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Natural products from synthetic biology

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DNA sequencing has uncovered microbial secondary metabolic potential that never surfaced in fermentation based screens. Deep and cheap sequencing of a genus such as *Streptomyces* can rapidly expose hundreds of metabolic genes and operons. Meanwhile, synthetic biologists, in their quest to engineer advanced biofuels, are mastering metabolic engineering. Natural products, a reliable source of new therapeutic leads for many years, have fallen into disfavor with drug discoverers partly because these molecules are rarely available as pure compounds and sourcing is often problematic. The convergence of next generation sequencing and synthetic biology, along with less spectacular progress in analytic technologies such as mass spectroscopy, opens the door to the creation of large, reliable libraries of pure natural products for drug discovery.

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Introduction

A starting point for drug discovery revival

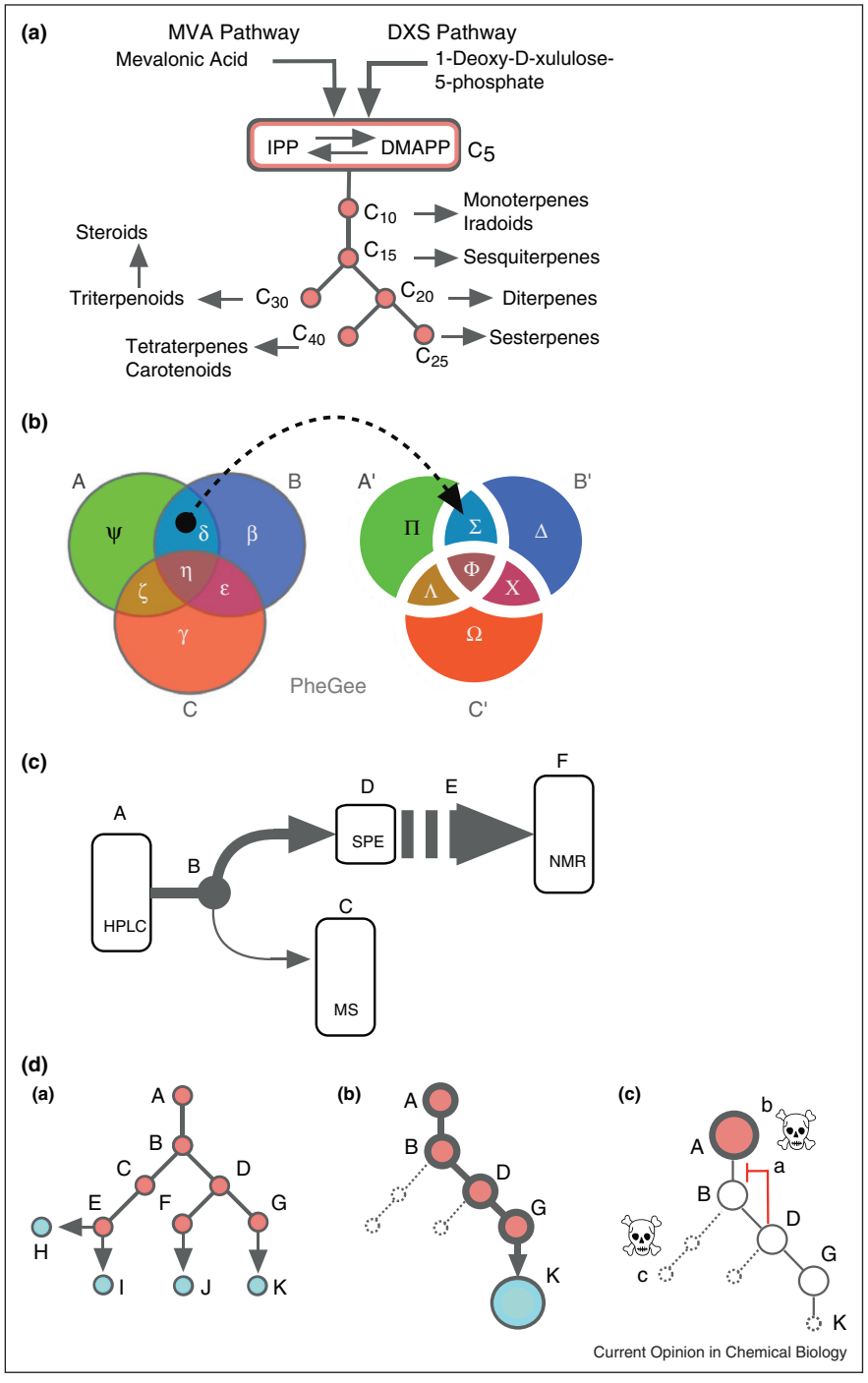
''. . ..there is plenty still to do.''
Sir David Hopwood [1]

Natural products (NPs), derived from the secondary metabolism of bacteria, fungi and plants [2], are chemically distinct from synthetic molecules. NPs have more chiral centers, more oxygen and less nitrogen, sulfur and halogens. The ratio of aromatic rings to total heavy atoms is lower in NPs, the number of solvated H-bond donors and acceptors is higher, and NPs have a broader distribution of mass, log *P* and a greater diversity of ring systems. Surprisingly only 10% of NP structures incur two or more Lipinski Rule of Five violations [3]. Perhaps because of these properties NPs are prominent in the allopathic pharmacopeia, yet the drug industry has moved away from them over the past two decades. Commercial interest in infectious disease indications, an NP stronghold, has flagged. Meanwhile technical innovations such as robotic high throughput screening of single protein targets and combinatorial synthesis have pushed NPs to the margin of the drug discovery process [3].

A renaissance in natural product based drug discovery would be timely. Mounting levels of bacterial drug resistance against existing antibiotics and a concomitant rise in nosocomial infection, especially by the ESKAPE pathogens, portend a genuine public health crisis [4•]. Meanwhile, the drug discovery engine is faltering, as demonstrated by the decline in five year averages for approved new molecular entities (1995–1999: 37.6; 2000–2004: 30; 2005–2009: 20.2) [5]. A 2009 joint study by the European Medicines Agency and the European Centre for Disease Prevention and Control found increasing levels of infection by multi drug resistant bacteria, leading to about 25,000 deaths per annum in the European Union, and, ''a particular lack of new agents . . . against multi-drug-resistant Gram-negative bacteria'' [6]. The Infectious Disease Society of America has set a target of 10 new antibacterial drugs by 2020 to address similar findings in the United States [7]. If this ambitious goal is to be reached, natural products probably will need to play a role. Historically, NPs have been a reliable source of small molecule therapeutics. Of 974 New Chemical Entities, excluding biologics, approved from 1981 to 2006, 39% were NP, NP derived, or fully synthesized NP pharmacophores [8], and natural products continue to deliver. Spirotetrahydro-b-carbolines (spiroindolones) are a class of compounds that have recently produced a novel, potent, orally administered single daily dose anti-malarial candidate. Significantly, the starting point for this discovery was a collection of ~12,000 pure natural products and synthetic compounds with NP-like properties [9•]. Combinatorial libraries that run to over a million compounds are not uncommon, but these collections appear to lack the diversity and enrichment for 'privileged scaffolds' that would typify even a much smaller library of natural compounds [3]. Large collections of pure NPs are rare because they are hard to build by classical fermentation methods, an obstacle that may fall before developments in metabolic engineering.

Synthetic Biology (SB) is the repurposing of living systems for useful ends. SB, philosophically rooted in the engineering paradigm, aims to reduce complex 'natural'

Figure 1



(a) Isoprenoid pathway. Two upstream pathways feed into the pool of the isoprenoid C_5 building blocks, isopentenyl diphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Eukaryotes use the Mevalonic Acid pathway to build IPP and DMAPP. Plants have a second route, the D-xululose Pathway. Most prokaryotes only have DSX. Pools of C_{10}, C_{15}, C_{20}, C_{25}, C_{30}, and C_{40} intermediates feed downstream pathways to produce various classes of secondary metabolites. One goal of SB is to build standard chasis optimized for production of NPs originating at each of these nodes.**(b)** Conceptual phenotype–genotype mapping in the PheGee algorithm. The Wenn Diagrams A and A′ illustrate the fact that given a set of genomes, orthologous genes will distribute into sets that are unique to one genome (Ψ, β, λ), are shared between some genomes but not others (δ,ε,ζ), or are ubiquitous (η). Sets of phenotypes distribute similarly ((Π,Δ,Ω),(Σ,X,Λ),(Φ)). Despite confounding effects such as convergent evolution, redundancy, and horizontal gene transfer, the method is surprisingly good at linking molecular phenotypes to candidate genes in prokaryotes.**(c)** A split flow system for natural product structure determination. Extracts from natural or engineered producer strains are separated, for example by HPLC (A). Peaks are captured for analysis, and split into two channels (B), ~5% going to on-line MS analysis (C), the balance diverted solid phase extraction (SPE) media (D), for collection and subsequent off-line analysis by MicroCryoProbe equipped NMR (F). SPE allows analyte to be concentrated by

(i.e. evolved) systems to simplified, reliable, quality-controlled modules, or 'parts', that can be mathematically modeled, manipulated by computer aided design (CAD), 'abstracted' (passed between loosely coupled design and production layers), bolted together to achieve predictable results, and fabricated on an industrial scale [10]. There are compelling reasons to believe SB will strongly influence the bioresearch agenda in the 21st century and in fact may be the vehicle that moves biotechnology into the economic mainstream [11•,12]. Momentum in SB is being driven by so-called 'advanced biofuel' research (cellulosic and algal derived but excluding bioethanol), with very substantial resource inputs from venture capitalists, public equity markets, government subvention and the traditional energy sector totaling well over a billion U.S dollars (USD) [13–16]. Although the focus of the SB biofuel community is the metabolic reengineering of lipid pathways for the production of diesel, jet, automotive fuels and other industrial oils, as a collateral effect the biofuels campaign will enable *generic* synthetic biology technologies including the creation of a technical metabolic engineering knowledge base, the training of a human cohort of practitioners skilled in the art, and the discovery of practical solutions to high level problems in metabolic engineering and their dissemination as general principles.

Artemisinin: an exemplar

The remarkable achievement of the Artemisinin Project provides a compelling example of Synthetic Biology's potential to reinvigorate natural product therapeutics. Artemisinin is a botanical antimalarial isolated from *Artemesia annua*, a wormwood related to *Artemesia absinthium*, and a Traditional Chinese Medicine remedy (*qinqhaosu*, 青蒿素). Like other NPs, artemisinin is biosynthesized in multiple, sequential steps by a suite of functionally related enzymes (a 'pathway') that in bacteria is often recapitulated by a colocalized cluster of corresponding genes (an 'operon'). By transferring plant genes for the artemisinin pathway into a fermentable 'chasis organism' and forcing production of the artemisinin precursors amorpha-4,11-diene and artimisinic acid [17,18], the cost of artemisinin was cut in half, opening access to Artemisinin Combination Therapy (ACT) to low income malaria victims in developing countries [19].

Ingenious general purpose solutions undergird this project's success. Because the isoprenoid artemisinin belongs to a class of compounds that includes many therapeutically interesting members, these advances have broad implications. Isoprenoid biosynthesis branches from a series of precursors that are multimers of C_5 isoprene units [Figure 1a], formed either via the mevalonate dependent MVA pathway (in all eukaryotes) or, via the mevalonate independent DSX pathway (in plants, which have 2 routes to isoprene, and in most prokaryotes). Increasing production of artemisinin precursors, a requirement for increased yield of the downstream product, was complicated by uncharacterized regulatory feedback loops in the circuitry of the native DSX pathway. To overcome this the eukaryotic MVA pathway was introduced into *E. coli* [20], resulting in the hoped for accumulation of the isoprenoid feedstock isopentenyl pyrophosphate (IPP) in engineered cells when grown in mevalonate supplemented medium. However, having bypassed indigenous control, it was then important to re-regulate biosynthesis, since healthy growth and optimal secondary metabolite production result from properly balanced pathway flux. Rational pathway regulation was achieved by codon optimization of imported genes, combinatorial engineering of intergenic regions in target genes [21], by altering enzyme levels through modulation of gene copy number, and by mimicking natural substrate channeling in multizyme complexes by precisely bundling rate limiting enzymes on molecular scaffolds [22••]. Scaffolding is a general solution that has been successfully applied to other systems, including glutaric acid production in an engineered *E. coli* chasis [23].

The artemisinin triumph is not unique. To cite only salient examples, substantial progress has been made in bacterial production of taxol [24••], penicillin has been produced in yeast [25], and a quarter century of inspired work has built up not only a large physical inventory of engineered polyketides and non-ribosomally synthesized peptides, but also a singular understanding of the rules governing their biogenesis [26,27••,28–30].

Blueprint

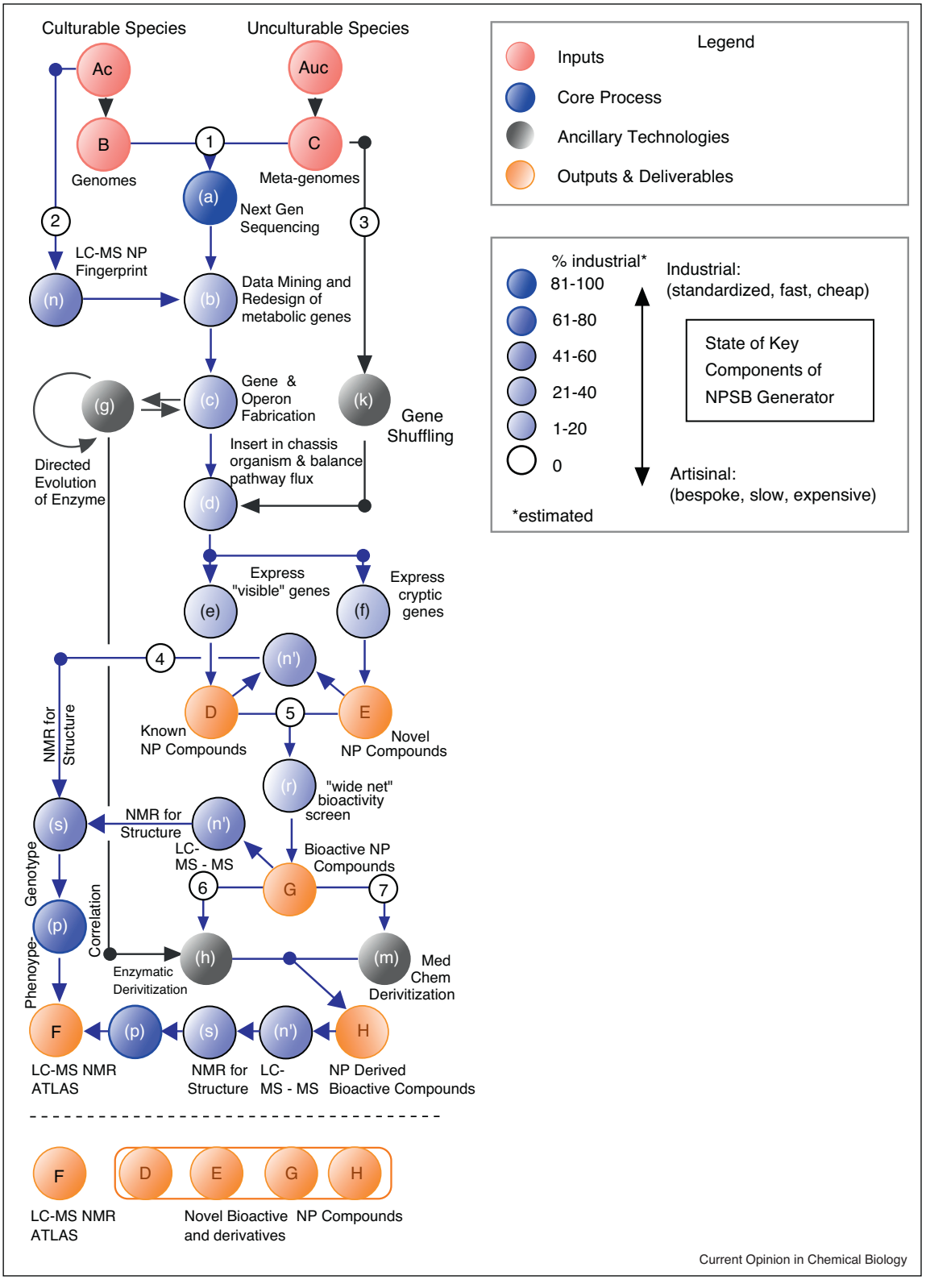
A synthetic biology generator for a natural product 'chemicopia'.

Natural products from synthetic biology

SB promises to do for NP metabolic engineering what automated sequencing and standardized cloning systems have done for molecular biology, triggering a revolution in speed, scale and convenience that will leverage the archive of knowledge accumulated by natural product scientists into the framework of a resurgent NP biology.

(Figure 1 Legend Continued) multiple HPLC runs, thus providing sufficient product for NMR (E). **(d)** Metabolic engineering, the ideal and the reality. (a) An idealized secondary metabolism network forms a tree (a directed acyclic graph), with nodes representing compounds and edges representing reactions, mostly enzyme mediated (intermediates are shown as red circles, end products as blue circles). (b) Conceptually it is straightforward to prune off tree branches leading to undesired products, B–C–E and D–F, thus increasing flux through the path A–B–D–G leading to the desired product, K. (c) In practice this is confounded by many factors. The true graph probably is not acyclic; for example there might be an unexpected negative feedback edge from D to A–B (shown in red at 'a'). High concentrations of D might shut down the pathway upstream and lead to decreased level of the desired product K. Turning off A–B could also cause A to accumulate, and this might be toxic to the host ('b'). Also, the pruning itself might be deleterious. For example if the products of E are essential then deleting E will be fatal ('c').

Figure 2



A process flow diagram for NPSB, Natural Product Compound Library Production using Synthetic Biology. Process inputs are NP producing species (Ac) and their genomic DNA (B), and unculturable NP producers (Auc) and their meta-genomes (C). The backbone process begins with acquisition of whole genome sequence by NGS (path 1, (a)). The secondary metabolome of culturable organisms is sampled by LC–MS fingerprinting to prioritize

How can this momentum be directed most profitably? In terms of drug discovery a concrete goal might be to build a collection of pure NP compounds, reliably and reproducibly sourced in reasonable yield from engineered producer strains, an NP Chemicopia. Such a propitious starting point for drug discovery would be a measurable and useful goal around which resources can be rallied, talent recruited, imaginations engaged and definite plans drawn. A synthetic biology generator to power the engineered biosynthesis of pure NP compound libraries is a realistic goal. Such an engine will combine next generation sequencing, synthetic biology, and small molecule analytics, tightly integrated with complementary informatics and computational design resources. Some components of this engine are fully realized and even commercially available. Others are still in development. But–strikingly–no part of the engine is mere vaporware. It can be built today, albeit with plenty of room for improvement in certain subsystems [Figure 2].

Streptomyces sp.

The genus *Streptomyces* has been the single richest source of NPs beginning with the discovery of actinomycin (*Streptomyces sp*. 1940) and streptomycin (*S. griseus*, 1943) [31], and is an excellent focal point for an initial NPSB campaign. The genus includes over 500 species and thousands of strains and isolates with distinct secondary product capabilities. Thus, the twenty-eight *Streptomyces* genome sequences available [http://www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_taxtree.html] represent a mere fraction of the genetic and metabolic potential. Between 50% and 90% of secondary metabolism genes identified bioinformatically, amounting to 5% of the very large (~8 megabase) *Streptomyces* genomes, were unknown from previous biochemical surveys [32]. We can conclude that collectively *Streptomyces* probably harbor a considerable genetic potential for secondary metabolism that has been undetected by the classic methods of fermentation and extract screening. Large blocks of genetic dark matter mask the real extent of genetic metabolic potential in the genus.

Next generation sequencing

Next generation sequencing has brought microbial genomics to the lab, and nearly to the benchtop [[33], http://blogs.forbes.com/matthewherper/2011/04/13/ion-torrent-gives-its-dna-sequencing-box-a-boost/] The recent proposal to sequence 1000 bacterial species from prominent NP producing genera is a sound idea [27••]. Arguably, *Steptomyces* alone can consume this effort with good returns. *In lieu* of a large set of fully sequenced and annotated *Streptomyces* genomes, their potential compound space has been estimated from a mathematical model of NP discovery that used a discrete logistic equation modified to include positive and negative feedback (additional screening is encouraged or discouraged by recent success or failure). When fit to an historic time series of NP compound discovery the model's *K*-parameter, corresponding to the total number of discoverable NPs, was estimated at about 150,000 [34•]. One thousand genomes will capture a substantial part of the genes supporting NP metabolism in the genus only if the total potential compound discovery space is much smaller than this and the genomes are non-redundant at these loci (neither likely to be true). A hypothetical 1000 *Streptomyces* Genomes Project therefore should be seen as a preliminary reconnaissance, although one that certainly will expose ample secondary metabolic gene clusters for a robust test of the NPSB engine. Looking beyond the first thousand, sequencing of additional genomes should continue until a plot of novel metabolic gene discovery as a function of the number of genomes sequenced approaches zero slope (or some minimum rate to be decided). Sequencing is cheap. It makes sense to continue sequencing out to the edge of *Streptomyces sp.* diversity. With nearly 100,000 strains in some collections, the project initially can rely on culturable strains and species for material. In the long run, NPSB will want to tap into the much greater genomic potential of the unculturable microbes that make up at least 99% of diversity. This can be accomplished by 'metagenomic' sequencing of environmental DNA. As an example of what can be accomplished, a functional screen of a 4.7 gigabase metagenomic library uncovered three genes that improved the growth of *E. coli* 5.7–6.9 fold in the presence of inhibitors produced in biomass conversion [35]. This is a valuable finding, but the discovery rate is low, an observation that underscores the advantage of the more efficient NPSB scenario where only rationally selected genes and operons will be fabricated.

(Figure 2 Legend Continued) genomes (path2, (n)). Transcriptional data can also be gathered on culturable organisms under different induction conditions (not shown). Next, genomic content is mined to extract metabolic operons and genes; at this step LC–MS fingerprints can be correlated roughly with genomic content. Genes of interest are then redesigned and optimized for expression using the evolving set of synthetic biology design principles (b). The reconfigured operons and genes are sent for physical fabrication (c). Genes for metabolic enzymes can be shunted to a parallel process to extend their synthetic capabilities by directed enzyme evolution (g) and either returned to the backbone process or, employed as derivatizing agents (h). Shuffled metagenomic DNA (path 3, k) can be piped into the process at this point. Physical genes and operons are 'bolted' to an appropriate chassis organism and pathway flux is tuned to optimize production of secondary metabolites (d). In some percentage of cases operons direct the biosynthesis of known or previously unknown metabolites (D and E) that are purified if present at a threshold concentration empirically determined on the basis of convenience, or simply processed as extracts The LC–MS Fingerprinting module is employed again on these compounds or extracts (n′), ideally in conjunction with NMR (s), to create much tighter phenotype–genotype correlations that are systematically accumulated in a database (path 4, (p) and F). The D/E compound set can be filtered by a 'wide net' bioactivity screen such as live cell phenotype screening, and the presumably smaller set of bioactive compounds (path 5, G – not discussed in this review) then purified, and further expanded by medicinal chemistry (path 7, m) or through the action of evolved enzymes (path 6, h) to produce a more diverse set of compounds (H) that will be characterized by LC–MS-SPE-NMR.

Genome mining

The purpose of genome mining is to identify genes responsible for secondary metabolism for subsequent metabolic engineering. Microbial genomics can expose the genetics underlying the biosynthetic potential hidden in cryptic and unexpressed operons ('cryptons'). However, the function of a large percentage of genes, 20–40% in a typical bacterial genome, will be of unknown function, so metabolically interesting cryptons must be culled by bioinformatic analysis. Where protein homology and motif analysis do not suffice, a phenotype to genotype algorithm can be useful. With *n* genomes sequences in hand the cooccurrence of gene homologs define subsets of genomes that range in size from a single member (for a gene found in a single genome) to all *n* members (for a gene present in all genomes). A straightforward mapping between shared homolog subsets and experimentally defined phenotype positive subsets link observed traits to a small number of candidate genes that can then be tested experimentally [Figure 1b]. This simple approach is surprisingly good at identifying gene candidates for molecular traits in bacteria [36]. The transcriptome can be used as a 'genotype' to narrow the list of genes potentially responsible for the strain's secondary metabolic profile (the phenotype), providing both data sets are generated under the same conditions. NGS is emerging as the method of choice for measuring levels of cDNA (mRNA) in prokaryotes, despite the absence of 3′ poly adenylation on their transcripts [37]. *In extremis* very sensitive and sophisticated Hidden Markov Model based tools for the statistical prediction of secondary metabolism genes are also available [38].

Overall, data mining is effective as shown by the rapid exposure of two cryptic sesquiterpene synthases in the recently sequenced *S. clauligerus* genome [39].

Fingerprints and Atlases

The key analytical technologies of this platform, chromatography (usually HPLC), mass spectroscopy (MS), and nuclear magnetic resonance (NMR), have been challenged to meet the demands of accurate NP characterization of bacterial extracts in non-targeted metabolomic screens. Although these technologies are not improving as rapidly as the omic equipment, there is progress. MS for example, while laborious, is obviously many times faster and cheaper than it was just a few years ago. As more genomic sequence is collected, along with corresponding low resolution MS finger prints of bulk metabolites, it may become possible to correlate the two, at least at the level of prioritizing species for deeper scrutiny. MS has also been used repeatedly to generate molecular fingerprints for phenotype–genotype mapping [40••] even in investigations more challenging than NPSB (where a small number of known genes are being investigated in a defined host). Informatics plays a key role. If we assume that initially most NPs will be novel, then a large NMR effort will be required for structure elucidation. As compounds are purified, their MS–MS profile can be determined and stored in an MS–MS profile database (or 'Atlas'). In future rounds of experimentation, routine MS–MS analysis will catch progressively more known structures, taking some of the burden off NMR [41]. MS can also be combined with genomic information and isotopic labeling ('genomisotopics') to link metabolites back to orphan operons as part of the genome mining process [42].

NMR, the lowest throughput component of the NPSB engine, is also the process that demands collection of the most material, leading to longer experimental cycles, and higher cost per compound identified. A technical NMR advance that reduces the sample requirement is the microcryoprobe. Using a 600 MHz instrument and a 1.7 mm microcryoprobe it was possible to identify bacterial derived trisoxazole macrolides extracted from a single host sponge [43]. Methods are evolving that should allow tighter integration of MS and NMR and more automation of the coupled process. A general approach is to split the LC eluent stream when a peak is detected, sending a small percentage to MS and the bulk to Solid Phase Extraction (SPE) media, where compounds are trapped, and potentially concentrated by repeated LC runs, for offline NMR processing [Figure 1c] [44,45]. Not every advance need entail a new machine. Systematic investigation of NP extraction conditions revealed that addition of ammonium fluoride improved ESI-MS performance, with a 2-fold increase in detectable features and 3.5 fold increase in hits in compounds databases [46].

Fabrication

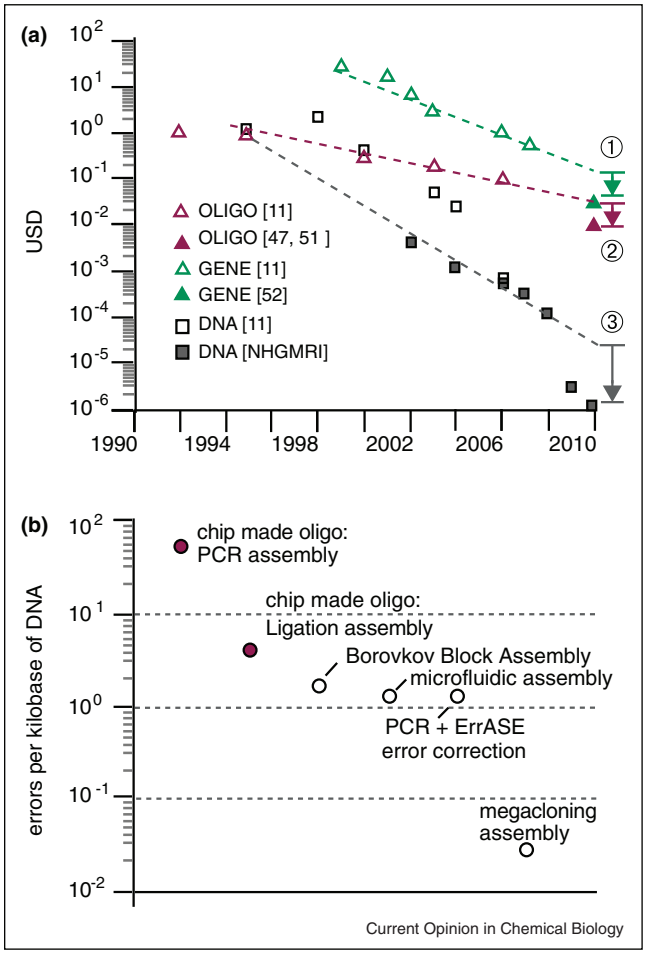
The ability to design and routinely manufacture cheap and reliable DNA components, at least up to the size of bacterial operons, is a fundamental premise of synthetic biology and an absolute requirement of NPSB. Significant progress has been reported. Fabrication has two components: the chemical synthesis of DNA oligo building blocks approximately one hundred bases long and their subsequent assembly into long multi-gene sequences thousands and tens of thousands bases long. The obvious advantage compared to cloning from genomic DNA or cDNA is the total control exerted over the design and sequence of the product, features such as restriction sites and promoters can be inserted or deleted almost at will. More importantly, a fabricated construct can be codon optimized for good expression in a heterologous host, for example in an *E. coli* chasis, an issue especially pertinent to *Streptomyces* where GC content is high. Although the cost of DNA synthesis has not yet followed DNA sequencing down to the vanishing point, recent developments indicate a corner has been turned, and economical gene synthesis now looks feasible [Figure 3a].

The classic method for oligo nucleotide synthesis, 3′ to 5′ addition of nucleoside phosphoramidites tethered to a silica bead substrate, is too expensive for operon scale synthesis. Automated platforms such as the 'MerMade', brought DNA synthesis by this method down to about 0.50 USD per base by 2006, approximately the cost of the underlying chemical reagents. That price is still prohibitive. An ultra miniaturized microfluidic oligo synthesis platform has been reported, which will minimize reagent usage and further lower price, but probably not as low as array synthesized oligos [47] (see below). Cost aside, there are technical limitations in the length and purity of resin-based oligo synthesis that limit their usefulness for SB. Failure at any synthetic step of an individual oligonucleotide truncates further downstream addition lowering total yield for full length oligonucleotides, necessitating labor intensive purification by HPLC, or gel electrophoresis to reliably acquire the desired product.

Assembly of oligos into larger, functional genes and operons is even more expensive. Assembly follows one of two basic strategies [Figure 4a,b]. One method is to design oligo building blocks with unique overlapping ends that will hybridize to form a skeletal, partially double stranded template that then can be filled in by Polymerase Chain Reaction (PCR) to complete the construct. Alternately, a complete spanning set of oligos can be made, then hybridized and stitched together by a ligase enzyme. The second route requires a larger investment in oligos, but has the advantage of bypassing the error prone second-strand fill-in by PCR. *In vitro* constructs up to 500,000 base pairs have been created using refinements of this method [48]. These very large constructs can also be assembled and maintained in yeast, and efficient shuttle tools have appeared, including a three-way yeast/*E. coli*/'other' system [49].

Oligos synthesized on chips by photoactivation or ink jet are much less expensive than resin synthesized polymers, as little as 0.01 USD per base. However, chip synthesis is also inherently more error prone, and, the product oligos are present individually in low concentrations and therefore must be amplified by PCR thus introducing additional sequence defects. Furthermore, pools of chip synthesized oligos are highly complex mixtures promoting inappropriate cross hybridization and misassembly. These problems have been overcome by meticulous computational design of flanking sequences that allowed a total pool of 55,000 unique oligos to be 'amplification sorted' into 10 subpools, each potentially encoding about 100 different genes. The sub pools were individually amplified by sequential, differential PCR, then assembled into complete genes at an final cost of less than 0.10 USD per base. This is a significant, order of magnitude economy [50••,51].

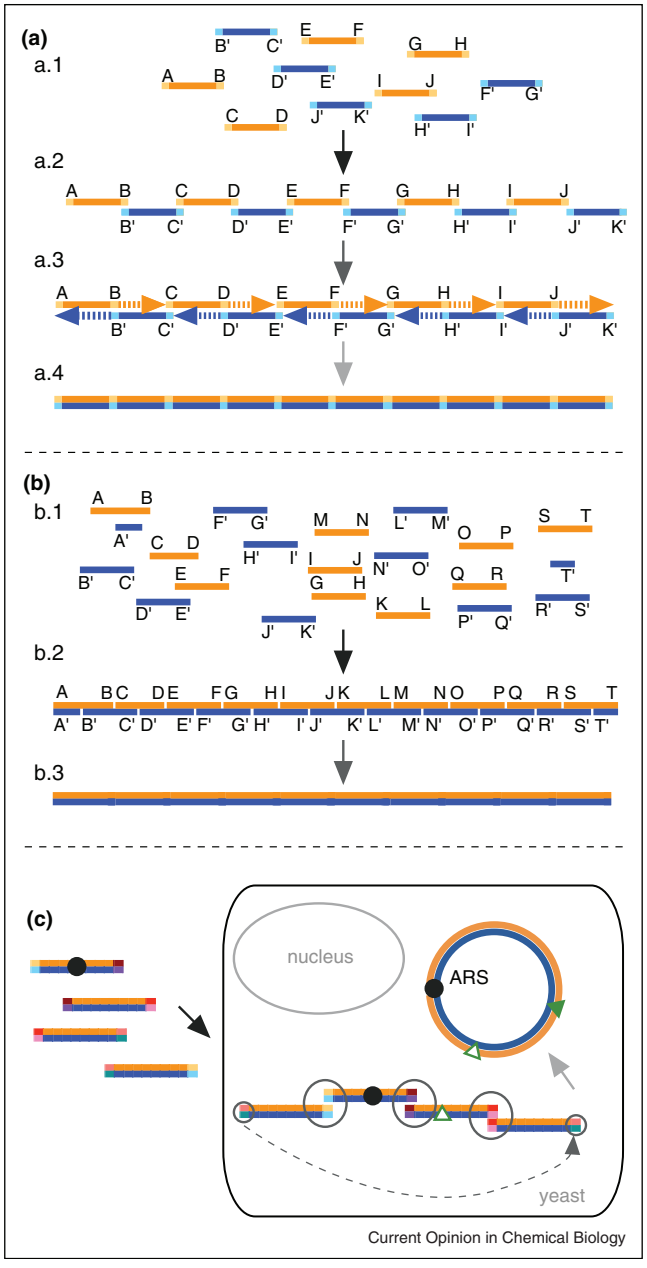
Figure 3



(a) The cost of sequencing, oligo manufacture and gene assembly is declining. Next Generation Sequencing (NGS), shown in open black squares [11•] or closed black squares [Wetterstrand KA. DNA Sequencing Costs: Data from the NHGRI Large-Scale Genome Sequencing Program, www.genome.gov/sequencingcosts. Accessed 06042011] has pushed the cost DNA sequence down to negligible levels. Oligo manufacturing costs, shown in open red triangles [11•] or closed red triangles [47,51] only declined slowly through about 2005 but have dropped an order of magnitude in the past two years. The cost of assembled genes, shown in open green triangles [11•] or closed green triangles [52••] also dropped by an order of magnitude over the same period. These recent declines are marked by downward pointing arrows drawn from the projected trend lines (based on about a decade of data) to the 2010 data points (1,2,3).
(b) Error rates in assembled gene constructs are shown on a per kilobase basis for various error correction technologies. Error rates for uncorrected assemblies are shown as solid purple circles: chip synthesized oligos with PCR assembly and ligation assembly. Error rates for various strategies are shown as open circles: block assembly [52••], microfluidic assembly [47], PCR assembly with ErrASE [54•], and megacloning [53••].

Error propagation is a serious problem for any gene assembly strategy because the final product must direct accurate transcription/translation of a viable protein. Deletions or insertions are likely to cause frame shifts,

Figure 4



Three strategies for assembling genes, operons and genomes from oligonucleotides. **(a)** (A.a.1) Polymerase Assembly. A series of oligos are designed relatively short, overlapping complimentary regions, adhering to a narrow Tm range. Complimentarity is indicated in the figure by a capital letter-letter prime pair, for example oligos A–B and B′–C′. (A.a.2) A linear network of oligos is formed by hybridization, for example A_B–B′_C′–C_D . . . (A.a.3). The single stranded gap regions are filled-in by a polymerase. **(b)** Ligase Assembly. (B.b.1) A spanning set of oligos is designed represent complete coverage of both strands. (B.b.2) The oligos are hybridized to form a double-stranded construct with unjoined 'nicks' at the oligo ends. (B.b.3) The construct is stitched together with ligase enzyme. **(c)** Whole Genome Assembly. Very large constructs (>500,000 base pairs), containing genetic modifications engineered into the constituent oligonucleotides (open green triangle) can be assembled in yeast by homologous recombination (open grey circles and dotted arrow); by including a yeast ARS (solid black circle). These constructs can be maintained as Yeast Artificial Chromosomes, and further modified using the yeast molecular biology tools kit (solid green triangle).

and even frame-preserving non-synonymous substitutions can render the construct useless if the altered amino acid disrupts protein function. The JVCI genome transplantation project was derailed for several months by a single base deletion in the essential replication protein *dnaA* that dysfunctionalized an entire 500,000 base pair whole genome construct [52••]. The workaround has been to painstakingly purify the starting oligos, to sequence the final construct extensively to identify errors, then to use PCR repair, and finally to functionally screen the constructs for activity. These techniques are labor intensive, require skilled practitioners, and drive up the price of finished genes and operons (nearly 1.00 USD per base in 2007 [Figure 3a]). Errors can be *suppressed* pre-assembly by using the highest possibly quality oligos, or errors can be *removed* post-assembly. In a gene assembly strategy called 'megacloning' next generation sequencing (NGS), reimagined, is used as a preparative technique to purify perfect oligos and maximize error suppression [53••]. For post assembly an efficient error correction system called ErrASE has been developed and will be distributed commercially by Life Technologies [54•]. Overall, progress in driving down error rates in assembled genes is impressive [Figure 3b] meaning that far fewer constructs need to be sequenced and functionally tested. As fabrication is a fundamental SB procedure, these advances are crucial.

Metabolic engineering

The importance of balancing the activities of multiple enzymes along a pathway has already been emphasized [Figure 1d]. This also can be accomplished at the level of translation. Riboswitches are natural RNA aptamers that modulate gene expression or translation in response to ligand binding. Often the ligand is a product of the gene circuit being regulated, thus forming a feedback loop. The potential of riboswich control is apparent in the successful reengineering of the add-A mRNA element that operates by closing or opening access to the ribosome binding site in response to binding by adenosine. An orthogonal add-A* was created that was unresponsive to adenine but highly responsive to ammeline and azacytosine, ligands inactive on native add-A [55]. A completely different approach to regulatory engineering has been taken in which parallel evolution of multiple ribosomal binding sites is orchestrated by the addition of mutagenic oligos complimentary to the lagging strand of DNA synthesis that are introduced through cycles of growth and electroporation [56•]. Another approach to translation tuning has been demonstrated in yeast where series of hairpin RNA substrates have been designed that have a

spectrum of susceptibility to the yeast RNase III cleavage enzyme Rnt1p. This spectrum of activity correlated with protein levels when the hairpins where inserted in the 3′ untranslated region (UTR) of a test gene, indicating that the system shows promise as an 'off the self' kit to tune protein expression [57].

Conclusion

Ungent. Urgent. Opportune.

'Snakeoil' has come into English as a derogatory shorthand for 19th century patent medicines that were at best useless, and often frankly harmful. But snake oil or *sheyou* (蛇油) is in fact a standard remedy still in use in Chinese Tradition Medicine (TCM), a *bone fide* natural product, albeit a non-standardized, non-crystalline, molecularly uncharacterized admixture. Just the features that made NPs unattractive to drug developers in the first place. Oil of a different sort is fueling explosive growth in synthetic biology. I have argued here that an SB engine can be built and used to assemble libraries of purified NP compounds that will offer excellent starting points for a revival in drug discovery. Whether *sheyou* itself is or is not efficacious, the persistence of TCM, Ayurveda and other traditional pharmacopeiae [58–60] remind us that NPs represent a rich therapeutic lode waiting to be mined. The creation of pure natural product and natural product derived collections, a natural product Chemicopia, is urgently needed, is feasible, and is opportune.

To move forward the community should embrace a definite and immediate program linked to concrete objectives. A manifesto for the first phase of such a campaign might have the following platform:

1. Sequence *Streptomyces* to the edge of new secondary metabolic gene discovery, 1000 genomes to start.
2. Use synthetic biology to build a collection of pure *Streptomyces* derived natural products, 10,000 to start.
3. Collaborate with medicinal chemists to derivatize these compounds where appropriate to make additional pure semi-natural products, 5000 to start.
4. Make these compounds widely available for screening in drug discovery programs.

Conflict of interest statement

The author is unaware of any conflicts of interest that would materially influence the content of this work.

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