Metabolic engineering in chemolithoautotrophic hosts for the production of fuels and chemicals

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Abstract

The ability of autotrophic organisms to fix CO₂ presents an opportunity to utilize this ‘greenhouse gas’ as an inexpensive substrate for biochemical production. Unlike conventional heterotrophic microorganisms that consume carbohydrates and amino acids, prokaryotic chemolithoautotrophs have evolved the capacity to utilize reduced chemical compounds to fix CO₂ and drive metabolic processes. The use of chemolithoautotrophic hosts as production platforms has been renewed by the prospect of metabolically engineered commodity chemicals and fuels. Efforts such as the ARPA-E electrofuels program highlight both the potential and obstacles that chemolithoautotrophic biosynthetic platforms provide. This review surveys the numerous advances that have been made in chemolithoautotrophic metabolic engineering with a focus on hydrogen oxidizing bacteria such as the model chemolithoautotrophic organism (*Ralstonia*), the purple photosynthetic bacteria (*Rhodobacter*), and anaerobic acetogens. Two alternative strategies of microbial chassis development are considered: (1) introducing or enhancing autotrophic capabilities (carbon fixation, hydrogen utilization) in model heterotrophic organisms, or (2) improving tools for pathway engineering (transformation methods, promoters, vectors etc.) in native autotrophic organisms. Unique characteristics of autotrophic growth as they relate to bioreactor design and process development are also discussed in the context of challenges and opportunities for genetic manipulation of organisms as production platforms.

Keywords: CO₂ fixation, biofuels, biochemicals

37 **1. Introduction**

38 The vast majority of biological activity on our planet derives its energy from the sun.
39 Heterotrophic organisms rely on the primary productivity of photosynthetic organisms to provide
40 reduced carbon. This photosynthetic activity is divided into the process of photon capture to
41 release high energy electrons (light reactions) and the channeling of those electrons to their use
42 in the reduction of carbon dioxide (dark reactions). Electrons can be made available by a variety
43 of sources (wind, hydroelectric, etc.) where the most analogous to photosynthesis is the
44 production of electricity from photovoltaics. In Figure 1, this analogy is used to illustrate the
45 concept of ‘replacing’ the light reactions of biological photosynthesis with photovoltaics and
46 utilizing those electrons to reduce CO₂. The path traversed by these electrons defines broad
47 classes of microorganisms. Since free electrons are rapidly reacted, the reducing power is often
48 shuttled by other compounds. The prevalence of dihydrogen (H₂) from numerous biological or
49 geochemical reactions has provided motivation for biological utilization of this form of reducing
50 power to fix CO₂, and can be viewed simplistically as a form of autotrophic growth where H₂ is
51 used as a substitute for light, and the dark reactions of CO₂ fixation proceed as they do in
52 photosynthetic organisms. Not surprisingly, nature has found numerous other pathways to fix
53 CO₂ (Berg, 2011); however, the basic concept of capturing available reducing power into central
54 metabolism remains the basis of autotrophic growth.

55 Included in the ‘dark reactions’ of Figure 1 is also the process of splitting water, which
56 can be achieved external to the cell by electrolysis, or with the assistance of electron carriers that
57 might be reduced either inside or outside the cell. As will be discussed in more detail below,
58 some organisms can directly accept the reducing power from an electrode (Nevin et al, 2010),

and the nature of configuring bioreactors to accommodate this broad range of electron delivery becomes an integral component of an eventual process to use the reducing power within metabolically engineered organisms to produce biochemicals (conceptually depicted in Figure 2). The scope of metabolic engineering of autotrophs therefore spans from improving the ability of an organism to accept electrons into metabolism and fix CO₂ to the introduction of biosynthetic pathways into this diverse class of organisms that already possesses chemolithoautotrophic metabolism. Since the metabolic engineering of facile heterotrophic organisms such as *E. coli* or yeast is highly advanced, it is tempting to pursue introducing autotrophic metabolism into these model chassis. This remains an exciting possibility, particularly if the host chassis is an extremophile or industrially robust organism, however this is currently limited by the tremendous complexity of both the associated enzyme systems and their regulation. As it is not possible to cover this material in exhaustive detail in one document, a list of relevant reviews referenced throughout the text is summarized in Table 1 for the reader's convenience.

2. Background

By definition, autotrophic growth encompasses any organism that can 'feed itself' from non-organic carbon sources – namely CO₂. Since there are microorganisms that can utilize essentially any reduced chemical as a component of their metabolism, it is useful to briefly review this classification to define the scope of this review. Photoautotrophs utilize light to extract hydrogen from water (Figure 3), and are not the focus of this review. Many organisms can utilize H₂ including the anaerobic methanogens and acetogens, as well as aerobic hydrogen oxidizing bacteria that are often referred to as Knallgas bacteria (Schwartz et al, 2013). The

methanogens are largely excluded from this review because their ubiquitous presence in association with anoxic breakdown of organic material (sediments, wastewater, animal gut) require its own specific review (Demirel & Scherer, 2008; Thauer et al, 2008). Acetate production from CO₂ and H₂ is of interest in this review in part because acetate can readily be converted to alternative biochemicals.

The aerobic hydrogen-oxidizing bacteria are of particular interest for this review because of their demonstrated potential as production platforms. The model organism *Ralstonia eutropha* (used here in preference to the new classification of *Cupriavidus necator*) has been the subject of study for over 100 years (Kaserer, 1906). Its ability to accumulate biomass and poly-3-hydroxybutyrate (PHB) to very high levels both heterotrophically (Ryu et al, 1997) and autotrophically (Tanaka et al, 1995) demonstrated its ability to be competitive with *E. coli* as a production platform. Considerable efforts have been made to conduct scale-up and optimization studies. *Rhodobacter capsulatus* is also a noteworthy chemolithoautotrophic (Madigan & Gest, 1979) candidate due to its study as a model organism and diverse growth modes (Hunter et al, 2009). Obligate anaerobic acetogens, like *Clostridium ljungdahlii*, are attractive hosts because of their ability to use synthesis gas (syngas) or CO₂ and H₂ for growth. *C. ljungdahlii* naturally produces acetate, ethanol, butanol, and 2,3-butanediol; companies such as IneosBio, Coskata and LanzaTech are actively commercializing it for production of variety of chemicals from syngas (Köpke et al, 2011a). A more in-depth examination of some of the characteristics of these model, sequenced organisms provides some additional perspectives on considerations for metabolic engineering of biochemicals production. The autotrophs which can utilize reduced inorganic compounds (NH₄⁺, H₂S, Fe²⁺) are also of notable interest because of either utilization of an industrial waste stream or the potential of the substrate to undergo cyclic reduction at a cathode

as the basis of delivering the reducing power. Finally, the potential for direct electron feeding from a biocathode (sometimes referred to as microbial electrosynthesis) (Lovley & Nevin, 2013) represents the most direct route for the delivery of reducing power from electricity to a microorganism.

2.1 Carbon Fixation in Chemolithoautotrophs

2.1.1 Calvin-Benson-Bassham (CBB) Pathway and Rubisco

The predominant mechanism for CO₂ capture in aerobic chemolithoautotrophs is via the photosynthetic dark reactions of the Calvin-Benson-Bassham (CBB) pathway (Figure S1). This pathway is present in many of the model autotrophic organisms including *R. eutropha*, *Rhodobacter*, and some species of *Nitrosomonas*, and *Acidithiobacillus*. The first committed step of the CBB pathway is a carboxylation reaction involving CO₂ and ribulose-1,5-bisphosphate (RuBP) by ribulose bisphosphate carboxylase/oxygenase (Rubisco), which many autotrophs compartmentalize into organelles called carboxysomes. Regeneration of RuBP involves enzymes of central metabolism, a sedoheptulose bisphosphatase (SBPase), that converts a 7-carbon sedoheptulose-1,7-bisphosphate to sedoheptulose-7-P, and phosphoribulokinase (PRK), that converts ribulose-5-P to RuBP. Enzymes Rubisco, SBPase and PRK are considered to be unique to the CBB pathway. Rubisco is responsible for most of the CO₂ captured on the planet and is the CO₂-fixing enzyme in the majority of aerobic chemolithoautotrophs. The extensive studies of energetic and catalytic efficiency of Rubiscos are of particular interest to metabolic engineering. Three forms of Rubisco exist, which differ in catalytic efficiency (k_{cat}) and CO₂-specificity (S_{CO_2}) – important properties which generally represent trade-offs in functionality. Variants of these forms are found in chemolithoautotrophic bacteria (Badger & Bek, 2008).

Sometimes more than one variant exist simultaneously in a single bacterium and are generally differentially regulated in response to CO₂ concentration (Berg, 2011).

An affinity for O₂ is the biggest disadvantage of the Rubisco enzymes and causes the phenomenon called photorespiration in plants, which is the tendency of Rubisco to oxidize RuBP, rather than carboxylating it. The resulting need to ‘recover’ the oxidized product is energetically very expensive and involves loss of CO₂ and consuming the amino acid glycine to give ammonium. Photorespiratory CO₂ loss is estimated to be 21% of the net CO₂ assimilation in plants (Berg, 2011). In bacteria, the product of Rubisco oxidation is metabolized more efficiently than plants, where 2-phosphoglycolate is dephosphorylated to glycolate, which is either secreted from the cell or recycled back to central metabolism (Berg, 2011). Both of these mechanisms waste carbon and energy, thus there is room for improvement in chemolithoautotrophic bacteria as well.

The Rubisco forms have evolved for optimized performance (by a balance of k_{cat} and S_{CO_2}) in their respective niches with varying degrees of CO₂ and O₂ availability; therefore the target platform organism may not have the appropriate properties for the intended bioreactor environment. Therefore the choice of the Rubisco form for metabolic engineering will be largely dictated by the O₂ and CO₂ operational conditions. For example, choice for a Form II Rubisco (having lower S_{CO_2} but higher k_{cat}) can be made in an autotroph if high catalytic rate is desired by increasing CO₂ and limiting O₂ reactor concentrations. Carbon concentrating mechanisms (CCM) and carboxysomes found in bacteria are a reminder that the bioreactor operating conditions can be significantly modified by the microbial chassis. Rubisco and associated genes needed for functional CO₂ fixation are nearly always located in *cbb* operons (Figure S1). These operons reflect that a functional CBB pathway requires at least four genes including a

transcriptional regulator (CbbR) while at the same time a tremendous diversity has evolved. These carbon-fixing operons tend to be 'complete' by the principle of the 'selfish operon' (Lawrence & Roth, 1996), where the likelihood of a gene being passed along to subsequent host is enhanced by the clustering of genes in an operon that encodes a complete function. This clustering of genes by function continues to be a significant asset to metabolic engineering efforts that has been accelerated by next generation sequencing and associated reverse genetics.

2.1.2 Other Carbon-fixation Pathways

Due to the diverse habitats of chemo-/photoautotrophic bacteria, various other CO₂ fixation pathways have evolved. In addition to CBB, at least five other CO₂ fixation pathways have been characterized (Figure 4): (1) the reductive tricarboxylic acid cycle (rTCA), (2) reductive acetyl-CoA or Wood-Ljungdahl pathway (rAC), (3) 3-hydroxypropionate bicycle (3HP), (4) 3-hydroxypropionate-4-hydroxybutyrate cycle (HP/HB), and (5) dicarboxylate-4-hydroxybutyrate cycle (DC/HB) (Berg, 2011). rTCA cycle has a wide distribution in chemolithoautotrophic and photosynthetic bacteria. It involves running the TCA cycle in reverse. Wood-Ljungdahl pathway is limited mostly to obligate anaerobic acetogens of *Clostridial* genera and methanogenic *Archaea* and fixes CO₂ as well as CO via a linear pathway. Distribution of 3HP pathway is very limited to within green non-sulfur bacteria with *Chloroflexus aurantiacus* being the most heavily studied. The DC/HB and HP/HB are found in archaea - mostly aerobic/microaerobic thermophiles. Although the HB arm is common to both the cycles, the enzymes appear to have evolved independently of each other. With the interest in using CO₂ as a substrate for biochemical production, these pathways are being intensely studied including

efforts to enhance or even transplant these pathways to create new autotrophic chassis for metabolic engineering.

2.2 Hydrogen Utilization by Chemolithoautotrophs

Hydrogenases are metal-containing enzymes that are common in bacteria (Vignais & Billoud, 2007). They are classified based on the H₂-binding sites as the [FeFe] and [NiFe], with a third rare [Fe]-only type found only in methanogens (Schick et al, 2012). [FeFe] hydrogenases, have simple monomeric forms, consisting of only the catalytic subunit, which in principle would be easier to introduce into a heterologous host. [NiFe] hydrogenases are composed of small and large subunits, and generally have relatively large number of maturation and assembly accessory proteins. Hydrogenases are also subdivided into membrane bound (MBH) (two varieties – H₂-uptake or H₂-evolving types), and bidirectional soluble (SH) forms (Vignais & Billoud, 2007). Of the two membrane bound hydrogenases, the uptake hydrogenase is of particular interest in genetic engineering of autotrophs as it is involved in the consumption of H₂. The uptake MBH is generally attached to *cytochrome b* complex and ultimately involved in ATP generation. The SH contains subunits that bind to soluble cofactors such as NAD or NADP. They are able to catalyze the evolution or consumption of H₂, subsequently oxidizing or reducing the cofactors, depending on the physiological conditions (i.e. NAD(P)/NAD(P)H ratio, H₂ partial pressure etc.). Some SH also act as H₂ sensors, being part of the regulatory mechanism and not H₂ activation. Further discussion of the details of hydrogenases is beyond the scope of this review and are thoroughly reviewed elsewhere (Lubitz et al, 2014). A schematic representation of an aerobic chemolithoautotroph in terms of energy generation, utilization, CO₂-fixation and regulation is presented in Figure 5.

The genes for hydrogenase synthesis, assembly and maturation are usually conveniently organized onto operons which facilitates their heterologous expression (Rousset & Liebgott, 2014). In fact, *Alcaligenes hydrogenophilus* contains the hydrogenase activity (Hox⁺) on a megaplasmid that allowed early identification of hydrogenase genes by mobilization into other species which conferred ability to grow on hydrogen (Yagi et al, 1986). The transitions of this work into the current bioinformatic era is discussed further in Section 4.3. These historical studies on the genetic transfer of the Hox⁺ phenotype, were followed by a period of intense study of hydrogenases for the production of bio-hydrogen (Lubitz et al, 2014; Rousset & Liebgott, 2014), providing considerable genetic resources and experience in heterologous hydrogenase expression.

2.3. Microbial Electrosynthesis via Direct Electron Feeding

The capacity of microorganisms to serve as electron donors for a microbial fuel cell (MFC) has been extensively studied, for example via anode reduction of wastewater coupled to abiotic hydrogen production. The relatively new development of microbial biocathodes as electron acceptors for reduction of CO₂ merits brief discussion in this review. The ability of hydrogenase to reduce NAD⁺ mediated by electrode potential was demonstrated over 20 years ago (Cantet et al, 1992). Biohydrogen and biomethane production subsequently dominated the study of electrosynthesis where the typical feedstock included organic-laden wastewater streams. The first production of hydrogen from a biocathode was accomplished in 2008 (Rozendal et al, 2008), while the electrosynthesis of methane by a methanogen was first demonstrated in 2009 (Cheng et al, 2009). Microbial electrosynthesis (MES) of organic compounds is recognized and recently reviewed (Rabaey & Rozendal, 2010; Lovley & Nevin, 2013). While most microbial

electron feeding involves undefined consortia of bacteria, recent reports describe some axenic cultures of acetogens (belonging to *Spormusa*, *Clostridium* and *Moorella* genera) capable of accepting electrons from a cathode and fix CO₂ into acetate (on the order of 0.1-1 mM over several days) (Nevin et al, 2011). Electrosynthesis of acetate and hydrogen gas was recently reported by a microbial consortia (dominated by *Acetobacterium* spp.) for production of acetate to 175 mM (17.25 mM d⁻¹) and hydrogen to the equivalent of 1164 mM (100 mM d⁻¹) was demonstrated (Marshall et al, 2013). More recently, an *Acetobacterium*-dominated consortia achieved acetate production of >50 mM d⁻¹ at -800 mV vs. SHE, in addition to >1000 mM d⁻¹ H₂ (LaBelle et al, 2014). To date we are not aware of any genetically engineered microbial electrosynthetic system.

3. Metabolic Engineering of Natural Chemolithoautotrophic Organisms

The diversity of strategies for metabolic engineering in chemolithoautotrophs is as diverse as the range of autotrophic hosts. The status of genetic engineering is very organism-dependent with the two major categories of aerobic H₂-oxidizing bacteria and anaerobic acetogens. The current focus of metabolic engineering study is largely defined by considerations for the level of basic science study, existing metabolism, genetic engineering tools and ease/danger of culture conditions (using H₂ and CO). Study of model chemolithoautotrophic organisms including *R. eutropha*, *Rhodobacter* sp. and *C. ljungdahlii*, provide a useful basis for discussing key characteristics of autotrophic metabolisms. An additional constraint to interpretation, is that although performance of a metabolically engineered chemoautotroph is most relevant when tested under autotrophic growth conditions, testing is often only presented for heterotrophic conditions because it is much easier to implement.

3.1. Status and Constraints for Metabolic Engineering in Chemolithoautotrophs

R. eutropha is a model chemolithoautotroph that has very high aerobic autotrophic productivity when grown on H₂, and accumulates large quantities of PHB where its knockout in principle could provide extensive flux within central metabolism via acetyl-CoA. As a result of relatively mature genetic engineering tools (transformation, chromosomal modifications, plasmids), *R. eutropha* has been the biotechnological platform of a number of commodity biochemicals and biofuels. *R. capsulatus* is also a robust hydrogen-oxidizing chemolithoautotroph that benefits from the extensive study of its closely related species *R. sphaeroides*, which can also be coerced into chemolithoautotrophic growth as well (Paoli & Tabita, 1998). The study of its diverse metabolism including anaerobic photoheterotrophy has resulted in extensive understanding of its basic physiology and genetics. *Rhodobacter* also has moderate capacity for PHB accumulation as well as isoprene metabolism associated with photosynthetic pigments.

On the other hand, due to the anaerobic nature of acetogens, these organisms display natural accumulation of fermentative end-products of industrial interest from CO₂, including ethanol, butanol and even acetone and 2,3-propanediol. *C. ljungdahlii* has emerged as a front-runner for further development as a metabolically engineered biochemical platform in part since the genetic tools are becoming available relative to other candidates. Acetogenic bacteria also have a high autotrophic flux to acetyl-CoA which makes them attractive candidates for autotrophic production of a variety of fuels and chemicals (Hu et al, 2013).

This range of model organisms represents a gradual transition of metabolism from highly O₂ tolerant to highly O₂ sensitive. This distinction is quite important from the standpoint of

metabolic engineering of chemolithoautotrophs due to energetic considerations. Anaerobic organisms are generally more efficient at energy capture because of the inherently smaller available thermodynamic free energy to drive heterologous metabolism (Bar-Even et al, 2012).

For this same reason the anaerobic rate of energy capture is quite constrained particularly for the production of highly reduced molecules (Fast & Papoutsakis, 2012). Acetogenesis from H_2/CO_2 proceeds via the stoichiometry

and is thus one of the most constrained modes of energy conservation known (Drake & Daniel, 2004). Energy is conserved through the Wood-Ljungdahl pathway by a combination of substrate level phosphorylation (production of acetate from acetyl-CoA) and chemiosmosis (Mayer & Müller, 2014). Diverting acetyl-CoA flux away from acetate production therefore puts severe energy limitations on the cell, and stoichiometric models predict that this will drastically limit the yield of the target molecule (Fast & Papoutsakis, 2012). This may explain why attempts to produce highly reduced chemicals through the Wood-Ljungdahl pathway to-date have met with limited titers and yields. Unlike genetic systems, which can be gradually improved and refined over time, these thermodynamic considerations are immutable, and may ultimately constrain the commercialization of these microbes.

On the other hand, aerobes are capable of capturing large amount energy from an electron donor because of the ability to use O_2 as an electron acceptor, and thus drive high rates of productivity of energy-dense molecules at the expense of energy capture efficiency. In addition to the inherently less efficient energy capture in aerobic chemolithoautotrophs, the presence of O_2 poses numerous additional problems (see Section 4.1.2 for further discussion). Almost all the key enzymes for CO_2 -fixation and H_2 -activation are either permanently or temporarily

deactivated by O₂ or have undesired side reactions that are energetically wasteful. Natural systems have solved this problem by evolving a balance among these antagonistic effects of O₂. Where light is available in high quantity, higher photoautotrophs use the abundance of ATP to circumvent the wasteful photorespiratory branch of photosynthesis and use carbon concentrating mechanisms to exclude O₂ from reaction centers. Bacteria that do not have this option, carry out a slightly more efficient (but still wasteful) cycling mechanism to counteract this non-specific reaction of Rubisco. Rubiscos and hydrogenases in nature have also evolved highly ‘optimized’ forms based on the organism’s natural habitats. Catalytically faster Rubiscos are less specific towards CO₂. Catalytically faster [FeFe] hydrogenases are highly sensitive to O₂ and deactivate permanently, while [NiFe] SH and MBH display gradual tolerance towards O₂ and corresponding reduced catalytic activity. The goal of metabolic engineering is to develop organisms to produce chemicals at high rates of productivity and using low energy as both of these contribute towards the cost – biofuel being the most extreme example where both of these conditions have to be simultaneously met. From this standpoint, the engineering needs to capture the best of both worlds – through genetic, metabolic and bioreactor engineering.

3.2. Metabolic Engineering in Aerobic Chemolithoautotrophs

The native biosynthetic capacity of *R. eutropha* to produce PHB at greater than 70% of dry weight, even in presence of carbon monoxide (Volova et al, 2002), has motivated the engineering of this organism for even higher levels of PHB and other biopolymers. Fukui and co-workers engineered non-native poly[(R)-3-hydroxybutyrate-co-3-hydroxypropionate] polyesters in *R. eutropha* by introducing malonyl-CoA reductase and 3-hydroxypropionyl-CoA synthetase genes from *C. aurantiacus* (Kichise et al, 1999). Most metabolic engineering strategies of

heterologous pathways in *R. eutropha* have similarly incorporated some form of knock-out of the PHB synthesis pathway, to divert a portion of that carbon flux. However, in recent work producing methyl ketones from a truncated lipid β -oxidation pathway, there was surprisingly no enhancement observed for the $\pm$ knockout comparison, and the highest level of chemolithoautotrophic production was only 180 mg/L. Hydrocarbon biosynthesis engineering in *R. eutropha* using decarboxylation of fatty acids also used β -oxidation mutants, and was executed in a manner to explore promoters, origins of replication and ribosomal binding (Bi et al, 2013). Where broad host range pBBR and arabinose-inducible P_{BAD} outperformed other typical vectors, an unexpected combination of a high copy number mutant of pCM62 combined with a synthetic RBS gave 6 mg/L in this generally low expression testing.

Only *R. eutropha* PHB knockout strains were tested under heterotrophic growth conditions in efforts to produce branched-chain isobutanol and 3-methyl-1-butanol production (Lu et al, 2012). Batch production was over 300 mg/L and repeated media removal for 50 days achieved a cumulative production totaling 14 g/L. In a subsequent effort from the Sinskey laboratory, the systematic optimization of isopropanol production demonstrated substantial effects for codon usage, and gene dosage and was able to achieve 3.44 g/L in batch cultures with less than 1 gDW/L. This represents one of the highest specific productivities achieved to date for metabolic engineering of *R. eutropha* (Grousseau et al, 2014). However, this was achieved on fructose, and remains to be demonstrated under chemolithoautotrophic conditions. As part of a demonstration of integrated electrolytic reduction of CO₂ to formate (with subsequent uptake and formation of CO₂ and NADH), Liao laboratory (Li et al, 2012) executed an autotrophic fermentation (80:10:10, H₂, O₂, CO₂) to produce 1 g/L alcohols (~50:50 isobutanol:3-methyl-1-butanol). This work included a PHB knockout and the ‘Ehrlich pathway’ in conjunction with

enhanced α -keto acid flux toward the branched valine/leucine amino acid synthesis pathway. The elevated production is partly assisted by the ability of autotrophic growth to achieve high cell concentrations of ~12 gDW/L ($OD_{600}=24$).

Purple non-sulfur bacteria, such as *R. sphaeroides* and *R. capsulatus*, naturally synthesize large amounts of carotenoids as well as increased lipid metabolism during intracellular membrane production under O_2 /light limitation (Beekwilder et al, 2014). Utilizing the endogenous IPP/DMAPP flux of *R. sphaeroides*, valencene production (57 mg/L) was achieved by simply expressing *Callitropsis nootkatensis* valencene synthase (CnVS) which was 40-fold higher than the same construct in *Saccharomyces cerevisiae*. Further enhancement to 352 mg/L was observed upon introduction of the mevalonate (MVA) pathway from *Paracoccus zeaxanthinifaciens*, indicating that the CnVS enzyme was substrate limited. We took an alternative approach of enhancing the endogenous *R. capsulatus* methylerythritol phosphate (MEP) pathway to produce the C_{30} triterpenes, squalene and botryococcene, using the triterpene synthases from the colony algae *Botryococcus braunii* race B (Niehaus et al, 2011). This was accomplished by expressing high activity avian FPS to catalyze conversion of IPP/DMAPP to farnesyl diphosphate (FPP), along with expressing the rate-limiting MEP enzymes 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and isoprenyl diphosphate isomerase (IDI) from pBBR vectors (Khan et al, 2015). As observed for most other biochemicals tested, autotrophic production was comparable to screening results on complex heterotrophic media, indicating a robust metabolism under fully chemolithoautotrophic growth conditions. Autotrophic bioreactors achieved 110 mg/L when biomass reached 6.6 gDW/L and subsequent continuous operation stabilized at 60 mg/L (at 2.6 gDW/L). This represents a specific productivity of $0.5 \text{ mg} \cdot \text{gDW}^{-1} \cdot \text{h}^{-1}$ which is comparable to the average alcohol specific productivity of $0.67 \text{ mg} \cdot \text{gDW}^{-1} \cdot \text{h}^{-1}$ (though

this work of Liao displayed a growth-dissociated burst of $3.3 \text{ mg} \cdot \text{gDW}^{-1} \cdot \text{hr}^{-1}$ after 3 days of culture). These results are still an order of magnitude lower than the $93 \text{ mg} \cdot \text{gDW}^{-1} \cdot \text{hr}^{-1}$ isopropanol reported for growth on fructose (Grousseau et al, 2014). Comparative evaluations of productivity are often difficult to interpret in the absence of details of bioreactor operational strategy, which is addressed in some additional detail in Section 6 of this review.

3.3 Metabolic Engineering in Acetogens

Metabolic engineering of acetogens is in its infancy, largely due to the limited genetic tools available for these microbes. In contrast, the native productivity of these organisms for producing their final metabolites is well beyond metabolic engineering efforts in aerobic chemolithoautotrophs. Since acetate is the precursor to a variety of important chemical compounds, for example polyvinyl acetates, the ability to generate monomer precursors autotrophically instead of from petrochemicals continues to be developed, including the use of genetic engineering. For example, the acetogenic capacity of *Acetobacterium woodii* was recently improved by overexpression of native pathway enzymes. Transformation of plasmids carrying either the phosphotransacetylase (PTA) and acetate kinase (ACK) or the four THF-dependent enzymes in the Wood-Ljungdahl pathway was achieved by methylating these vectors using an *E. coli* strain expressing the *C. ljungdahlii* DNA methylation genes prior to electroporation (Straub et al, 2014). The PTA-ACK strain had significantly higher specific productivity of $0.9 \text{ g} \cdot \text{gDW}^{-1} \cdot \text{hr}^{-1}$ and final acetate titer 50.5 g/L compared to the already impressive ‘wild-type’ production strain performance ($0.85 \text{ g} \cdot \text{gDW}^{-1} \cdot \text{hr}^{-1}$ and 44.7 g/L). In addition to acetate, syngas-fermenting organisms natively produce several other compounds, including ethanol, butyrate, butanol, and 2,3-butanediol (Liew et al, 2013; Daniell et al, 2012; Köpke et al, 2011b). Three companies:

Coskata, INEOS and LanzaTech are commercializing autotrophic ethanol production (Schiel-Bengelsdorf & Dürre, 2012). LanzaTech is also working on 2,3-butanediol production (Köpke et al, 2011b) and although no results are yet available in the literature, numerous patents have been filed in the last year by LanzaTech in this space.

The first example of an engineered acetogen for non-native production involved transplanting the butanol biosynthesis pathway from the heterotrophic acetogen *Clostridium acetobutylicum* into *C. ljungdahlii* via the pIMP1 shuttle vector. The recombinant strain produced 0.15 g/L (5 kJ/L) butanol from synthesis gas in mid growth-phase, but this was subsequently consumed by the end of the fermentation (Köpke et al, 2010). More recently Derek Lovley's group improved the transformation protocol for *C. ljungdahlii*, reporting high efficiency replicating plasmids, as well as integrative vectors and the ability to generate knockouts (Leang et al, 2013). The genetic toolkit was further expanded by the discovery that the lactose-inducible promoter and glucuronidase reporter on the pAH2 vector originally designed for *Clostridium perfringens* functioned in *C. ljungdahlii*. The inducible system was used to drive expression of genes coding for acetone biosynthesis, leading to the successful production of acetone from syngas at a titer of ~0.87 g/L (26.8 kJ/L) (Banerjee et al, 2014), a substantial improvement over 9 mg/L acetone production previously obtained in *C. aceticum* (Schiel-Bengelsdorf & Dürre, 2012).

Butyrate production in *C. ljungdahlii* was recently enhanced using plasmid-based expression of the 8-gene butyrate synthesis pathway from *C. acetobutylicum* resulted in 0.74 g/L for H₂/CO₂ and 1.24 g/L for CO/CO₂ (Ueki et al, 2014). By chromosomal integration of the butyrate pathway and shutting down Pta-dependent acetate, AdhE1-dependent ethanol and Ctf-dependent fatty acid synthesis pathways, the production improved to 1.35 g/L for H₂/CO₂ and

1.48 g/L for CO/CO₂. A notable result of this effort is that chromosomal knockouts predicted to eliminate ethanol and acetate could not abolish accumulation, indicating the existence of additional unidentified genes/pathways for these competing molecules. In addition, for the initial plasmid introduction the acetate/ethanol levels were essentially unchanged from wild type at roughly 7 g/L, where the 0.64 g/L enhancement in butyrate was accompanied by a 5.9 g/L decline in these alternative acetate/ethanol fluxes. This indicates that much needs to be learned about the pathways and compensatory energetics of these organisms to effectively utilize them as biochemical production platforms.

Genetic systems have also recently been developed for the promising acetogen *Moorella thermoacetica*. First, a pre-methylated suicide vector was used to generate a uracil auxotroph by inactivation of the *pyrF* gene, selected for with 5-FOA. Uracil prototrophy was then restored along with integration of a lactate dehydrogenase gene behind the GAPDH promoter, that allowed production of lactate at 0.61 g/L from fructose (Kita et al, 2012). Later, a kanamycin resistance gene was integrated into the same locus, conferring resistance to 300 µg/mL (Iwasaki et al, 2013). A functional antibiotic resistance marker opens up the ability to perform integration into any site within the genome. Earlier this year, Evonik (in collaboration with LanzaTech) announced the production of 2-HIBA, the precursor for Plexiglas, from syngas using an engineered strain (<http://corporate.evonik.com/en/media/focus/Pages/bacteria-syngas.aspx>). Although the details of the engineering are not publically available, the work likely takes advantage of a recently discovered CoA-carbonyl mutase enzyme (Rohwerder & Müller, 2010).

Recently, a series of studies by Tyurin and co-workers at Syngas Biofuels Energy, Inc. reported engineering several *Clostridium* sp. strains for the continuous high level production of several heterologous compounds such as acetone, n-butanol, mevalonate etc. (Kiriukhin &

Tyurin, 2014; Berzin et al, 2013; Kiriukhin & Tyurin, 2013; Berzin et al, 2012). By elimination of key processes in the cell, such as spore formation, acetate and acetaldehyde production, they have reported very high specific productivity of these molecules in continuous syngas or CO₂/H₂ fermentations of recombinant systems. However, strong doubts have been expressed on the validity of these results, and they should be considered with caution (Bengelsdorf et al, 2013).

3.3 Metabolic Engineering in Other Chemolithotrophs

Nitrosomanas europaea is an example of a chemolithoautotroph that utilizes the reducing power of ammonium to reduce CO₂ (Khunjar et al, 2012). The Scott Banta group investigated the engineering of isobutanol into *N. europaea*; however, it exemplified the challenge of working with non-model organisms. Although preliminary genetic tools were developed by the group, and GFP expression and isobutanol production were achieved, the slow growth and excessive energetic penalty for transport and oxidation of ammonia by this bacteria ultimately resulted in this effort being abandoned in favor of an iron-oxidizing chemolithotroph (personal communication). Utilizing the bioreactor concept of an electrochemical reactor to repeatedly reduce an electron carrier, this effort has transitioned to ferric-ferrous reduction at a cathode as basis of autotrophic CO₂ reduction by *Acidithiobacillus ferrooxidans* (Li et al, 2014). Although this work is still quite new with initial work demonstrating introduction of isobutyrate production (Banta & West, 2014) but is expected to continue to more detailed study including autotrophic heptadecane production.

4. Using Metabolic Engineering to Improve / Creating New Chemolithoautotrophs

While genetic engineering in existing autotrophic systems has shown promise and demonstrated a wide range of compounds produced directly from CO₂, the limitations of tools development combined with the likelihood of desirable characteristics for an industrial platform from a non-autotrophic host has motivated the alternative approach of improving or transplanting pathways for carbon and reducing power capture. Besides avoiding homologous regulation, heterologous expression often provides insights into scientific details obscured in the native host.

4.1. Engineering Carbon Capture

4.1.1. Engineering CBB pathway

The limitations of rate and CO₂/O₂ specificity of Rubisco have made this the target for improving primary productivity in agriculture by extensive research efforts that are chronicled in many reviews (Mueller-Cajar & Whitney, 2008). Although these efforts have largely ‘failed’ as stated in a recent evaluation “ ... although the challenge of making a ‘better Rubisco’ has exceeded the grasp of career of many scientists” (Whitney et al, 2011) this must be qualified by understanding that the context of the goal of agricultural productivity is very different from the metabolic engineering goal that is the basis of this review. There is both tremendous natural diversity associated with evolution ranging from aerobic to anaerobic conditions, as well as presence / absence of carbon concentrating mechanisms. Screening assays have improved both k_{cat} , affinity and specificity where tradeoffs have largely precluded improved overall performance. *R. capsulatus* has been used as a host to screen for improved Rubisco by creating a large subunit deletion mutant with impaired photoautotrophic growth, that was then used in complementation studies with a mutated cyanobacterial Rubisco (Smith & Tabita, 2003). The defined conditions of a bioreactor provide a clearly defined objective to exploit the extensive

research on Rubisco. For example, an improved affinity for CO₂ can allow a higher H₂ partial pressure with associated improved gas transport rates. Similarly, there is an opportunity to manipulate wasteful RuBP oxidation through bioreactor operation and control, particularly under the continuous steady-state operation that is pragmatically the only means of achieving economic feasibility for an autotrophic process (Khan et al, 2014). This emphasizes the importance of integration of metabolic engineering efforts with bioprocess design. It seems highly likely that an ‘improved’ Rubisco would be component of an autotrophic production platform.

In addition to Rubisco, other components of the CBB pathway can also be limiting. It was observed that overexpression of SBPase from the cyanobacteria *Synechococcus* and *Arabidopsis thaliana* in tobacco (Miyagawa et al, 2001; Lefebvre et al, 2005) and from *Chlamydomonas reinhardtii* in *Dunaliella bardawil* (Fang et al, 2012) improved photosynthetic CO₂ fixation by 20-100%. A gene found on bacterial cbb operons, *cbbZ*, encodes for phosphoglycolate phosphatase (PGP) that dephosphorylates phosphoglycolate (formed due to the oxidation of RuBP; analogous to photorespiration in plants). This allows glycolate to recycle back to CBB cycle via the D-glycerate pathway while also relieving phosphoglycolate inhibition of triosephosphate isomerase (Berg, 2011). By defining a production platform organism and operating condition, the relevance of such potentially rate-limiting steps within the CBB pathway becomes a much clearer optimization objective.

4.1.2 Engineering Other Carbon Capture Pathways

Engineering the CBB pathway may not be the most logical choice from biotechnological perspective where kinetic and energetic efficiency are critical criteria. The CBB pathway in algae and plants evolved under conditions where the supply of ATP and NADPH were not typically

limiting. In aerobic systems, robustness of CBB enzymes to O₂ appears to be a dominant criterion, where co-evolution of carbon concentrating mechanisms further protected the kinetic shortcomings of this pathway. The five other known carbon fixation pathways have varying degrees of kinetic and energetic efficiencies and O₂ tolerance (Bar-Even et al, 2012; Fast & Papoutsakis, 2012) and computational analysis suggests that superior pathways may be possible based on several quantitative criteria (such as pathway specific activity, ATP and NADPH cost, number and compatibility of the enzymes with existing network etc.) These existing alternative pathways have also been subject to the various natural selective pressures for their respective niche (Berg, 2011), which may require modifications for optimal biotechnological application.

Reconstituting a functional heterologous carbon fixation pathway in a non-autotrophic organism will be the next major step towards creating a synthetic chemolithoautotrophic production platform. The 3-HPA bicycle (from *Chloroflexus aurantiacus*) has been largely installed into *E. coli* by Pam Silver laboratory (Mattozzi et al, 2013). Although each component of the pathway was demonstrated to be functional, the complete pathway failed to achieve autotrophic growth. By introduction of a portion of the HP/HB pathway from *Metallosphaera sedula* into the hyperthermophile *Pyrococcus furiosus*, partial chemolithoautotrophy was conferred to this archaeon to produce 3-hydroxypropionic acid, which represents a major advance toward a process-rationalized host rather than a convenient genetic model (Keller et al, 2013). As an example of altering carbon fluxes through expression of heterologous CO₂ fixation pathways, an “autotrophic bypass” was engineered into yeast by introducing Rubisco and PRK from the chemolithoautotroph *Thiobacillus denitrificans* (Guadalupe-Medina et al, 2013). The associated CO₂ fixation created an electron sink to decrease glycerol production by 90% and increase ethanol production by 10%. These recent

results, combined with the broadened emphasis toward using biological metabolism of CO₂ as a component of reducing GHG emissions is poised to utilize metabolic engineering to create more carbon efficient biochemical production processes.

4.3 Engineering improved H₂-utilization

In contrast to the challenge of transplanting CO₂ fixation, conferring the ability to utilize H₂ is comparatively easy, with the initial successes preceding modern genomics as a result of the transmission of plasmids containing complete functional hydrogen utilization operons (Umeda et al, 1986). This does not mean that hydrogenase function is by any means simple, as it involves metal incorporation and maturation accessory proteins that vary considerably by hydrogenase type and organism (Lubitz et al, 2014). The role of metabolic engineering is parsing through the available hydrogenase diversity, including the focus on biohydrogen production, to identify the appropriate platform candidate. To date, there has not been a transgenic hydrogenase strain that was characterized in terms of improved biomass or product yield.

In chemolithoautotrophic bacteria, oxidation of molecular H₂ provides the reducing equivalents and ATP needed for CO₂-fixation. The mechanism for energy conservation in MBH are generally less efficient than SH. SH can provide the reducing equivalents directly to the redox carriers such as NAD(P) or ferredoxin (Fd) and ATP is generated via proton-motive force. On the other hand, although ATP generation is facilitated more directly by MBH through membrane linked electron transport chain, less efficient reverse electron transport is generally required to generate reducing equivalents (Vignais & Billoud, 2007). A practical example of the relative effect of SH and MBH is *R. eutropha*, where doubling time increased significantly for SH knockout mutants (12 h vs. 3.6 h for wild-type) while only slightly for MBH mutants,

indicating that reductant supply is growth-limiting. Furthermore, transfer of *R. eutropha* SH genes into *Pseudomonas facilis* (containing MBH) reduced its doubling time from 12 to 9 h (Friedrich & Schwartz, 1993). This is likely also reflected in apparent H₂ yield of *R. eutropha* of 6.4 gDW/mol-H₂ (Bongers, 1970) versus only 2.7 for *R. capsulatus* (Siegel & Ollis, 1984). Energetic efficiency is also affected the specific redox carrier used. For example, Fd is more efficient than others because of its lower reduction potential (Bar-Even et al, 2012).

The catalytic properties of hydrogenases are of relatively minor importance compared to carboxylating enzymes where some approach ‘kinetic perfection’ (Berg et al, 2002), being limited by the diffusion rates of H₂ (see Supplementary Table S1 for rates). On the other hand O₂ tolerance is an important factor, [NiFe] MBH are the most O₂ tolerant, followed respectively by [NiFe] SH and [FeFe] hydrogenases, with the catalytic properties generally reflecting an evolution that varies inversely with their oxygen tolerance. Therefore, choices of hydrogenases and cofactors are ultimately dictated by the consideration of whether the chemolithoautotrophic platform is being designed for aerobic or anaerobic conditions. Although [FeFe] hydrogenases are easier to genetically engineer due to relatively small number of maturation factors and are also generally faster and more efficient, both the hydrogenases and the cofactor Fd are very sensitive to O₂. However, if bioreactor operating conditions permit maintaining low dissolved O₂ (e.g. to avoid explosion hazard), there may be added advantage to using [FeFe] versus [NiFe] hydrogenases.

Much of the work related to engineering hydrogenases in heterologous hosts was motivated by the production of hydrogen (Rousset & Liebgott, 2014) rather than the creation of better autotrophic organisms. None-the-less, the tools, and in many cases, the outcomes of these efforts are equally useful for improvement to organisms as autotrophic production platforms. In

fact, at bioreactor operating conditions where partial pressure of H_2 is anticipated to be above 0.5 atm (Bongers, 1970), it is likely that most hydrogenases will act as uptake hydrogenases, because at typical ratios of cellular redox pairs ($NAD(P)H/NAD(P)$ or Fd_{red}/Fd_{ox}) the equilibrium partial pressure for H_2 uptake appears to be quite low (<1000 Pa) (Veit et al, 2008). Therefore, studies seeking to increase the efficiency of H_2 production via improved O_2 tolerance (Rousset & Liebgott, 2014) or channeling substrate and reductant flow (Kontur et al, 2012) may directly translate to enhanced H_2 uptake rates.

4.4 Metabolic Engineering of Alternative Electron Delivery Pathways

Although there is no demonstration of a engineered organism directly accepting electrons from a cathode to produce non-native chemicals, components of this system have been demonstrated separately, namely electron delivery to autotrophic organisms (Cheng et al, 2009; Villano et al, 2010; Nevin et al, 2011), some of which have been genetically modified to produce various compounds (Ueki et al, 2014). However, the volumetric rates of product formation are extremely slow (compared to the performance of the same organisms in other systems), likely due to the very small cell densities (depending on the surface area of the electrode only) and/or the rate of electron delivery to the cells. The mechanism for electron delivery to the cell is not yet fully understood. Genetic engineering has been used to improve the electron transfer in microbial fuel cells (MFC) via overexpression of electron carriers or synthesis of novel electron carriers (Sydow et al, 2014), an approach which may be applicable to microbial electrosynthesis. A summary of the status, challenges and potential for improving the electrosynthesis platform is available (Rabaey & Rozendal, 2010). The choice and tradeoffs between aerobic versus anaerobic is reiterated in terms of productivity and yield. The field is very new, however, with

much to be learned about the potential of microbial electrosynthesis for the autotrophic production of fuels and chemicals.

5 Metabolic Engineering Toolbox for Existing Autotrophs

For those trained in molecular techniques using only facile organisms such as *E. coli*, transitioning to work with most autotrophs requires patience, persistence and attention to detail as there are fewer tools and methods are less forgiving and reproducible. As details were largely omitted in the review of metabolic engineering advances (Section 3), the critical importance of the metabolic engineering toolbox warrants some additional discussion.

5.1 Genetic Transformation

Transformation by mating and counter-selection against the conjugal donor is still a dominant method utilized for transformation of both *Ralstonia* and *Rhodobacter*. Relative to heat shock or electroporation in *E. coli*, this method increases the time required for a given transformation by five or more. Transformation procedures also often require a period of developing mastery, which adds additional delay in achieving results that would otherwise seem rather mundane. Although recent successes provide methods to achieve metabolic engineering in chemolithoautotrophs, these tools are still under active development, where new methods are slow to be adopted. It is therefore useful to compile some of the recent advances for manipulating chemolithoautotrophic organisms – particularly for acetogens that have only very recently yielded to reliable transformation.

5.1.1 Transformation of Aerobic Chemolithoautotrophs

Relatively routine techniques are available for transformation of *Ralstonia* and *Rhodobacter*. Both species are amenable to conjugal plasmid transformation, electroporation

and homologous chromosomal transformation. One of the seminal developments in engineering a wide range of chemolithoautotrophs was the establishment of intergeneric conjugation from specialized *E. coli* donor strains in a bi- or tri-parental mating (Ditta et al, 1985). These systems take advantage of broad host range vectors that feature an origin of transfer (*oriT*), which can be mobilized from *E. coli* strains harboring the RK2 mobilization machinery, either integrated into the chromosome (i.e. *E. coli* S17-1, diparental mating) or harbored on the pRK2013 plasmid, (triparental mating). The recipient strain preferably exhibits a drug resistance to counter-select the *E. coli* host, such as rifampicin resistance for *R. capsulatus* SB1003 or gentamicin for *R. eutropha*. This adds considerable additional constraints on vector design. Homologous recombination in gram-negative bacteria has been available for over 20 years utilizing the efficient *sacB* counter-selection (Quandt & Hynes, 1993). These methods provide both for deletion, gene modification and insertion chromosomal insertion (Jaschke et al, 2011) as noted for the most recent high-level expression systems based on the T7 polymerase vectors described in the next section.

One of the ongoing challenges concerning genetic engineering of chemolithoautotrophs is the recalcitrance of many host strains to electroporation of foreign DNA, which if successful would circumvent the need for time-consuming conjugation protocols. Improved vectors that utilize electroporation continue to be developed for *R. eutropha* (Solaiman et al, 2010). Restriction systems are believed to be responsible for recalcitrance by rapidly degrading foreign DNA. Recently, the *rshI* endonuclease gene of *R. sphaeroides* was knocked out, which subsequently made the strain amenable to electroporation (Jun et al, 2013), a method which is described in more detail below for acetogens.

5.1.2 Genetic Transformation of Acetogens

The primary limiting factor for acetogen metabolic engineering is the lack of robust genetic tools. Transformation in these organisms is frequently hindered by extensive restriction modification systems (Leang et al, 2013; Straub et al, 2014; Kita et al, 2012; Tsukahara et al, 2014). There are a number of strategies to combat this. In a strategy named Plasmid Artificial Modification Systems (PAMS), the transformation vector is purified from an *E. coli* host heterologously expressing the native methyltransferases, increasing transformation efficiency (Yasui et al, 2009; Suzuki & Yasui, 2011). The methylation domains of these enzymes are readily identified bioinformatically, thus the strategy can be implemented based solely on the sequenced genome of the target organism. Alternatively, the specific methylation pattern, or ‘methylome’ of the target organism can be established via Single Molecule Real Time (SMRT) sequencing, and then avoiding these sites in the design of transformation vectors (Murray et al, 2012; Flusberg et al, 2010). Even when DNA digestion has been abated, the plasmid must either successfully replicate in the host, or integrate into the genome. Toward the first aim, a modular *Clostridia* plasmid system, with multiple origins of replication and antibiotic resistance markers has been generated by the Minton lab (Heap et al, 2009) that facilitates rapid screening of potential vectors. Integration via the universal homologous recombination mechanism occurs with low efficiency in *Clostridia* (Al-Hinai et al, 2012), hypothesized to be partially due to the absence of the resolvase enzyme in many of these species (Rocha et al, 2005). This scheme as well as generally implementing knock-outs and knock-ins is limited by the availability and effectiveness of negatively selectable markers. A summary of the breadth of transformation methods currently being used for chemolithotrophs is provided as Supplemental Table S2.

In extensive work with homologous recombination in *C. ljundalhi*, the Lovley lab has noted an inability to get any of the three negative selections to work (e.g. pyrF, galK, mazF)

(Ueki et al, 2014) thereby preventing marker reuse of the limited selectable markers using the Cre-LOX excision procedures (Marx & Lidstrom, 2002). Despite the noted limitations, transformation systems now exist for the three model acetogens: *Moorella thermoacetica*, *Clostridium ljungdahlii*, and *Acetobacter woodii*, facilitating both metabolic engineering and studies to expand and improve the genetic systems.

5.2 Plasmids, Promoters and Vectors of Particular Utility for Chemolithoautotrophs

The most heavily utilized broad host range plasmids for engineering of gram-negative bacteria are the *IncP*-based pRK plasmids (Ditta et al, 1985) and the pBBR1MCS vectors (Kovach et al, 1995). Most *IncP*-based vectors rely on light-sensitive tetracycline selection, although pRKD418 employs a tetrahydrofolate reductase gene for trimethoprim resistance (Mather et al, 1995). The largest drawback of most plasmid-based expression in a production environment is the need for antibiotic supplementation for constant selection pressure. Recently, the narrow-host range plasmid, pIND4, was maintained in *R. sphaeroides* without selection pressure (Ind et al, 2009). This vector incorporates several attractive features, including an IPTG-inducible *lacIq* cassette and the repressible hybrid $P_{A1/A0/A3}$ promoter and stable MG160 replicon (Inui et al, 2003). This plasmid has also been used to build a BioBricks® toolbox for *R. sphaeroides* with expression levels (of DsRed fluorescent protein) comparable to an *E. coli* P_{lac} system (Tikh et al, 2014). Recognizing large plasmid performance differences in related strains, we note in our work leading to triterpene expression (Khan et al, 2015), we observed that pIND4 displayed poor stability in *R. capsulatus*.

A *hoxA*-mediated ‘plasmid addiction’ system was recently developed specifically for *R. eutropha* lithoautotrophic growth (Lütte et al, 2012). The chromosomal gene for the HoxA hydrogenase operon transcriptional regulator is essential for utilization of hydrogen. The

transgenes of interest are provided on the pLO11 plasmid which complements the *hoxA*-
mutation and renders the plasmid indispensable, thereby eliminating the need for a selectable
marker under chemolithoautotrophic conditions. A similar obligatory plasmid system based on
the KDPG aldolase (*eda*) system (Voss & Steinbüchel, 2006) is not applicable to
chemolithoautotrophic growth because the enzyme, KDPG aldolase, is part of the Entner-
Doudoroff pathway which is only active during heterotrophic growth – although this
demonstrates the generality of the approach. The mobilization efficiency and stability of four
different plasmid systems (based on incompatibility groups *IncP*, *IncQ*, *IncW* and *pBBR*) capable
of replicating in *R. eutropha* were recently assessed (Gruber et al, 2014). They showed that RP4
and RSF1010 based mobilization sequences are respectively about 50000 and 5000 times more
efficient than pBBR1 mobilization sequence. Notably, the inclusion of a 2.3 kb *par* region of
RP4 onto other plasmids nearly completely prevented plasmid loss for up to 96 hours in the
absence of antibiotic selection. This *par* region apparently has broad utility for plasmid retention
as it previously conferred a high degree of stability to *E. coli* plasmids in absence of antibiotic
selection for up to 200 generations (Gerlitz et al, 1990). It is now believed that *par* region
encodes for proteins that stabilize plasmid retention based on site-specific recombination that can
resolve plasmid multimers. A similar new high-stability plasmid for *R. eutropha* has been
generated by cloning *oriV28* and *parABS28* regions from pMOL plasmid of *Ralstonia*
metallidurans into an *E. coli* cloning vector which also permitted transformation by
electroporation (Sato et al, 2013). The relative copy number for the four plasmids having
common *mob* and *par* regions was assessed and the pBBR based vector was found to be about 2
and 4 times higher than those of RSF1010 and pSa respectively.

Early promoter engineering efforts in *R. eutropha* developed native promoters to drive expression of acetoin, polyhydroxyalkanoate, and pyruvate biosynthetic genes (Delamarre & Batt, 2006). While most of these fusions with an artificial polyhydroxyalkanoate operon succeeded in producing PHA, the overall yields were less than 10%, reflecting an innate limitation of this method. Orthogonal promoters for *R. eutropha* gene expression under chemolithoautotrophic conditions have the potential to bypass the host's innate genetic regulation machinery, where many of the transcription initiation signals were adapted from *E. coli*. In comparison of heterologous promoters it was found that the T5 phage-derived promoter P_{j5} enhanced expression in *R. eutropha* by 4 to 5-fold relative to P_{tac} and P_{lac} (Gruber et al, 2014). The P_{cbbL} promoter which is induced under autotrophic conditions (Kusian et al, 1995), was effective in increasing flux to the amino acid oligomer cyanophycin (Lütte et al, 2012).

The T7 RNA polymerase (T7 RNAP) system that is used to drive high level expression in *E. coli* vectors has been adopted to *R. eutropha* and *R. capsulatus*, although most metabolic engineering efforts may not require ultra-high protein expression. In *R. eutropha* the T7 RNAP gene was placed under the control of *phaP* promoter of the PHA biopolymer operon and the *oph* gene for organophosphohydrolase (OPH) target protein under the control of T7 promoter (Barnard et al, 2004). This approach expressed OPH at 6% to 18% of total soluble protein. A similar T7 RNAP expression system has been developed for *R. capsulatus* resulting in upwards of 80 mg/L of yellow fluorescent protein (Katzke et al, 2010).

The anhydrotetracycline-inducible promoter system based on high-affinity binding to the tetracycline repressor/operator (TetR/O; Lutz and Bujard 1997), has recently been adapted for tunable gene expression in *R. eutropha* by screening for variable expression of TetR (Li & Liao, 2014). An order of magnitude dynamic range of tunable expression was achieved. Although this

is two orders of magnitude smaller range than the analogous *E. coli* system, it was sufficient to alleviate toxicity associated with an intermediate accumulation in the isobutanol metabolic engineering in *R. eutropha*. These recent advances in genetic toolbox development should accelerate progress in metabolic engineering which is just beginning to tap into the potential of chemolithoautotrophic organism as illustrated in the next section.

6. Status of productivities and scale-up considerations for metabolic engineering

Design of metabolic engineering strategies are affected by scale-up considerations, where there are additional challenges for autotrophs as compared to traditional organisms. Downstream factors such as product separation, genetic stability, induction of gene expression and growth/non-growth-associated product formation, all affect the choices for metabolic engineering approach. The choice of platform organism is also quite critical and is coupled with the energetic efficiency (growth yield and maintenance coefficients) (Khan et al, 2014), aforementioned scale-up considerations and various other factors such as product toxicity. Many of these issues relate specifically to the target product and are beyond the scope of this review. In this section we will briefly discuss the general performance characteristics of autotrophs during bioreactor culturing and the relevance to metabolic engineering.

Table 2 contains a compilation of the productivities reported in the literature for genetically engineered autotrophic hosts for the conditions noted (substrate and operational mode) and includes production of native metabolites by the same hosts (non-engineered systems) for comparison. Productivities of model non-autotrophic systems are also included to provide a benchmark for industrial application. Specific productivity [g/gDW-h] represents biosynthetic capacity of an organism, while volumetric productivity [g/L-h] represents the overall

performance of the organism and culture conditions. Titer levels [g/L] indicate the ease of downstream processing and starts to become industrially relevant at >50 g/L (Sun & Alper, 2014). All of these parameters affect the capital and operating cost of a process and are therefore provide a comparative basis for different hosts and products.

Production of native metabolites by autotrophs represent the high capacity of these organisms for producing biochemicals from CO₂. The volumetric productivity levels reflect not only the biosynthetic capacity, but the permissiveness of the organism to be cultured at high density. The production of ethanol by *S. cerevisiae* (12.7 g/L-h, >100 g/L EtOH) (Bayrock & Ingledew, 2005) serves as a useful productivity benchmark for industrial applicability. Heterotrophic cultures of *R. eutropha* have been shown to routinely exceed 200 g/L (with 70-80% PHB production) (Ryu et al, 1997), while a high k_{La} (~3000/h) autotrophic culture has been shown to reach biomass densities close to 100 g/L (~70% PHB) (Tanaka et al, 1995). Therefore, given sufficient metabolic flux, getting even higher autotrophic productivity would largely be a challenge of reactor design and optimization of gas composition.

It is noteworthy that the highly productive PHB (Choi et al, 1998) and ethanol (Dumsday et al, 1999) could be largely replicated in *E. coli* for which the most advanced metabolic engineering tools exist. By comparison, productivities of nonnative compounds (such as isopropanol, botryococcene or butyrate) in engineered autotrophs are generally two to three orders of magnitude lower than native compounds (such as ethanol, acetate or PHB; see also Figure 6). This situation is also generally not improved for the engineered strains even for heterotrophic conditions, suggesting that the current ability to manipulate fluxes in autotrophs is still tapping into only a fraction of the potential of these organisms. This would be expected, in part because autotrophs have not been optimized for biotechnological application as has been

done with *E. coli* or yeast. There is also insufficient understanding of key areas of chemoautotrophic metabolism, such as the sensitive coupling of energy metabolism and CO₂ fixation. Developing metabolic engineering strategies that integrate target product formation tightly with existing cellular machinery for energy generation and regulation, while eliminating non-essential competing pathways are needed to increase product flux.

Figure 6 is a visual representation of engineered and non-engineered autotrophic productivities on a common axis. The time courses (Figure 6A) represent ‘best case’ data presented in the literature with the case of PHB (Tanaka et al, 1995) outpacing other chemolithoautotrophs by a large margin (and comparable to the best results of even *E. coli*). Acetate and ethanol production by *M. thermoacetica* and *C. ljungdahlii* are also quite impressive (Hu et al, 2013) as compared to engineered metabolites. In contrast, the best case for metabolically engineered butyrate production is still quite low. Figure 6B presents a final reminder of the fundamental differences between aerobic and anaerobic chemolithoautotrophic organisms as production platforms. While the theoretical maximum energy efficiency of acetate and ethanol production are the highest for acetogens (using Wood-Ljungdahl pathway), they are severely limited in the production of higher energy molecules such as butanol, due to ATP limitation (Fast & Papoutsakis, 2012). Energy efficiency of aerobic systems are lower in general but have much improved capacity for producing high-energy molecule since they are not limited in ATP production. Actual energy efficiency of acetogens for acetate and ethanol production are about 70-80% of this theoretical maximum (Phillips et al, 1994). Unfortunately, the energy efficiencies of engineered systems are not reported to make a useful comparison. In contrast, carbon efficiency (fraction of CO₂ fixed in product) can be calculated relatively easily from reported values of biomass and product densities and ranges from 5-25% of theoretical

maximum. At this time the capability of metabolic engineering in autotrophs is so far from realizing its yield potential that using such arguments for excluding one chemolithoautotrophic platform over the other is not warranted.

7. Conclusion

The status of metabolic engineering of chemolithoautotrophs is very dependent on the specific organism, as the genetic tools available for different chemoautotrophs range from reasonably well-developed to almost non-existent. The results of metabolic engineering efforts can be generally characterized as falling far short of tapping into the native fluxes of these organisms by several orders of magnitude. On one hand, the genetic tools developed in model organisms have provided for comparatively rapid advances of methods. The unique characteristics of chemolithoautotrophic behavior has provided opportunities such as the Hox-addiction plasmid, but have experienced limited use since the majority of metabolic engineering testing is carried out under convenient heterotrophic conditions. At this early stage, metabolic engineering strategies are understandably driven by proof-of-concept studies. However, the next generation of strain development must be designed with autotrophic operating conditions and scale-up in mind with performance testing under autotrophic conditions. Progress in autotrophic metabolic engineering is currently limited by the availability of robust genetic tools. Whereas full pathways can be assembled, transformed into *E. coli* and tested in the time-scale of days, comparable efforts routinely require weeks for even the most tractable autotrophic hosts. Methods for generating multiple gene knockouts are well-established in *E. coli* (Datsenko & Wanner, 2000), but the ability to generate a double-crossover mutant in *Clostridium acetobutylicum* was elusive for twenty years, and remains so for *C. ljungdahlii*. While laboratory

815 strains of *E. coli* have been continually improved over the last forty years to make them more
816 genetically tractable, with features such restriction modification systems and recombinase
817 activity deleted, engineering efforts with most autotrophs use strains with no such adaptations.
818 The recent development of important genetic tools, including isothermal assembly (Gibson et al,
819 2009) and CRISPR/Cas9 gene editing techniques, should accelerate the development of
820 “designer” chemoautotrophs. Interestingly, CRISPR/Cas9 was engineered to cleave targeted
821 RNAs in *P. furiosus* by Hale and co-workers (Hale et al, 2012).

822 If the full capability of autotrophs is to be harnessed, more work is needed to reduce the
823 timescales associated with genetic manipulation. This will facilitate not only the rapid
824 introduction and screening of heterologous pathways for chemical and fuels production, but also
825 further basic genetic studies that are needed to more fully understand their physiology and
826 biochemistry, which are of paramount importance when designing a production strain. The
827 problem is that the development of even basic genetic tools can take many years, and is a largely
828 trial-and-error process, incompatible with the demands placed on researchers by granting
829 agencies and thesis committees for rapid results. This leads to a self-perpetuating tendency for
830 those in the metabolic engineering and synthetic biology communities to focus instead on the
831 model organisms.

832 We believe that to fix this dilemma, an effort should be made to gain a more fundamental
833 understanding of the process of bacterial transformation, so that each attempt to develop tools for
834 a novel species can be rationally guided, rather than an isolated exercise in statistical
835 experimental design. Fortunately, such efforts are underway. The ability to rapidly determine the
836 methylation pattern of any microbial species via SMRT sequencing, for example, has the
837 potential to significantly increase transformation efficiencies in numerous microbes (Murray et

al, 2012). Once the barrier to entry for genetic manipulation of autotrophs has been eliminated, we believe this will bring these organisms into the focus of mainstream metabolic engineering and synthetic biology.

The alternative route to developing chemoautotrophic production platform from existing model organisms, will require a much deeper understanding of the metabolic fluxes and regulatory networks of autotrophic metabolism. Computational models for some well-studied chemoautotrophs are available (Nagarajan et al, 2013; Park et al, 2011; Golomysova et al, 2010), that provide useful insights (Braakman & Smith, 2014) and should allow the quantification of specific hypothetical changes. While transplanting H₂-utilization to more facile model organisms is straight-forward, the goal of conferring either CO₂-fixation or enhanced direct electron uptake has not yet been reported.

Creation of an ‘optimal’ chemolithoautotrophic production platform should also include considerations for bioreactor operating conditions where the enzymes chosen can reflect achieving appropriate controlled gas transport rates. Oxygen concentrations are of particular importance in case of aerobes because dissolved oxygen appears to be one of the dominant evolutionary drivers for the key chemolithoautotrophic enzymes. The efficiency advantage of anaerobes appears to come with an inherent constraint on energy generation, the impact of which is also dependent upon the operational strategy where continuous high density culture provides the highest productivity at minimum cost. Chemolithoautotrophic systems have the potential to utilize an inexpensive carbon source as well as interface to thermochemical deconstruction of renewable biomass (via synthesis gas for example) with associated environmental benefits. This opportunity will continue to drive commercialization efforts and overcome the scientific and technical challenges presented by these useful carbon-fixation pathways.

861

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870

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Figure captions

Figure 1: Conceptual schematic of the delivery of electrons into metabolism for the reductive capture of CO₂ - analogy between direct photosynthetic growth and indirect photosynthetic growth based on the feeding of electrons as reducing power to reduce CO₂. XH denotes any electron carrier molecule, where X is recycled.

Figure 2: An examination of the CO₂ reduction reactions from the perspective of the mode of transport of the reducing power to either the reactor, or to the inside of the cells as a major determinant of the application of these for biotechnological purposes. Light cannot penetrate dense cultures (Beer-Lambert Law), where gas transfer rates (GTR) face limitations of solubility in the liquid phase (Henry's Law). Electrons are particularly challenging to deliver (Ohm's Law) either to a reactor electrode or into the cell although this can be facilitated by the reduction of a 'carrier' (X-H).

Figure 3: A summary of autotrophic growth modes with the common theme of reducing CO₂ to biomass (carbohydrate) using various forms of reducing power. These different autotrophic growth modes are often associated with other classification names that are listed. These common name classifications are useful, but also lead to problems typical of generalizations.

Figure 4: Representation of various CO₂-fixation pathways in relation to each other: CBB (black $\equiv$), Wood-Ljungdahl (red $\equiv$), Reverse TCA (rTCA; yellow - -), 3-hydroxypropionate (3-HPA; blue —), 3-hydroxypropionate/4-hydroxybutyrate (HP/HB; green $\cdots$), dicarboxylate/4-hydroxybutyrate (DC/HB; purple — $\square$ $\square$). The carboxylating enzymes are indicated by numbered circles: 1, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco); 2, bifunctional carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS); 3, formate dehydrogenase; 4, 2-oxoglutarate synthase (Fd-dependent); 5, isocitrate dehydrogenase; 6, pyruvate synthase; 7, phosphoenolpyruvate carboxylase; 8, bifunctional acetyl-CoA/propionyl-CoA carboxylase.

Figure 5: Schematic representation of carbon and energy metabolism, regulation and gene expression in a typical aerobic chemoautotroph. Both membrane bound (MBH) and soluble

(SH) hydrogenases generate reducing equivalents. MBH also channels electrons to the membrane-bound Electron Transport Chain for the production of ATP. SH facilitates ATP production via the NADH oxidoreductase (Complex I). The energy and reducing power are used by the carbon fixation pathway (typically CBB). The Regulatory Network is a complex interaction among external substrates as well as internal metabolites and cofactors, which eventually controls the transcription of the hydrogenase operons (*hox*, *hup*, *hyp* etc.) and CBB operons. There is direct regulation of these operons as well (e.g. H₂).

Figure 6: (A) Maximum reported productivities in grams of carbon fixed in products per liter (gC-fixed/L) in autotrophic systems. Native pathways: (I) PHB by *R. eutropha*, (Tanaka et al, 1995); (II) acetate by *M. thermoacetica*, (Hu et al, 2013); (III) ethanol by *C. ljungdahlii*, (Phillips et al, 1993). Engineered pathways: (IV) botryococcene by *R. capsulatus*, (Khan et al., 2015), (V) methyl ketone by *R. eutropha* (Müller et al, 2013); (VI) butyrate by *C. ljungdahlii*, (Ueki et al, 2014). All are autotrophic timecourses adapted from literature reports presented in Table 3. (B) Maximum theoretical yield (using values reported by Fast & Papoutsakis, 2012) on H₂ of acetate, ethanol, PHB and butanol using aerobic CBB and anaerobic Wood-Ljungdahl (acetogen) pathways.

Figure 1
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Figure 2
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Figure 3
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Figure 5
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Figure 6
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Table 1: List of reviews referred in this article

(Thauer et al, 2008)	Methanogenic archaea: ecologically relevant differences in energy conservation
(Demirel & Scherer, 2008)	The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: a review
(Badger & Bek, 2008)	Multiple Rubisco forms in proteobacteria: their functional significance in relation to CO ₂ acquisition by the CBB cycle
(Shively et al, 1998)	Something from almost nothing: Carbon dioxide fixation in chemoautotrophs occurrence in bacteria
(Berg, 2011)	Ecological aspects of the distribution of different autotrophic CO ₂ fixation pathways
(Bar-Even et al, 2012)	A survey of carbon fixation pathways through a quantitative lens
(Mueller-Cajar & Whitney, 2008)	Directing the evolution of Rubisco and Rubisco activase: first impressions of a new tool for photosynthesis research
(Lovley & Nevin, 2013)	Electrobiocommodities: powering microbial production of fuels and commodity chemicals from carbon dioxide with electricity
(Hawkins et al, 2013)	Biological conversion of carbon dioxide and hydrogen into liquid fuels and industrial chemicals
(Schwartz et al, 2013)	H ₂ -metabolizing prokaryotes
(Lubitz et al, 2014)	Hydrogenases
(Vignais & Billoud, 2007)	Occurrence, classification, and biological function of hydrogenases: an overview
(Rousset & Liebgott, 2014)	Engineering Hydrogenases for H ₂ Production: Bolts and Goals
(Böck et al, 2006)	Maturation of hydrogenases
(Bengelsdorf et al, 2013)	Bacterial synthesis gas (syngas) fermentation
(Schiel-Bengelsdorf & Dürre, 2012)	Pathway engineering and synthetic biology using acetogens
(Cho et al, 2014)	Metabolic engineering of clostridia for the production of chemicals
(Rabaey & Rozendal, 2010)	Microbial electrosynthesis - revisiting the electrical route for microbial production
(Sydow et al, 2014)	Electroactive bacteria--molecular mechanisms and genetic tools

1 **Table 2. Comparison of productivities of different compounds in various autotrophic and**
 2 **non-autotrophic hosts. Productivities have been calculated using data reported in the cited**
 3 **works.**

Substrate	Organism	Compound	Mode of operation	P_v , avg (max) [g/L-h]	P_s , avg (max) [g/gDW-h]	Density, biomass (product) [g/L]	Reference
Autotrophic hosts – engineered systems							
H ₂ /O ₂ /CO ₂	<i>R. eutropha</i>	Methyl ketones	Batch	0.0017 (0.0036)	0.0019 (0.0046)	1*	(Müller et al, 2013)
H ₂ /O ₂ /CO ₂	<i>R. eutropha</i>	Cyanophycin	Fed-batch	0.0097	0.00041	25.4*	(Lütte et al, 2012)
H ₂ /O ₂ /CO ₂	<i>R. capsulatus</i>	Botryococcene (C ₃₀ H ₅₀)	Continuous	0.001 (0.0023)	0.0003 (0.0006)	7 (0.11)	(Khan et al, 2015)
CO/CO ₂	<i>C. ljungdahlii</i>	Butyrate	Batch	0.011	0.028	0.8*	(Ueki et al, 2014)
H ₂ /CO ₂	<i>A. woodii</i>	Acetate	Fed-batch	0.56 (1.2)	0.28 (0.9)	2 (51)	(Straub et al, 2014)
Glucose	<i>R. eutropha</i>	OPH	Fed-batch	0.24 (0.31)	0.0026 (0.0034)	113	(Barnard et al, 2004)
Fructose	<i>R. eutropha</i>	Isopropanol	Batch	0.076	0.093	1.25*	(Grousseau et al, 2014)
Yeast extract	<i>R. sphaeroides</i>	Valencene (C ₁₅ H ₂₄)	Batch	0.0049	-	-	(Beekwilder et al, 2014)
Fructose/Yeast extract	<i>C. ljungdahlii</i>	Acetone	Batch	0.016	0.019	0.885	(Banerjee et al, 2014)
Autotrophic hosts – non-engineered systems							
H ₂ /O ₂ /CO ₂	<i>R. eutropha</i>	PHB	Fed-batch	1.55 (5)	0.05 (0.18)	91.3 (61.9)	(Tanaka et al, 1995)
CO/CO ₂	<i>M. thermoacetica</i>	Acetate	Fed-batch	0.55	0.14 (0.4)	3.92	(Hu et al, 2013)
H ₂ /CO/CO ₂	<i>C. ljungdahlii</i>	Ethanol	Continuous	0.322	0.184	4	(Phillips et al, 1993)

Glucose	<i>R. eutropha</i>	PHB	Fed-batch	3.14 (9.6)	0.06 (0.14)	281 ()	(Ryu et al, 1997)
Sucrose	<i>Alcaligenes latus</i>	PHB	Fed-batch	4.94 (5.13)	0.38 (0.9)	111.7 ()	(Wang & Lee, 1997)
Model, non-autotrophic systems							
Glucose (LB)	<i>E. coli</i> (engineered)	PHB	Fed-batch	4.63	0.088	194.1 (141.6)	(Choi et al, 1998)
Glucose (LB)	<i>E. coli</i> (engineered)	Ethanol	Continuous	0.57	0.18	3.2*	(Dumsday et al, 1999)
Glucose	<i>S. cerevisiae</i> (non-engineered)	Ethanol	Continuous	12.7	1.41	9 (106)	(Bayrock & Ingledew, 2005)

P_V = volumetric productivity, P_S = specific productivity.

Non-engineered systems indicate production of native compounds by hosts that have not been otherwise genetically engineered. Engineered systems indicate production of heterologous compounds by hosts that have been genetically engineered. Non-autotrophic, engineered systems indicate production of heterologous compounds by non-autotrophic hosts that have been genetically engineered.

* Estimated using a conversion factor of 0.5 g/L/OD