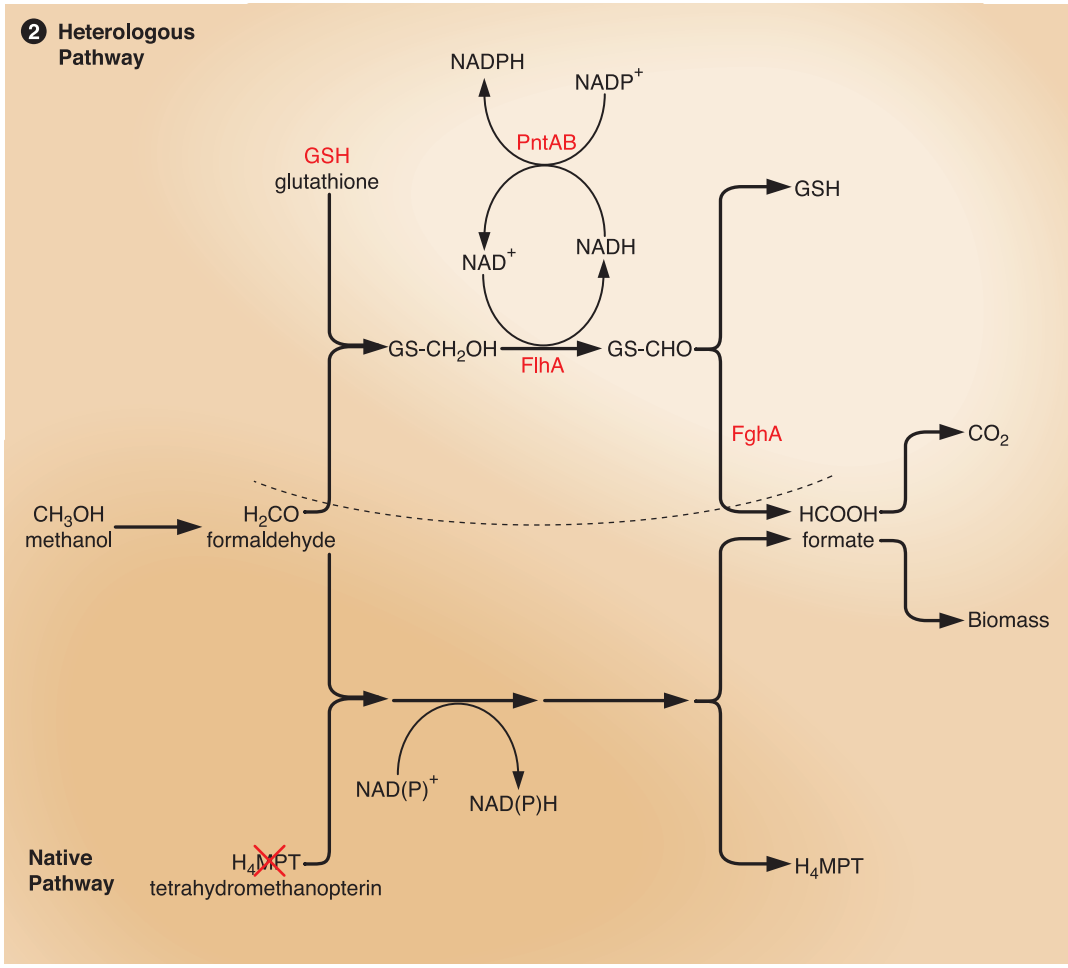
One of the most dramatic consequences of HGT is the rapid spread of antibiotic resistance determinants among bacterial species and strains. Acquisition of a new resistance determinant often carries a fitness cost to the recipient cell. When patients are being treated with antibiotics, the benefits of resistance outweigh these costs. In the absence of the antibiotic, however, strains frequently acquire compensatory mutations that enable them to maintain resistance while minimizing those fitness costs.

When resistance determinants are carried on plasmids, mutations that lessen the plasmid carriage costs can occur in the host bacterium, the plasmid, or both. Similarly, resistance genes with an inherent fitness cost may acquire regulatory segments that block expression until the bacterial cell becomes exposed to that antibiotic, thus minimizing costs to the cells. In other cases, mutations that disrupt antibiotic binding can provide resistance but at a substantial fitness cost, until compensatory mutations restore fitness while maintaining resistance. These kinds of post-transfer refinements within the pathogen have significant medical consequences, since eliminating the fitness cost of resistance can prevent such bacteria from reverting to antibiotic sensitivity in the absence of antibiotic treatment.

Genes for Other Horizontally Transferred Pathways May Undergo Refinements

By introducing metabolic pathways for degrading novel substrates into new host microbes, HGT can enable recipients to occupy new environmental niches. For example, several bacterial strains can grow naturally on dichloromethane (DCM) as the sole source of carbon and energy. In the first step of this catabolic pathway, DCM is converted into formaldehyde plus two molecules of hydrochloric acid. The associated dehaloge-

FIGURE 2



Replacement of the native methanol oxidation pathway in *Methylobacterium extorquens* with an alternate pathway from *Paracoccus denitrificans* imposes several burdens on the recipient: enzyme overexpression, glutathione depletion, and redox cofactor imbalance. Effective use of the alternate pathway for growth on methanol requires alleviating these stresses by decreasing expression of the heterologous enzymes (FlhA and FghA), increasing glutathione biosynthesis, and overexpressing a transhydrogenase to interconvert NADH and NADPH (PntAB).

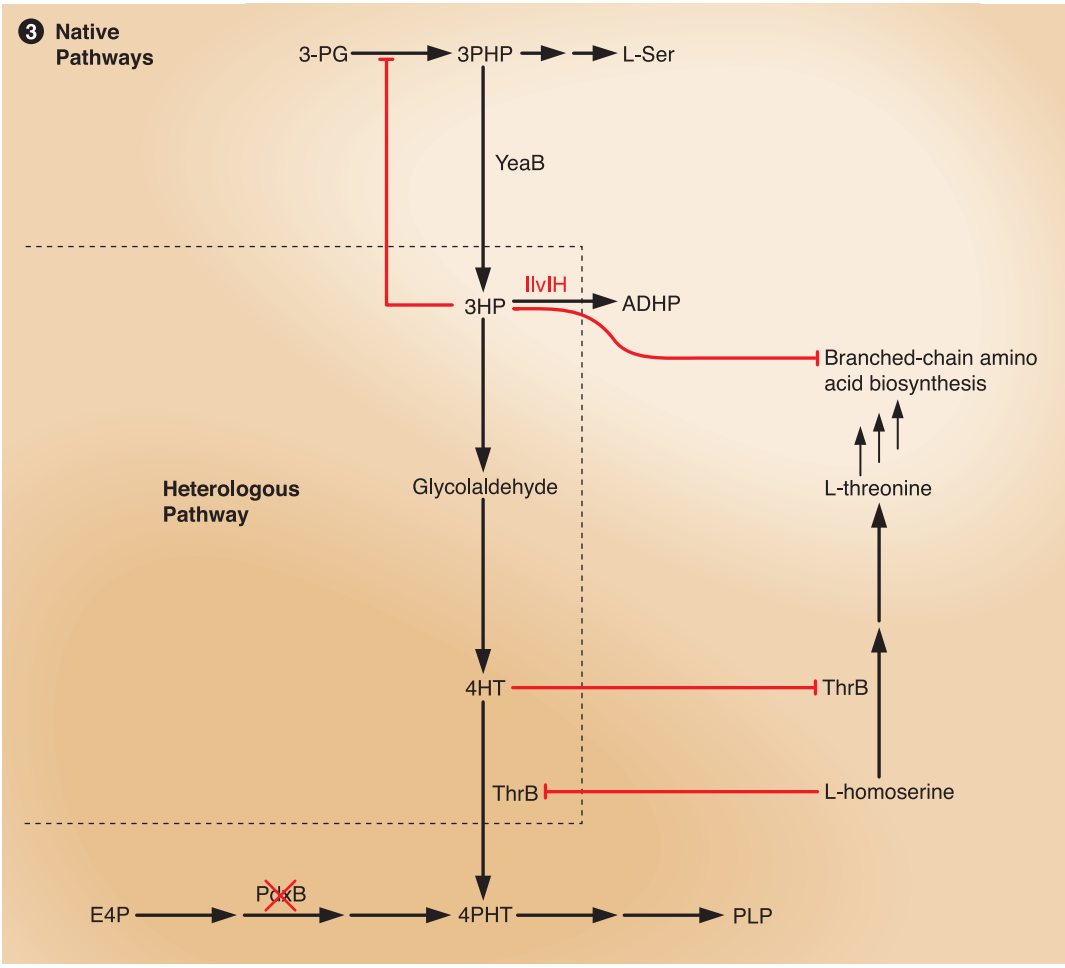
nase gene, *dcmA*, is found in distantly related strains and is usually flanked by IS elements, observations that are consistent with ready dissemination by HGT.

Although the DCM catabolic pathway is found in several different bacterial strains, it is very difficult for recipients to exploit their new ability. Among other challenges, this catabolic pathway produces toxic formaldehyde and hydrochloric acid, as well as a highly mutagenic intermediate, *S*-(chloromethyl) glutathione.

To learn more about what makes such transfers difficult and how to overcome those difficulties, we transferred the *dcmA* gene from *Methylobacterium extorquens* DM4 into six other *Methylobacterium* strains. When the transconjugants were fed DCM, none grew as well as the reference strain, and one of those strains was completely unable to grow on DCM. Each strain functionally expressed the dehalogenase, suggesting that pathway-related stresses of some kind limit growth on DCM.

We then used experimental evolution procedures to identify mutations that could improve the ability of the *dcmA*-containing strains to grow on DCM. Mutations in four loci improve the

FIGURE 3



Overexpression of native enzymes allows *Escherichia coli* to bypass an otherwise essential gene knockout. However, this novel pathway has several deleterious interactions with native metabolic and regulatory networks. For example, repurposed enzymes maintain their original regulation, even when this regulation interferes with their new function (e.g., ThrB); the new pathway produces novel intermediates that react with native enzymes to form unproductive metabolites (e.g., IlvIH); and these novel intermediates can misregulate native metabolic pathways (e.g., 3HP).

fitness of transconjugants during growth on DCM, and all of these mutations are linked to increased chloride efflux. Of note, several mutations increase expression of the chloride/proton antiporter, ClcA. Using this knowledge, we demonstrated that two independent DCM-degrading environmental isolates of *M. extorquens* both contain mutations in the *clcA* promoter. These promoter mutations are necessary and sufficient to permit effective growth on DCM.

Thus, for a *Methylobacterium* strain to grow on DCM requires not only a functional version of *dcmA* but also appropriate refining mutations. Remarkably, the adaptive steps we observed in our experiments appear to be the same as those that arose on multiple, independent occasions in nature. Putting this finding in engineering terms, organisms that are designed to degrade organochloride compounds are also likely to benefit from mutations that increase chloride export. More specifically, overexpressing ClcA appears to satisfy this goal without compromising viability in natural environments.

Replacing Metabolic Pathways Involves Co-Evolution of the Host and Pathway

Beyond transfers of novel pathways, examples of HGT include the replacement of a viable, functioning pathway with a homologous or orthologous alternative. Strikingly, these transfers can include highly-connected housekeeping functions such as RNA polymerase subunits, ribosomal proteins, or core metabolic enzymes. We constructed an example of this process by replacing a portion of the pathway for methanol catabolism in *M. extorquens* AM1 with an alternate metabolic route. Thus, instead of oxidizing methanol via the native tetrahydromethanopterin-dependent pathway, our modified strain contains genes for a glutathione-dependent pathway from *Paracoccus denitrificans*. Initially, this change led to a strain with a roughly threefold decrease in growth rate on methanol. Laboratory evolution of the modified strain yielded isolates that closed this fitness gap.

Analysis of one improved glutathione-dependent isolate revealed three main adaptations that enable it to better accommodate its new pathway for degrading methanol. One mutation reduces expression of the novel pathway, and subsequent work demonstrated that these enzymes are produced at unnecessarily high levels, with a substantial fitness cost for the modified cells. Another host mutation led to increased glutathione biosynthesis, accommodating increased demands for glutathione by the new pathway. The third mutation led to increased expression of a transhydrogenase, which interconverts NADH and NADPH. The methanol pathway in the parent strain produces a mixture of NADH and NADPH when methanol is oxidized, while the new pathway produces just NADH, and the transhydrogenase apparently adjusts for this difference. Similar to post-transfer refinement of DCM catabolism, replacement of a portion of the core methanol oxidation pathway requires significant refinement of both the host and the new pathway in order to function efficiently. As will be discussed below, similar strategies for balancing enzyme expression or increasing cofactor biosynthesis are common in metabolic engineering.

Overexpression of Promiscuous Enzymes Mimics Some Challenges of HGT

In addition to acquiring new metabolic pathways through HGT, microbes are hypothesized to extend their metabolic networks by coopting promiscuous catalytic activities of native enzymes. In *Escherichia coli*, several knockouts of genes that are otherwise essential for growth on minimal media can be complemented by overexpression of enzymes from unrelated pathways. Promiscuous catalysis by these overexpressed enzymes produces novel metabolic pathways that bypass the deleted reactions. However, these new pathways lead to many of the same challenges that are seen in cases of HGT, since in both situations the host cells may be unprepared for the new regulatory and metabolic interactions that result.

For example, overexpressing either YeaB or ThrB, the former from an unknown metabolic pathway and the latter involved in threonine biosynthesis, enables *E. coli* cells to grow after deletion of *pdxB*, a gene encoding an enzyme involved in pyridoxal phosphate (PLP) biosynthesis. Overproducing either enzyme enables mutants to grow by redirecting intermediates from serine biosynthesis into the PLP biosynthetic pathway, downstream from the reaction catalyzed by PdxB.

However, that growth is limited because the novel pathway introduces two new intermediates, 3-hydroxypyruvate and 4-hydroxythreonine, that inhibit enzymes involved in amino acid biosynthesis. Moreover, ThrB continues to be regulated by threonine, even when this regulation conflicts with its new role in PLP biosynthesis. Finally, native enzymes such as IlvH can react with these new intermediates and reduce PLP production.

In cases involving HGT, enzyme amplification, or synthetic biology, introducing new metabolites and enzyme activities can disrupt both the novel function and the native pathways of the host. Developing new tools to identify and eliminate these deleterious interactions would aid in both the analysis of HGT and the construction of synthetic organisms.

Heterologous Enzymes Put New Stresses on Host Cells

Metabolic engineers frequently transfer genes from natural isolates into recipient bacterial species that are considered more genetically tractable but may be only distantly related to the source species. Just as in HGT, the metabolic intermediates being produced in such engineered pathways can impose stresses on the recipient strains that

interfere with production of the target metabolite. For example, transfer of the gene encoding an engineered P450 monooxygenase from *Bacillus megaterium* into *Saccharomyces cerevisiae* led to only moderate conversion of caffeine into the bronchodilator theophylline. Decreasing expression of that enzyme, however, increased both the growth rate and levels of theophylline in the engineered yeast strain, suggesting that the pathway was placing a stress on this host. Measurements of mRNA transcript levels confirmed that enzyme expression severely depletes heme in such cells. Accordingly, boosting heme biosynthesis in the yeast host increases both monooxygenase levels and theophylline yields.

Heterologous enzymes, whether transferred by HGT or synthetic biology, often increase demand for native cofactors or require the introduction of new cofactors. Learning to recognize the signatures of increased cofactor demand will aid in the analysis of environmental isolates, while identifying mutations that increase cofactor production without overly burdening the host will be valuable tools for metabolic engineering.

Similar to the enzyme amplification example discussed above, deliberately introducing an engineered metabolic pathway may produce intermediates that interfere with the native metabolism of the new host. For example, several genes were introduced into *E. coli* cells, enabling them to make a precursor for artemisinin, a valuable drug for treating malaria. However, when the first three genes in the pathway are overexpressed, a non-native intermediate, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) quickly accumulates and inhibits growth. Further analysis shows that elevated levels of HMG-CoA inhibit fatty acid biosynthesis in the engineered cells. This problem can be overcome, however, by supplementing the growth medium with fatty acids or by modifying expression of the enzymes in the pathway to prevent accumulation of HMG-CoA.

In both natural and engineered systems, including these examples of artemisinin production and glutathione-dependent methanol oxidation, differences between donor and recipient cells may lead to metabolic pathway imbalances in the new host cells. In some cases, reducing the levels of the introduced enzymes will overcome the burden of protein overexpression and also avoid any accumulation of toxic intermediates.

Joshua K. Michener is a postdoctoral fellow in the Department of Biological Engineering at the Massachusetts Institute of Technology, Cambridge, Christopher J. Marx is an Associate Professor in the Department of Biological Sciences at the University of Idaho, Moscow.

Suggested Reading

Akhtar, M. K., and P. R. Jones. 2014. Cofactor engineering for enhancing the flux of metabolic pathways. Frontiers Bioeng. Biotechnol. **2:**30.

Andersson, D. I., and D. Hughes. 2010. Antibiotic resistance and its cost: is it possible to reverse resistance? Nature Reviews Microbiol. **4:**260–271.

Chou, H.-H., H.-C. Chiu, N. F. Delaney, D. Segre, and C. J. Marx. 2011. Diminishing returns epistasis among beneficial mutations decelerates adaptation. Science **332:**1190–1192.

Kim, J., and S. D. Copley. 2012. Inhibitory cross-talk upon introduction of a new metabolic pathway into an existing metabolic network. Proc. Natl. Acad. Sci. USA **109:**E2856–2864.

Kizer, L., D. J. Pitera, B. F. Pfleger, and J. D. Keasling. 2008. Application of functional genomics to pathway optimization for increased isoprenoid production. Appl. Environ. Microbiol. **74:**3229–3241.

Michener, J. K., J. Nielsen, and C. D. Smolke. 2012. Identification and treatment of heme depletion attributed to overexpression of a lineage of evolved P450 monooxygenases. Proc. Natl. Acad. Sci. USA **109:** 19504–19509.

Michener, J. K., A. A. Camargo Neves, S. Vuilleumier, F. Bringel, and C. J. Marx. 2014. Effective use of a horizontally-transferred pathway for dichloromethane catabolism requires post-transfer refinement. eLife 2014;10.7554/eLife.04279.

Ochman, H., and N. A. Moran. 2001. Genes lost and genes found: evolution of bacterial pathogenesis and symbiosis. Science **292:**1096–1099.

Popa, O., and T. Dagan. 2011. Trends and barriers to lateral gene transfer in prokaryotes. Curr. Opinion Microbiol. **14:**615–623.

Shapiro, B. J., et al. 2012. Population genomics of early events in the ecological differentiation of bacteria. Science **336:**48–51.