Metabolic engineering for the microbial production of 1,3-propanediol
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Improvements in the biological production of 1,3-propanediol, a key component of an emerging polymer business, have been realized. Utilizing genes from natural strains that produce 1,3-propanediol from glycerol, metabolic engineering has enabled the development of a recombinant strain that utilizes the lower cost feedstock D-glucose. This accomplishment bodes well for future metabolic engineering efforts and, ultimately, for increased societal benefit obtained through the production of chemicals from renewable resources.

Over the past few decades, there has been growing interest in 1,3-propanediol as an industrial chemical. It has long been known that a polymer prepared from terephthalic acid and 1,3-propanediol has highly desired properties for large volume markets (e.g. fiber for use in apparel and carpet applications). The belief that improved chemistries, including both traditional petrochemical and biological routes, could enable the production of 1,3-propanediol with the economy required by these competitive markets has fueled large efforts in this arena [6,7]. Biological efforts include fermentation optimization of the natural glycerol-utilizing process and an ambitious metabolic engineering effort, directed towards a more economical process, to build a single microorganism capable of utilizing the lower cost feedstock D-glucose. As aspects of the natural fermentation process from glycerol have been reviewed recently [5**], the subject of this review is the metabolic engineering of the novel D-glucose pathway and recent developments relating to that effort.

Introduction
1,3-Propanediol is produced in nature by the fermentation of glycerol. Since it was first observed in the 19th century [1], numerous aspects of this niche metabolic pathway have served as focal points in the areas of chemistry, enzymology, genetics, microbial physiology and biochemical engineering [2,3,4**,5**]. For a large number of bacteria, including Citrobacter, Clostridium, Enterobacter, Klebsiella and Lactobacillus species, a consequence of anaerobic growth on glycerol is the generation of excess reducing equivalents in the form of reduced nicotinamide adenine dinucleotide (NADH) (Figure 1). Regeneration of oxidized nicotinamide adenine dinucleotide (NAD⁺) necessitates the formation of a by-product to serve as an electron sink. In a seemingly unlikely solution to this redox requirement, glycerol undergoes a difficult chemical rearrangement to 3-hydroxypropionaldehyde (3-HPA) followed by an NADH-dependent reduction to 1,3-propanediol. Specialized enzymatic pathways for both the oxidation (associated with carbon incorporation into cell mass) and reduction of glycerol are segregated at the DNA level and are employed only in the rare event that glycerol is present but alternate carbon sources (e.g. D-glucose) are not. To satisfy a conditional requirement of microbial growth, a complex but robust process for the anaerobic production of 1,3-propanediol from glycerol has evolved.

Abbreviations
3-HPA 3-hydroxypropionaldehyde
PEP phosphoenolpyruvate
PTS phosphotransferase system

Engineering 1,3-propanediol pathways
The role of coenzyme B₁₂ reactions
Glycerol dehydratase (EC 4.2.1.30) is a coenzyme B₁₂-dependent enzyme composed of three polypeptides that catalyzes the free radical mediated conversion of glycerol to 3-HPA. As a consequence of the normal catalytic cycle with glycerol, the coenzyme B₁₂ is occasionally rendered inactive (B₁₂-inact). The B₁₂-inact remains tightly bound to the dehydratase and catalysis ceases. An auxiliary enzyme, glycerol dehydratase reactivase, facilitates the dissociation of the B₁₂-inact to form glycerol dehydratase free of cofactor (apoenzyme). The resultant apoenzyme rebinds coenzyme B₁₂ and glycerol conversion to 3-HPA resumes. In yet another coenzyme B₁₂ related activity, coenzyme B₁₂ is regenerated from B₁₂-inact. Clearly, there are potential issues that confound the use of glycerol dehydratase as a foundation upon which to build a metabolically engineered strain for 1,3-propanediol production. The ability to dissect natural strains is essential for engineering improved strains. Fortunately, the multiple genes that encode these and other functionally related proteins...
activities are physically linked, being tightly clustered in what is known as the dha regulon. Similarly, diol (1,2-propanediol) dehydratase (EC 4.2.1.28), an isoenzyme of glycerol dehydratase with overlapping substrate specificity, is also associated with a gene cluster (the pdu operon) that also comprises genes encoding reactivase and B\textsubscript{12}\,-regenerating activities.

Identification and engineering of new dehydratases

New, naturally occurring bacterial strains, including novel species, that are able to produce 1,3-propanediol continue to be identified: for example, lactic acid bacteria, species of Clostridia and a thermophile (Caloramator viterbensis) [8–10]. Historically identified by physiological tests performed on pure cultures, the association between anaerobic 1,3-propanediol (or 3-HPA) production and conserved regions of DNA has allowed molecular biology screening techniques to be developed for the identification of these organisms. Ultimately, for the purposes of metabolic engineering, dissection of the genetic organization [11,12] and biochemical characterization of relevant enzymatic steps [13\textsuperscript{*},14–16] must follow strain identification. The combined effort provides additional tools to identify limiting steps in relevant pathways and increases the knowledge base from which solutions arise once limiting steps are identified.

The generation of environmental DNA libraries, by-passing the traditional isolation of pure cultures, allows the evaluation of specific aspects of microbial diversity. Such libraries were obtained for dehydratase (mixtures of glycerol and diol dehydratase) genes and screened using dehydratase-specific DNA probes and activity assays (growth on glycerol) in a specially constructed Escherichia coli strain [17\textsuperscript{*}]. The objective of this work was the isolation of dehydratase variants with higher resistance to inactivation by glycerol and inhibition by 1,3-propanediol. Leading to a particularly interesting result, a partial diol dehydratase gene set was isolated and the incomplete portion was complemented with the corresponding DNA from Salmonella enterica. The fusion protein that resulted had greater resistance to inhibition by 1,3-propanediol than control dehydratases. This suggests a potential role for protein engineering in improving the performance of particular enzymes that may limit the overall performance of a pathway. Independently, an effort to generate mutant libraries of dehydratase genes that were screened using high-throughput techniques has provided significantly improved dehydratase enzymes (X-S Tang, personal communication). Recent crystallographic studies of dehydratase enzymes might also aid protein engineering efforts [18–20].

The reactivase system

The glycerol dehydratase reactivase reactions from Klebsiella pneumoniae and Citrobacter freundii have been characterized biochemically [14,15]. Like the previously characterized diol dehydratase reactivase system, the reaction is ATP-dependent and the reactivase has been proposed to serve as a molecular chaperone. Recent crystallographic evidence revealed structural similarity to known molecular chaperones, supporting this hypothesis [21]. The pdu\text{O} gene, obtained from the pdu operon of S. enterica, has been shown to encode an ATP:chromalmin adenosyltransferase that is involved in B\textsubscript{12} regeneration [13\textsuperscript{*}]. By analogy, the previously unknown function of genes present in the dha regulon was proposed to be B\textsubscript{12} regeneration. In a related advance, a protein system that is likely to be involved in B\textsubscript{12} regeneration, upstream of the ATP:chromalmin adenosyltransferase reaction, has been identified [22\textsuperscript{*}].

Fermentation studies

Traditional glycerol-fed (and glycerol/D-glucose co-fed) fermentation studies incorporating balance of specific substrate consumption and by-product formation rates continue to provide practical information, particularly when coupled with enzyme assays [23–30]. A particularly nice example is exemplified by Saint-Amans et al. [27]. Glycerol dehydratase obtained during an examination of Clostridium butyricum glycerol fermentations was observed to have striking similarities to a B\textsubscript{12}-independent, oxygen-sensitive diol dehydratase previously isolated. Detailed biochemical evaluation confirmed its B\textsubscript{12}-independence.
and full molecular characterization will allow its evaluation in anaerobic processes for 1,3-propanediol production [31**].

**Production of 1,3-propanediol from D-glucose**

In considering the merit of D-glucose versus glycerol as feedstock for the production of 1,3-propanediol, it is immediately apparent that D-glucose is not without its drawbacks. The oxidation state of D-glucose, glycerol and 1,3-propanediol (on a carbon basis) is D-glucose > glycerol > 1,3-propanediol. An examination of the chemical transformation of D-glucose to 1,3-propanediol (Equation 1) versus the transformation of glycerol (Equation 2) illustrates that there is a penalty to be paid in using a feedstock further removed from product with respect to redox balance.

1/2 D-Glucose + 4e⁻ + 4H⁺ → 1,3-Propanediol + H₂O  

(1)

Glycerol + 2e⁻ + 2H⁺ → 1,3-Propanediol + H₂O  

(2)

A more relevant comparison emerges after imposing biological pathways, including constraints associated with sugar metabolism. While the carbon pathway from glycerol remains the same (Equation 3), the route from D-glucose incurs an additional penalty in the form of a high-energy phosphate bond (~P_i) which accompanies the phosphorylation of sugar by ATP or phosphoenolpyruvate (PEP) (Equation 4).

Glycerol + NADH + H⁺  
→ 1,3-Propanediol + NAD⁺ + H₂O  

(3)

1/2 D-Glucose + ~ P_i + 2NADH + 2H⁺  
→ 1,3-Propanediol + 2NAD⁺ + P_i + H₂O  

(4)

It is clear that D-glucose is more demanding in terms of stoichiometric requirements for balance (i.e. the use of D-glucose requires more co-reactants than the use of glycerol). Co-reactants are generated from the substrate (either D-glucose or glycerol), thus yield (on a carbon basis) favors the glycerol pathway. However, the great equalizer is feedstock cost. A comparison considering substrate cost and yield establishes the merit of a direct D-glucose route. In addition to yield, the fermentation issues that dominate the economics of 1,3-propanediol production are rate and titer. Given assumptions on yield and empirical values of specific D-glucose consumption rates, encouraging estimates for 1,3-propanediol production rates are obtained. Additionally, it is reasonable to assume that the titers currently demonstrated in anaerobic glycerol to 1,3-propanediol fermentations can be improved.

Parenthetically, the generation of any material from substrate — other than product and required co-reactant(s) — is deleterious to yield. As a consideration related to the supply of co-reactants, it is also apparent that, placing arguments of co-product value and overall process complexity aside, the yield benefits from elimination of acid by-product, thus implying aerobic metabolism.

**Developing a single organism catalyst**

The strategy and progress of an effort by DuPont and Genencor International, Inc. to design and build a single organism catalyst for the direct conversion of D-glucose to 1,3-propanediol has been revealed in a series of patents and applications [32–34,35**]. Most recently, this work was also presented at a conference on metabolic engineering (CE Nakamura and P Soucaille, *Metabolic Engineering IV: Applied Systems Biology*, 6–11 October 2002, Castelvecchio Pascoli, Italy) (Figure 2). The strain is based on an *E. coli* K12 strain, which is eligible for favorable regulatory status in the United States. The base strain has only weak capacity to produce glycerol and no capacity to produce 1,3-propanediol, thus the engineered strain relies on a predominantly heterologous carbon pathway that diverts carbon from dihydroxyacetone phosphate (DHAP), a major ‘pipeline’ in central carbon metabolism, to 1,3-propanediol. In direct contrast to processes that use naturally available organisms, the DuPont/Genencor process is aerobic. Moreover, significant modifications have been made to the host strain to provide optimally for the energetic (~P_i) and redox demands of 1,3-propanediol production.

The carbon pathway utilizes glycerol 3-phosphate dehydrogenase (DAR1) and glycerol 3-phosphate phosphatase (GP2) genes, obtained from *Saccharomyces cerevisiae*, to provide glycerol [36,37]. Glycerol dehydratase (*dhaB1, dhaB2, dhaB3*) and its reactivating factors (*dhaBX, orfX*), obtained from *K. pneumoniae*, enable the conversion of glycerol to 3-hydroxypropionaldehyde [32,34]. Surprisingly, a previously uncharacterized oxidoreductase endogenous to *E. coli* (*yqhD*) completes the pathway [35**]. The preference for YqhD over the more obvious DhaT (or similar dehydrogenases available from alternate *dha* regulons) stems from fed-batch fermentation results in which strains utilizing YqhD produce 1,3-propanediol titers of approximately 130 g/L; such high titers are not obtained in identical strains utilizing DhaT and are unprecedented in glycerol-fed fermentations using natural 1,3-propanediol-producing organisms. In contrast to DhaT, YqhD utilizes NADPH rather than NADH (MH Emptage, personal communication) and it is likely that the differences in the cofactor reduced/oxidized ratios contribute to the higher titer.

The D-glucose-utilizing strain incorporates gene deletions that eliminate non-productive reactions. For example, once formed, glycerol is prevented from re-entering central carbon metabolism by deletion of the genes encoding glycerol kinase (*gldA*) and glycerol dehydrogenase (*gldK*) [33]. It is notable that a triosephosphate
isomerase (tpi) deletion was employed at early and intermediate stages of catalyst development. The tpi deletion forces a 1:1 split of carbon flux at the fructose bisphosphate aldolase step of the Embden-Meyerhof-Parnas pathway: one branch being forced towards 1,3-propanediol formation and the other towards the tricarboxylic acid cycle. Yield increased nearly 40% with the incorporation of this deletion [35]. Although the tpi deletion imposes an artificial ceiling on yield (42.5% weight yield, 50% carbon yield; if all carbon passes through fructose bisphosphate aldolase), its utility in providing a flux control point that is independent of modulation of enzyme expression was invaluable in assembling the carbon pathway from DHAP to 1,3-propanediol, up to the point where the artificial ceiling on yield was approached.

The two most fundamental changes imposed on the natural reactions of the E. coli-based strain are the elimination of D-glucose transport by the phosphotransferase system (PTS) and downregulation of glyceraldehyde 3-phosphate dehydrogenase (gap) (CE Nakamura, GM Whited unpublished results). The former modification addresses the limitation that D-glucose phosphorylation in E. coli is largely PEP-dependent. If PEP is generated solely via the enolase reaction of the Embden-Meyerhof-Parnas pathway, this constraint imposes a second artificial ceiling of 42.5% weight yield in 1,3-propanediol production. Although alternate routes for the generation of PEP exist, glucose phosphorylation is energetically more efficient if the PEP-dependent system is replaced with ATP-dependent phosphorylation. As a result, the PTS system is replaced with a synthetic system comprising galactose permease (galP) and glucokinase (glk), both genes are endogenous to E. coli. The gap modification is motivated by the requirement to remove the artificial ceiling imposed by the tpi deletion (discussed above). Downregulation of gap, concomitant with reinstatement of tpi, provides an improved flux control point.

Several additional reactions, outside of the direct carbon pathway to 1,3-propanediol, were examined to maximize biocatalyst efficiency. The examination involved modulation of rationally targeted enzyme expression levels and as a result of these studies several further changes were incorporated into the host strain. Although disclosure of the details must await the publication of patent applications, the end result is a metabolically engineered organism that provides 1,3-propanediol at a rate of 3.5 g/L/h, a titer of 135 g/L, and a weight yield of 51% in D-glucose fed-batch 10 L fermentations. By contrast, typical maximum values for rate, titer and weight yield of 1,3-propanediol from the anaerobic fermentation of glycerol are 3.0 g/L/h, 78 g/L and 55%, respectively [5**].

**Conclusions**

Biology, when compared to traditional chemistry, offers alternate catalysts for the production of chemicals, often through synthetic pathways and using starting materials not available through traditional chemistry. Such is the case for the production of 1,3-propanediol. The direct
fermentation of glycerol allows the use of a renewable feedstock in contrast to heterogeneous catalysis routes that are dependent on petrochemical feedstocks, ethylene oxide or acrolein. Today, the value of 1,3-propanediol lies predominantly in its use in polymers prepared from 1,3-propanediol and terephthalic acid, demand of which is estimated to be one to two billion pounds per year in 10 years. Providing low-cost 1,3-propanediol will be the key to competitiveness in this new market. It is notable that a leader in the chemical industry has elected to pursue a biological route [38]; it is all the more notable that a genetic engineering effort, arguably unprecedented in scope, resulted in the catalyst that is the centerpiece of that process.

In fermentation, metabolic engineering expands the range of chemical process solutions and enables improved cost effectiveness of options based on natural biological routes. The story of 1,3-propanediol represents a benchmark in metabolic engineering. Expanding the glycerol-based natural process to a more efficient process based on lower cost carbon feedstock involved several steps: changing an anaerobic process to an aerobic one; replacing the feedstock uptake (transport) mechanism of the host organism; intergeneric transfer of complex metabolic pathways; and both the design and implementation of an optimum solution to the balance of carbon, redox and energy with respect to microbial growth and product formation. The expectation of success presupposed that sufficient biochemical, genetic and physiological knowledge was available for rational design and that the tools necessary to design and implement genetic improvements were available. What was essential, but unavailable, was anticipated to be made available by concerted effort. The success of the project bodes well for future metabolic engineering efforts and, ultimately, for increased societal benefit obtained through the production of chemicals from renewable resources.

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Papers of particular interest, published within the annual period of review, have been highlighted as:

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