E. coli culture expressing a His-tagged p19 and a target gene hairpin is established, a laboratory needs only appropriate growth media, nickel beads and a means for small-RNA purification to ensure a constant supply of pro-siRNAs directed against the target gene. These methods are cost-effective compared with the purchase of individual siRNAs or esiRNAs. The cost savings might even enable researchers to build libraries of pro-siRNAs containing multiple pools directed against each target gene, thereby further reducing off-target effects compared with esiRNA pools.

E. coli has already been used to transmit dsRNA *in vivo* in *Caenorhabditis elegans*[5,6], and *E. coli*–mediated delivery of RNAi to mammalian cells has also been achieved[7]. The latter approach, called trans-kingdom RNAi, has been employed only in a few follow-up studies but has the potential to become an RNAi-based therapeutic. Briefly, *E. coli* are engineered to express invasin, which allows *E. coli* to enter mammalian cells; listeriolysin O, which allows *E. coli* to deliver oligonucleotides to the cytosol; and a short hairpin (sh)RNA against a target gene of interest. Yet so far such trans-kingdom RNAi methods have transmitted only a single shRNA sequence, and pro-siRNAs might be a better choice. Provided that digestion of the long target-gene hairpin is complete, *E. coli* that have entered into target cells can deliver pro-siRNAs instead of an shRNA, leading to more specific and effective knockdown of a disease-relevant gene.

The new pro-siRNA technology is not without its limits, however. In particular, the yield of the pro-siRNA cultures described by Huang *et al.*[3] is modest. As a result, the generation of a whole-genome pro-siRNA library would require scales of culture that are not practical. For now, this technology is better suited to the production of pro-siRNAs specific for individual genes or of small-scale libraries. Further optimization of the system to improve yield may transform pro-siRNA into a viable tool for genome-wide library production. In addition, the requirement for a relatively long target-gene RNA hairpin may make generation of pro-siRNAs from cDNA libraries difficult given the challenge in creating and maintaining long inverted repeats in bacteria. Perhaps pro-siRNA production would be facilitated by using similar vectors as those used for dsRNA and esiRNA generation, which transcribe RNA off of convergent T7 promoters; these expression vectors would require single-step cloning of a noninverted cDNA insert. Nonetheless, these drawbacks could be temporary as the technology is expanded and improved. We expect that further development of pro-siRNA technology will allow laboratories to keep a cost-effective, renewable supply of potent and specific siRNA pools for screening and *in vivo* applications.

Finally, it should be noted that the findings of Huang *et al.*[3] are interesting from more than a methodological point of view. The observation that bacteria can produce fully processed siRNAs is in itself unexpected, and future studies may reveal basic biological roles for these nucleic acids.

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1. Fire, A. *et al. Nature* **391**, 806–811 (1998).
2. Yang, D. *et al. Proc. Natl. Acad. Sci. USA* **99**, 9942–9947 (2002).
3. Huang, L. *et al. Nat. Biotechnol.* **31**, 350–356 (2013).
4. Kittler, R. *et al. Nat. Methods* **4**, 337–344 (2007).
5. Timmons, L. & Fire, A. *Nature* **395**, 854 (1998).
6. Timmons, L., Court, D.L. & Fire, A. *Gene* **263**, 103–112 (2001).
7. Xiang, S., Fruehauf, J. & Li, C.J. *Nat. Biotechnol.* **24**, 697–702 (2006).

Compartmentalizing metabolic pathways in organelles

William C DeLoache & John E Dueber

The entire metabolic pathway for producing a biofuel is confined within a yeast organelle to increase yield over the endogenous pathway split between two compartments.

The road from a well-characterized metabolic pathway to production of a final product at high yield in an industrially tractable host is beset with hurdles, including accumulation of toxic metabolites, diversion of substrates into side products and insufficient cofactor availability. Recent studies have shown that many of these problems might be overcome by compartmentalizing metabolic pathways in subcellular organelles, such as the mitochondrion[1] or vacuole[2], where conditions would be more favorable than in the cytosol. In this issue, Avalos *et al.*[3] improve the production of isobutanol in *Saccharomyces cerevisiae* by colocalizing all five biosynthetic enzymes to the mitochondrion. This study establishes organelle compartmentalization as a viable metabolic engineering strategy.

Isobutanol is an advanced biofuel with a higher energy density and lower hygroscopicity than ethanol. Endogenous production of isobutanol in *S. cerevisiae* uses the first three enzymes of valine biosynthesis (ILV2, ILV5, ILV3) to generate the intermediate α-ketoisovalerate (α-KIV) from pyruvate. α-KIV is then converted into isobutanol by means of the two-enzyme (KDC and ADH) Ehrlich degradation pathway[4]. Although all five of these enzymes are present and expressed in *S. cerevisiae,* they are segregated into separate compartments, with the upstream enzymes (ILV2, ILV5, ILV3) in the mitochondria and the downstream enzymes (KDC and ADH) in the cytosol.

Previous attempts to overexpress these enzymes in yeast have met with limited success, owing at least in part to accumulation of intermediates in the mitochondria. Avalos *et al.*[3] observed a substantial increase in isobutanol production when feeding purified α-KIV to the cells, suggesting that this intermediate is rate-limiting in the cytosol. Further supporting this conclusion was the recent work of Brat *et al.*[5], in which isobutanol yield was improved by expressing the entire pathway in the cytosol, eliminating the need for α-KIV transport across the mitochondrial membrane. Curiously, achieving the higher isobutanol yields necessitated knocking out the endogenous copies of ILV2, ILV5 and ILV3, and growing the yeast on media lacking valine[5]. Nonetheless, these results showed that unavailability of α-KIV is at least partially to blame for the limited isobutanol yields obtained in yeast (3–7 mg per g glucose) versus *E. coli* (>200 mg per g glucose) when overexpressing the endogenous pathway[4,6,7].

Avalos *et al.*[3] took the opposite approach to Brat *et al.*[5] and targeted the downstream enzymes to mitochondria so that the entire pathway was compartmentalized within the organelle rather than in the cytosol. To quantify the effect of mitochondrial compartmentalization, they compared strains overexpressing all five enzymes in the mitochondria with those overexpressing the upstream enzymes in the mitochondria and

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the downstream enzymes (KDC and ADH) in the cytosol. The authors also tested several variants of KDC and ADH to identify the best combination. For all nine KDC/ADH combinations, isobutanol titers were highest when the enzymes were localized to the mitochondria, with the best strain producing 635 mg/L of isobutanol—3.25-fold more than the split-pathway control strain. Owing to different procedures, media and strain backgrounds, a direct comparison between yields from mitochondrially compartmentalized pathways and those previously engineered with the full pathway in the cytosol[5] is not possible. However, both strategies clearly provide considerable improvements in pathway performance over the two-compartment native pathway.

In addition to the presence of α-KIV, which in the study by Avalos *et al.*[3] proved beneficial for isobutanol production, the mitochondrion has several properties that may be attractive for a variety of engineered pathways. For example, the mitochondrion is the only site of heme biosynthesis in the cell and the primary site for efficient loading of iron-sulfur enzymes, such as ILV3, the third enzyme in the isobutanol pathway. Although ILV3 is sufficiently active in the cytosol to complement a knockout of the mitochondrial copy[5], it is likely that many iron-sulfur cluster enzymes would have higher activity in mitochondria. Other metabolites are also present in mitochondria at high concentrations, including intermediates from the tricarboxylic acid cycle, amino acid biosynthesis and fatty acid beta-oxidation, as well as the cofactors tetrahydrofolate, ubiquinone, aminolevulinic acid, biotin and lipoic acid. Mitochondria might therefore prove to be a suitable location for other engineered pathways that involve these intermediates and cofactors. Similarly, depending on the pathway being optimized, metabolic engineers would do well to consider other organellar environments, such as the low pH condition of the vacuole or the oxidative condition of the endoplasmic reticulum (**Fig. 1**).

Organellar compartmentalization has until now been an uncommon metabolic engineering strategy, but at least two engineered pathways in yeast have benefited from this approach. First, Bayer *et al.*[2] synthesized methyl halides in the vacuole because most of the substrate halide ions and the cosubstrate, *S*-adenosyl methionine (SAM), are located in this organelle. Targeting the methyl halide transferase enzyme to the vacuole resulted in an ~1.5-fold improvement in the methyl-iodide production rate. Farhi *et al.*[1] targeted terpene and farnesyl diphosphate synthases to mitochondria as a means of preventing competing reactions in the cytosol. Encapsulation of farnesyl diphosphate synthesis in mitochondria occurs in many organisms, including humans, plants, insects and yeast[8]. Impressively, this pool of farnesyl disphosphate was sufficient to achieve an approximate threefold and sevenfold increase of valencene and amorphadiene, respectively, when their terpene synthases were targeted to the mitochondria of *S. cerevisiae*.

The study by Avalos *et al.*[3] raises several interesting questions for further investigation. First, how generalizable a strategy is organellar compartmentalization? Isobutanol production in the mitochondria was possible because the upstream pathway metabolites are naturally located in that organelle. Before compartmentalization can be applied to pathways with substrates and cofactors that are not normally present in an organelle, new technologies for controlling metabolite transport across intracellular membranes must be developed. Methods for altering the pH, redox state and other environmental conditions within organelles might even allow cellular compartments to be modified to fit the needs of a pathway, rather than modifying a pathway to fit the native conditions within the cell. Improved techniques for measuring subcellular chemical environments will be necessary to enable these types of advances.

Another question for future research is whether the limited volume of most organelles will restrict the amount of flux that can be achieved for compartmentalized pathways. Although mitochondria are similar in size to bacteria, they take up only a fraction of the total volume of a yeast cell. Even if an organelle were to provide the optimal chemical environment for a particular pathway, overall productivity might be suboptimal owing to constraints imposed by the organellar volume. Engineering organelles with increased cargo capacity might be necessary to fully harness the potential of subcellular compartments for improving metabolic flux. Even without such engineered organelles, however, compartmentalizing multi-enzyme pathways in natural organelles to produce high effective concentrations of enzymes, substrates and cofactors is an exciting new tool for metabolic engineers.

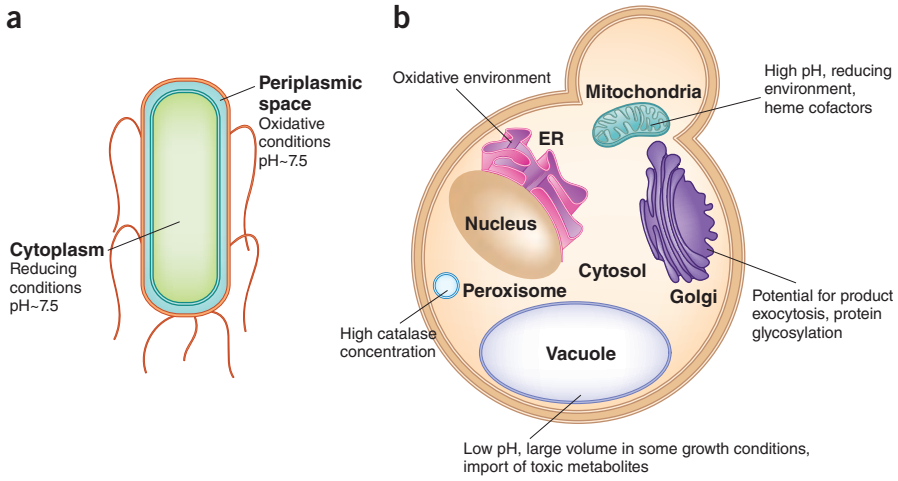

Figure 1 Eukaryotic organelles offer multiple environments distinct from the cytosol that may be attractive for engineering metabolism. (**a**) Bacteria such as *E. coli* have a simple, two-compartment system. Although the periplasm is largely devoid of metabolites, it has proven useful for certain engineering applications such as disulfide bond formation, owing to its oxidative environment[9]. (**b**) In contrast, eukaryotic organisms offer many specialized organelles, each with conditions distinct from the cytosol, that may provide a better environment for a range of metabolic pathways. ER, endoplasmic reticulum.

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1. Farhi, M. *et al. Metab. Eng.* **13**, 474–481 (2011).
2. Bayer, T.S. *et al. J. Am. Chem. Soc.* **131**, 6508–6515 (2009).
3. Avalos, J.L., Fink, G.R. & Stephanopoulos, G. *Nat. Biotechnol.* **31**, 335-341 (2013).
4. Atsumi, S., Hanai, T. & Liao, J.C. *Nature* **451**, 86–89 (2008).
5. Brat, D., Weber, C., Lorenzen, W., Bode, H.B. & Boles, E. *Biotechnol. Biofuels* **5**, 65 (2012).
6. Chen, X., Nielsen, K.F., Borodina, I., Kielland-Brandt, M.C. & Karhumaa, K. *Biotechnol. Biofuels* **4**, 21 (2011).
7. Kondo, T. *et al. J. Biotechnol.* **159**, 32–37 (2012).
8. Martín, D., Piulachs, M.-D., Cunillera, N., Ferrer, A. & Bellés, X. *Biochim. Biophys. Acta* **1773**, 419–426 (2007).
9. Denoncin, K. & Collet, J.-F. *Antioxid. Redox Signal.* published online, doi:10.1089/ars.2012.4864 (2 October 2012).