Production of coenzyme Q10 in recombinant *Schizosaccharomyces pombe* through metabolic engineering approaches

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Abstract

Coenzyme Q10 is an important cofactor in the electron transport chain, which powers oxidative phosphorylation. Similar to other cofactors such as vitamin B12, deficiency in coenzyme Q10 (CoQ) manifests as clinical symptoms. Hence, coenzyme Q10 can be used as a health supplement. But, difficulty in extracting it from natural sources and the high cost involved explains continuing interest in improving its production titer in various cellular hosts. A metabolic cofactor, and thus naturally of low cellular abundance, various genetic engineering approaches are explored for improving its concentration in heterologous hosts ranging from *Escherichia coli* to *Schizosaccharomyces pombe*. While the native pathway for CoQ biosynthesis feeds into that of cholesterol, and is conserved across the three domains of life, slight variation in pathway architecture exists. Specifically, some segments of the pathway are truncated for improving overall metabolic efficiency in hosts. Hence, choice of organisms as production host for CoQ10 is crucial and these typically exhibit high growth rates with ease for genetic manipulation such as heterologous expression of missing pathway genes. Whole cell metabolomics profiled by high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) coupled with metabolic flux analysis (MFA) and metabolic flux control (MCA), points to specific metabolic choke points where enhanced expression of enzyme increase the cellular concentration of important precursors and the final coenzyme product. One such choke point is the rate limiting enzyme, HMG-CoA reductase, whose over-expression improves CoQ titer. Systematic exploration of how fermentation parameters affect production yield is another important approach given myriad problems and challenges affecting the industrial use of recombinant CoQ production hosts. But CoQ production by recombinant routes usually results in CoQ of different side chain lengths; thus, efforts are directed towards tunable control of CoQ side chain moiety from the metabolic engineering perspective, which improves product quality and pharmaceutical properties. Another interesting basic science angle involves the conjugation of different cell localization signals for probing subcellular localization of CoQ. Finally, RNA interference (RNAi) mediated modulation of CoQ expression is a useful tool for indirectly assessing cellular energy state and redox balance, which opens up its use in studies examining how energy status affect physiological response to various nutrition and environment stressors. Collectively, much effort has focused on improving CoQ yield through metabolic engineering, and effecting tunable control of side chain length. However, important questions remain on understanding the subcellular localization of CoQ and examining the utility of tuning CoQ expression level for affecting cellular energy level, and its use as a physiology probe.

*Keywords*: metabolic engineering, flux balance, pathway analysis, metabolic flux analysis,
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Introduction

Coenzyme Q10 (CoQ or CoQ10) or ubiquinone (a member of the benzoquinone family) is an electron carrier of the electron transport chain (ETC) in mitochondria and some bacteria.¹ By cycling through its three oxidation states – fully oxidized (ubiquinone), semi-oxidized (semiquinone) and fully reduced (ubiquinol) – the enzyme mediates redox reactions by losing or gaining electrons. Given its importance in aerobic respiration (specifically, transferring electrons from the NADH dehydrogenase and succinate dehydrogenase to CoQ: cytochrome C reductase),² the cofactor is highly conserved across the tree of life. Besides respiration, the cofactor also participates in anti-oxidant removal, pyrimidine metabolism, formation of disulphide bonds, and sulphide oxidation.² In addition, CoQ also generates superoxide anion radicals through the autoxidation of semiquinone and antioxidant quenching of free radicals.³ Schizosaccharomyces pombe is a natural producer of CoQ, while its evolutionary brethren, Saccharomyces cerevisiae produces Coenzyme Q6 (CoQ6).² Escherichia coli, on the other hand, synthesizes CoQ8; but genetic engineering efforts have enabled the bacterium to coproduce CoQ10 and CoQ8.⁴

In humans, CoQ is synthesized in all cells from tyrosine (or phenylalanine) and mevalonate, and the highest concentrations are found in tissues with high energy turnover, such as the heart, brain, liver and kidney.⁵ Multiple mutations have been detected in various CoQ biosynthetic genes, which manifests clinically as a variety of neuro and early onset encephalomyopathies⁶; conditions which could be ameliorated through the provision of CoQ10 as a supplement and thus, the enzyme is gaining importance as a health supplement. Comprising 10 isoprene groups in the isoprenoid side chain, CoQ10 is also used as a food supplement.²³

In the context of electron transfer during oxidative phosphorylation, reduction of CoQ to its redox active form helps protect the mitochondria from oxidative damage, subsequently the respiratory chain enzymes convert the moiety back to its inactive form.³
Besides mediating electron transfer in oxidative phosphorylation, CoQ10 also participates as membrane-associated molecules for disulphide bond formation (in bacteria), oxidation of sulphide (in yeast), detoxification of reactive oxygen species, control of cellular redox state, generation of signaling molecules, and finally, gene expression. Other functions of the coenzyme include: regulation of mitochondrion permeability transition pore; activation of mitochondrion uncoupling proteins; regulation of membrane physicochemical properties; modulation of surface abundance of β2-integrins on red blood cells; and amelioration of endothelial dysfunction through increased provision of nitric oxide.

### Biosynthetic pathway of Coenzyme Q10

Native producers of CoQ10 include: *Agrobacterium tumefaciens; Paracoccus denitrificans; Crytococcus laurentii; Tricosporon sp.; Sporoboromyces salmonicolor;* and *Rhodobacter sphaeroides.* Essentially, CoQ10 shares a biosynthetic pathway with cholesterol. Broadly, the biosynthesis of CoQ10 involves the conjugation of the benzoquinone group to the hydrophobic isoprenoid side chain. The entire biosynthetic pathway comprises three parts: (i) synthesis of the quinonoid ring; (ii) decaprenyl diphosphate synthesis; and (iii) quinonoid ring modification. Isopentenyl diphosphate units constitute the isoprenoid side chain. The length of the side chain is determined by trans polypropenyl diphosphate synthases. In general, a side chain of less than 20 carbon atoms is unusual in natural CoQ since CoQ side chain tend to be more than 25 carbon atoms long. The methyl groups on the benzene ring are derived from S-adenosyl methionine. 4-hydroxybenzoate (pHBA) is the precursor of the quinonoid ring and is derived from the shikimate pathway; important for aromatic amino acid synthesis via the chorismate pathway. In the ring modification step, one decarboxylation, three hydroxylations, and three methylations occur in different sequence in prokaryotes and eukaryotes. Genes mediating CoQ synthesis in various organisms are shown in Table 1. Note that while all the genes of the pathway are known, the functions of Coq4, Coq8 and Coq10 remains elusive as of 2009. Unfortunately, the functional role of Coq9 remains unknown.

Isoprenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) – derivatives of pyruvate and glyceraldehyde-3-phosphate from the MEP pathway - are typical building blocks of CoQ10 biosynthesis. The CoQ10 biosynthetic pathway (Figure 1) comprises 9 steps mediated by enzymes, whose identities are elucidated in work done on *Escherichia coli* and *Saccharomyces cerevisiae* CoQ null mutants. With isoprenoid as the precursor, the pathway involves a series of methylation, decarboxylation and hydroxylation. In total, 10 genes (*dps1, ppt1, dlp1, and coq3-9*) are known to mediate CoQ biosynthesis in *S. pombe*. CoQ10 deletion mutants exhibit sensitivity to redox stress, require antioxidants for growth in minimal medium, produce a lot of sulphides, and do not survive stationary phase. Given that sulphide is a toxic compound, its concentrations in cells need to be tightly regulated through the action of sulphide:quinone oxidoreductase which detoxify high concentrations of sulphide. All CoQ- and respiration-deficient mutants of *S. pombe* accumulate sulphide. Supplementation of CoQ10 helps ameliorate detrimental impacts of sulphide accumulation in
CoQ10 deficient mutants since CoQ10 reduction in *S. pombe* is coupled to sulphide oxidation.⁸
Enlisting metabolic engineering approaches for enhancing CoQ10 production

Industrial production of this medicinal molecule is more effective through fermentation processes compared to chemical synthetic methods. For example, a maximum concentration of CoQ10 of 458 mg/L has been achieved in industrial fermentation with Agrobacterium tumefaciens as host. On the other hand, the highest titer obtained was reported in experiments using Rhodopseudomonas sphaeroides KY8598 (770 mg/L) and Spingomonas sp. ZUTEO3 (32.5 mg/gDCW dry cell weight). However, the relatively low product yield meant that the cost of CoQ10 remains high. Specifically, although production tier has been increased from 350 to 770 mg/L on volume scales from 50 to 100 kilolitres, the market price of CoQ10 remains high at between US$600-800. Production of other variants of CoQ has also been demonstrated; for example, CoQ5-CoQ10 can be produced in S. cerevisiae, where recombinant E. coli is used in producing CoQ7-CoQ10. Various organisms ranging from bacteria, Paracoccus denitrificans, Rhodobacter sphaeroides, Rhodopseudomonas palustris, to plants have been used in CoQ10 production, given its increasing importance as a food and medicinal supplement. Specifically, A. tumefaciens is a high producing strain of CoQ10, where experiments show that pH and dissolved oxygen are critical determinants of CoQ10 titer. Modulation of intracellular redox environment, principally through tunable NADPH availability, has been shown to be effective in enhancing CoQ10 production in E. coli BW25113. Specifically, heterologous expression of genes from different microorganisms is used in adjusting NADPH concentration, where a higher concentration enhances CoQ10 production. In addition, a change in growth medium from M9YG to SOB (with phosphate salt), as well as pH from 7 to 5.5 also results in elevated CoQ10 synthesis in E. coli; thus, suggesting that medium and pH are environment factors important to CoQ10 biosynthesis. In general, low copy number vectors are more efficient in producing CoQ10 in E. coli; highlighting that metabolic burden associated with expression of high copy number vectors negatively impact on product yield. Pathway-based metabolic engineering strategies are known to result in substantial variability (and instability) in CoQ10 biosynthesis in E. coli, which is linked to the metabolic burden associated with the heterologous expression of altered aromatic and mevalonate pathway on host primary metabolism. Expression of the Gluconobacter suboxydans decaprenyl diphosphate synthase (dds) gene (important for synthesizing the decaprenyl side chain of CoQ10) under the control of a E. coli BL21 constitutive promoter increases the biosynthesis of the metabolite even at low copy number of the introduced gene. The gene has been successfully expressed in S. cerevisiae for CoQ10 production. Replacement of the E. coli endogenous NAD-dependent gapA with NADP-dependent gapC from Clostridium acetobutyllicum, together with the latter’s overexpression is shown to increase CoQ10 biosynthesis through increasing NADPH availability. Heterologous expression of the DPS gene from either P. denitrificans or A. tumefaciens in E. coli has enabled the conversion of CoQ8 to CoQ10.
Heterologous expression of pathways in genetically engineered host typically results in a variety of problems such as metabolic imbalance, cellular burden, synthesis of nonfunctional proteins, and cytotoxicity resulting from intermediate products. Specifically, overexpression of multi-enzyme pathways is known to reduce target yield. To ameliorate the above problems, a self-regulating gene expression system based on trp/tac promoter together with engineering of ribosome binding site (RBS) is effective in tuning the expression of bottleneck mevalonate pathway genes in CoQ10 biosynthesis. Specifically, RBS sequence can be interpreted as a functional module for controlling protein synthesis given its effects on rate of translation initiation and mRNA stability. Reports have indicated changes in RBS sequence could result in multi-fold improvement in product yield. Results reveal that by overexpressing the enzymes DXS, DXR, IDI, and IspD under the trc promoter in the production host, Rhodobacter sphaeroides CoQ10 synthesis is improved through increase in the metabolic precursor, decaprenyl diphosphate. More importantly, the study demonstrates the utility of the self-regulating system in tuning the expression of four genes in a pathway; a feat not common in metabolic engineering, where modulating the individual expression levels of four genes concatenated in a pathway remains a significant challenge in synthetic biology.

Overexpression of HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA reductase), hmgR, the rate limiting enzyme in the mevalonate pathway in Schizosaccharomyces pombe, is useful in increasing the titer of CoQ10. In addition, expression of the gene could be induced by provision of arachidonic acid in the medium; thus, demonstrating an antibiotic-independent approach for regulating an exogenous gene. While production titer of CoQ10 is enhanced through increasing the availability of mevalonate, the partial divergence of flux to ergosterol synthesis meant that opportunities abound for further channeling the flux towards isoprene synthesis. Isoprene synthesis is reported as the rate limiting step of CoQ10 biosynthesis. S. pombe is the only unicellular eukaryote to harbor a single HMG-CoA gene; thus, greatly simplifying the targeted modulation of the enzyme expression and presents opportunities for understanding different facets of this enzyme’s activity, especially in regulating isoprenoid end-product synthesis. Other studies has focused on increasing CoQ10 titer in fission yeast through the use of directed evolution techniques comprising multiple rounds of mutagenesis and selection. However, random mutagenesis does not necessarily lead to higher CoQ10 titer given the probabilistic nature in which the gene expression space is search through the combinatorial library screening approach. On the other hand, metabolic engineering has provided a more rational (and targeted) approach towards increasing CoQ10 yield by modulating specific metabolic nodes deciphered through mass balance analysis. Unfortunately, myriad problems associated with metabolic imbalances, and unbalanced gene expression between consecutive genes in a concatenated pathway lead to lower productivity compared to those obtained through random mutagenesis. Finally, while increasing CoQ10 is the dominant goal of the field, the secondary issue of provision of adequate storage capacity for the lipophilic molecule produced is another important unresolved problem.

Besides modulating expression levels of pathway genes, efforts have also been devoted to tuning fermentation parameters for increasing CoQ10 titer. Effect of carbon and nitrogen
sources on CoQ10 synthesis has been explored using A. tumefaciens as an experiment model: specifically, sucrose, glucose, fructose, lactate, malate, xylose, galactose for carbon sources; and CSP, yeast extract, peptone, malt extract, casein, tryptone, and soytone for nitrogen sources.  

From the biotechnology perspective, coupled fermentation cum extraction has improved the yield of CoQ10 with Spingomonas sp. ZUTEO3 as the production host. Another study with mutant strains of Agrobacterium tumefaceins reveal that restricting oxygen supply and providing azide could increase intracellular CoQ10 content. Specifically, a reduction of dissolved oxygen content from 20 to 5% results in a 4-fold increase in CoQ10 production. Provision of azide at (0.45 mM) increases intracellular CoQ10 to 5.3 mg/g dw. Collectively, the data suggests that oxygen limitation induced electron flux reduction, and azide addition increases CoQ production by a feedback mechanism.

Analysis of metabolic choke-points in E. coli CoQ biosynthetic pathway reveals that DXS and DXR are critical enzymes in mevalonate pathway (MEP) for CoQ10 biosynthesis. In contrast to using the MEP pathway, another approach for modulating the mevalonate flux would be to introduce a foreign MVA pathway into E. coli. Three plasmids could be used to transfer all the pathway genes into E. coli; for example, the plasmids could encode the upper (phbA, mvaS and mvaA), lower (mvaK1, mvaK2, and mvaD along with idi), and finally, a single gene, DdsA (decaprenyl diphosphate synthase).
**Generation of CoQ with different side chains and assessment of their physiological impacts on Schizosaccharomyces pombe**

CoQ10 (with 10 repeats of the isoprene side chain) is the dominant (and perhaps the only) form of coenzyme Q synthesized by *S. pombe*. CoQ with differing side chains are found in different organisms, leading to efforts aimed at exploring the feasibility of isoprenoid side chain as a taxonomical marker.\(^{15}\) Previous work on heterologous production of CoQ10 in a variety of bacteria, plant and yeast species have established that the length of the CoQ isoprene side chain could be tuned by adjusting the expression level of the polyprenyl diphosphate synthase gene, or introducing specific prenyl diphosphate synthase for synthesizing CoQ with target length of isoprene side chains. For example, the decaprenyl diphosphate synthase gene specifies the synthesis of 10 isoprene units. Another study details the expression of a *Paracoccus denitrificans* (E)-decaprenyl diphosphate synthase capable of coupling 7 molecules of isoprenyl diphosphate with (E) farnesyl diphosphate for forming a C\(_{30}\) decaprenyl diphosphate in *E. coli*. While the synthesis of a CoQ with long isoprene side chain is an achievement in metabolic engineering, the general approach for enzyme-based synthesis of CoQ with specific length of isoprene side chain is not available. However, various metabolic engineering studies has illuminated the possibility of synthesizing a variety of CoQ with differing lengths of isoprene side chain. Utility of such a study include: (i) demonstrating the feasibility of synthesizing non-canonical CoQ in a model organism commonly used for studying eukaryotic cell cycle progression; (ii) evaluating the physiological impacts of CoQ with different side chain lengths; (iii) the efficiency at which CoQ of different isoprene length would localize to the respiratory chain and their effectiveness in mediating electron transfer between the different respiratory complexes during oxidative phosphorylation; and (iv) how individual CoQ affects the redox balance of the cell under different environmental stress and nutrition conditions.

Depending on whether the goal of the study seeks to synthesize a CoQ of specified isoprene chain length or a variety of CoQ with differing lengths of isoprene side chain, single or multiple polyprenyl diphosphate (*pds*) genes from a variety of microorganisms could be expressed in *S. pombe* by an expression vector system. In the case of a single *pds* gene, the target gene could be cloned into a vector with a suitable promoter. On the other hand, expressing multiple *pds* genes from different microbes would likely require the concomitant incorporation of organism-specific promoter into the expression vector for facilitating the transcription of the heterologous genes. Given the importance of ribosome binding site (RBS) in regulating ribosome binding efficiency and the initiation of translation, care must also be taken in designing appropriate RBS during vector construction. Relative expression levels of different *pds* genes could be modulated either by gene copy number, or by using promoters and RBS of different strengths. While expressing all *pds* genes at similar expression levels
would allow the generation of an assortment of CoQ with different isoprene chain length at similar relative abundance (which would facilitate the comparison of their physiological impacts), the alternative approach of tuning the specific expression level of each CoQ would enable specific questions concerning the biological role of specific CoQ (amidst a pool of different CoQ) to be examined in depth.

**Fusion of CoQ10 with different cell localization signals for exploring possible biological roles of this enzymatic cofactor**

Coenzyme Q10 is known to mediate most of its biological functions at the inner membrane of the mitochondria of human cells, the plasma membrane of unicellular eukaryotes, and inner membrane of Gram-negative bacteria. However, the question of what guides the specific membrane localization of CoQ10 is poorly understood. Specifically, while the hydrophobicity of the isoprene side chain may facilitate the accumulation of the cofactor within the hydrophobic segment of the phospholipid membrane, the preponderance of hydrophobic compounds and localities within the cell meant that non-specific subcellular localization of this molecule is theoretically possible. Recent observations and characterization of a CoQ10 binding proteins offer glimpses of a possible protein mediated targeting strategy where the binding protein serves as a CoQ10 carrier for transporting the cofactor to sites of biological function – in this case, the electron transport chain. Other studies have also demonstrated the utility of fusing CoQ10 with a lipophilic triphenylphosphonium cation for enabling the localization of the coenzyme to the lipophilic interior of cell membrane. The approach has led to the development of MitoQ, a medicine designed for oral delivery. However, given the presence of myriad lipophilic structures and compounds within the cytoplasmic milieu, the specificity of such a localization scheme would need to be assessed. Specific, non-specific localization of CoQ10 may result in off-target effects.

Identifying the sequence-based localization motifs in Coq10 would afford their subsequent translation to the design of peptide targeting sequences for localizing CoQ10 to specific locations in the cell. With the protein sequence of Coq10 known, a homology-based sequence search could be conducted to identify localization signals present in the protein sequence. Subsequently, homology-based structure prediction and/or molecular mechanics force fields could be used to obtain a coarse-grained structural model of Coq10 for preliminary assessment of concordance between putative localization motif and existence of any secondary structures that underpins function. Examining the plausibility of any structure recognition motif within the localization signal would add another level of specificity not available through enrichment of lipophilic amino acids residue at the sequence level.
Deletion of the motif in loss-of-function assay would be the next step in examining the postulated role of the identified sequence \textit{in vivo}.

After determining the localization sequence and validating its putative sequence and structure-based function, development of a specific conjugation strategy appropriate for the physiological environment would be the next major challenge of the project. While EDC-NHS and click chemistries are widely used in the \textit{in vitro} environment for surface conjugation and functionalization studies, the toxicities of the chemicals involved meant that they are generally not suitable for \textit{in vivo} cross-coupling experiments. Hence, the iconic biotin-streptavidin coupling mechanism would be the tool-of-choice for preliminary exploration of the feasibility of constructing a CoQ10-peptide localization sequence conjugate. In this framework, gene fusion constructs could be used in synthesizing a CoQ10-biotin construct, while an expression vector could harbor a gene segment for the peptide localization motif and green fluorescent protein (GFP reporter)-streptavidin construct. \textit{In vivo} assembly of the CoQ10 peptide localization motif would be mediated by the strong affinity between biotin and streptavidin.

Cell localization assays with fluorescent readout from GFP would provide critical information concerning the specificity of the localization strategy, in particular, answering the question of the severity of any physiological impacts due to off-target localization of CoQ10. Subsequently, the observed physiological impacts of such an expression system could be examined via a multitude of growth and physiological assays performed at the cellular, proteome, or transcriptome level, via next-generation sequencing or array-based readout.

The guiding principles learnt from the preliminary experiments could be employed in latter studies designed for examining the possibilities of generating other localization motifs for targeting CoQ10 to other subcellular locations. Such non-canonical localization of CoQ10 would afford a platform methodology for facilitating the interrogation of a series of questions concerning the myriad biological roles of CoQ10 beyond its main function as electron carrier in the respiratory chain. To this end, a rational design approach or library based screening could be employed for arriving at the localization motifs suitable for each subcellular targeting objective. Comparing the efficacy of the two approaches at the theoretical level, current state-of-the art in peptide structure prediction should enable a fairly accurate description of sequence-based protein structure characteristics for short peptides. In contrast, the library-based screening approach is a broad survey strategy which would yield unexpected sequence motifs that afford targeting of CoQ10 to non-target subcellular localities. Such serendipitous findings would add to the pool of information available for examining, at the correlation and maybe mechanistic level, the dependence (and degree of conservation) of particular sequences for specific localization of CoQ10.
Tunable expression of Coenzyme Q10 via RNAi

Multiple approaches are used in over-expressing genes of the CoQ10 synthesis pathway for increasing production titer. But, taken together, the goal of such studies is not directed towards fine-level control of CoQ10 production within a narrow physiological range. While metabolic engineering approaches have significantly increased CoQ10 yield, opportunities exist for exploring an alternative strategy of tailoring CoQ10 production at the post-transcriptional level. Similar to other studies aimed at increasing production of a target molecule, metabolic engineering of CoQ10 synthesis pathway revolves around the identification of specific metabolic nodes (or choke-points), followed by modulating the expression of either upstream or downstream genes around the nodes for directing metabolic flux towards a particular metabolic branch, and finally, the desired product. Modulation of gene expression typically occurs through the heterologous expression of genes from other organisms or via the coupling of a non-native strong promoter to specific genes or segment of a pathway. While transcription-level gene regulation is useful for achieving specific production goals, they are nevertheless unable to achieve fine-control of expression levels.

RNA interference (RNAi), in a variety of organisms, serves as an innate defence strategy for nullifying nucleic acids introduced by microbes, particularly viruses. Comprising a large family of small, non-coding RNA molecules, the sequence-dependent inactivation of protein translation from target mRNA regulates protein production at the post-transcriptional level. With no degradation of target mRNA in cases where Dicer or RISC (RNA-induced silencing complex) are not involved, the regulatory mechanism offers a potential way for reusing a given pool of target mRNA in reversible expression cycles via repeated on/off binding of 22-23 nucleotide microRNA to target sequence (or response element) on mRNA transcripts. Variables controlling expression levels include half-life of mRNA and amount of mRNA available. A RNAi-based method for modulating gene expression has recently been demonstrated in *Saccharomyces cerevisiae*.  

Given the multitude of genes present in the CoQ10 biosynthesis pathway, a number of microRNA (miRNA), long noncoding RNA (lncRNA) and short-hairpin RNA (shRNA) would be needed to modulate final CoQ10 titer in the envisioned approach. Using the eukaryotic cell cycle model organism, *Schizosaccharomyces pombe*, the study would first profile, at the genome-wide level, the range of miRNA, lncRNA and shRNA that bind to mRNA transcripts of genes in the CoQ10 synthesis pathway via chip-array high throughput screening assays. Functional validation of the gene modulation properties of the identified small RNAs (sRNA) would be subsequently conducted. Since it is likely that different sRNAs would have differential impact on the overall production of CoQ10, identification of the pathway genes most amenable to sRNA mediated modulation of expression level, and the concentration of sRNA required to achieve target levels of pathway requisite enzymes would comprise the major bulk of experimental work. Necessarily, optimization of the sRNA
transcript levels would require the possible over- or under-expression of genes encoding sRNAs. Alternatively, the more direct approach of supplying the sRNAs exogenously would avoid the problem of double modulation of transcript levels: sRNA followed by pathway genes. Nevertheless, given the instability of RNA in the extracellular milieu, the exogenous infusion strategy may be unable to deliver consistent reproducible results. On the other hand, the intracellular strategy would provide fundamental knowledge concerning the feasibility and ease of modulating multiple sRNA abundances for tuning the expression of genes in a pathway.

Such an experimental system amenable to tunable expression of CoQ10 would open up a swathe of research questions previously inaccessible to direct investigation. Firstly, given the importance of CoQ10 in facilitating electron transfer from Complex I and II to Complex III of the respiratory chain, under- or up-regulation of CoQ10 would be useful for understanding hitherto unknown dynamics of electron transfer between NADH dehydrogenase/succinate dehydrogenase to cytochrome C reductase. More important, by mediating electron transfer from NADH dehydrogenase, possibilities exist of using CoQ10 levels for modulating the available intracellular NADH pool and, by extension, the cellular redox balance. Redox balance is an indicator of cellular physiological stress, and potentiates entry into a range of cellular stress response mechanisms. Thus, with an experimental strategy capable of reversible modulation of cellular redox conditions, a variety of eukaryotic cellular processes – some of which related to aging and cancer – could be addressed at the in vivo level, where previously, the redox state of cells could not be effectively modulated intrinsically without chemicals or additives. Besides processes such as autophagy, metabolism, and proteasome-mediated enzyme degradation, the fundamental problem concerning the effect of cellular redox environment on protein folding could be investigated. Specifically, the mechanisms underpinning the “folding” of intrinsically disordered proteins is currently under intense academic debate, with many theories proposed for explaining observed data. Since redox state is a factor affecting protein folding through the formation of disulphide bonds, academic value can be derived from using the tunable CoQ10 enzyme level system for investigating the possible impacts of redox conditions on the structure and function of intrinsically disordered proteins within the intracellular milieu.

Secondly, the central role of the electron transport chain in producing ATP also meant that targeted modulation of CoQ10 levels could potentially be used as a lever for controlling cellular energy state. Either through enhancing or retarding the flow of electrons along the respiratory chain, ATP production and cellular energy level could be modulated. Existence of multiple energy sensors such as the quintessential AMPK (AMP activated protein kinase) helps the cell through the decision process necessary for responding to changes in nutritional and cellular energy levels. Cells in the energy-depleted state have been postulated to activate specific stress response pathways. Nevertheless, lack of tools for fine-tuning the energy levels of cells meant that the physiological processes could only be studied from the on/off

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perspective, where the effects of granulated changes in cellular energy on extent of cellular stress remain elusive to experimentation.

Finally, the study may also hold implications for modulating the electron transfer efficiency within the photosynthetic apparatus in cyanobacteria. Targeted tuning of photosynthetic activity affords the dual possibilities of understanding hitherto obscure aspects of electron transfer within the complex photosynthetic machinery, as well as providing an experimental system capable of precision modulation of electron flux necessary for synthesizing useful compounds via the solar energy to chemical precursor route.

Conflicts of Interest

The author declares no conflicts of interest.

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Simultaneous Measurement of Reduced and Oxidized Coenzymes Q9 and Q10 in Rat Tissues by LC-MS.

Appendix

Useful information

Database on Schizosaccharomyces pombe (http://www.pombase.org/)

Medium for cultivating Schizosaccharomyces pombe

Pombe minimum medium

Growth characteristics

Growth of S. pombe on medium with a non-fermentable carbon source is minimal.8

Strains

Strain of S. pombe capable of producing Coq10 (ATCC 24843)

Biosynthetic genes

Complementation assays reveal that PHB:polyprenyl diphosphate transferase is highly conserved from humans, yeast and S. pombe.2

Mutants of DdsA (decaprenyl diphosphate synthase) from Gluconobacter suboxydans, and IspB (octaprenyl diphosphate synthase) from E. coli are available.2

The genes (dxs, dxr, idi, and ispD) are assembled into pTrc99a, and conform to the BioBrick standard. The vector also comprises the endonucleases EcoRI, XbaI, SpeI and HindIII.7

Coq10 bind CoQ10 in S. pombe; a finding which holds implications on understanding of subcellular localization of the CoQ10 pool.8 Amongst the CoQ10 binding proteins identified, the S. pombe Coq10 is the smallest (164 aa).8 S. pombe deficient in Coq10 requires antioxidants for proper growth on minimal medium (specifically, growth is slower than wild-type cells), exhibits increased sensitivity to hydrogen peroxide, produce large amounts of H2S, and is deficient in respiration; all features associated with intracellular redox imbalance.8 Addition of anti-oxidant such as cysteine or glutathione could overcome this
growth defect. Finally, effective complementation of the deficiency through the expression of the human, yeast and Arabidopsis orthologs of Coq10 suggests that the enzyme is evolutionarily important and conserved across phylogenetic boundaries, and may have emerged early during the evolution of life. Collectively, the primary function of Coq10 may be in transferring CoQ10 from one membrane site to another.

Polyisoprenyl synthase in *S. pombe* is a hetero-tetramer comprising 2 subunits: decaprenyl diphosphate synthase, and D (aspartate rich motif)-less polyisoprenyl diphosphate synthase. This structural arrangement is similar to that in humans and mouse but differs from the homodimeric or heterotetrameric structure in *Saccharomyces cerevisiae*. Identity of the enzyme responsible for the decarboxylation step of coenzyme Q biosynthesis in eukaryotes remains an open question.

Expression of the *pptI* gene in *S. pombe* has been tried, but the results are equivocal.

Presence of kinase sequence motif led to the classification of Coq8 as a putative kinase. Evidence in support of this tentative functional annotation comes from the observation that Coq8 phosphorylates Coq3 – and thus, the enzyme could be considered as a regulator of Coq enzymes. On the other hand, while Coq9 is conserved across eukaryotes, it has no homology with known proteins; thereby, indicating that structural conservation occurs independently of sequence homology. Coq9 is shown to be part of a multisubunit enzyme complex.

### Table 1: Genes and enzymes involved in CoQ biosynthesis

| Genes  | Enzymes encoded by corresponding genes                                      |
|--------|------------------------------------------------------------------------------|
| *phbA* | Acetoacetyl-CoA thiolase (PhbA)                                               |
| *mvaS* | HMG-CoA synthase (MvaS)                                                      |
| *mvaA* | HMG-CoA reductase (MvaA)                                                      |
| *mvaK1*| Mevalonate kinase (MvaK1)                                                     |
| *mvaK2*| Phosphomevalonate kinase (MvaK2)                                              |
| *mvaD* | Mevalonate-5-pyrophosphate decarboxylase (MvaD)                              |
| *dxx*  | 1-deoxy-D-xylulose-5-phosphate synthase (DXS)                                |
| *dxr*  | 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR)                         |
| *ispD* | 4-diposphocytidyl-2-C-methyl-D-erythritol synthase (IspD)                    |
| *ispE* | 4-diposphocytidyl-2-C-methyl-D-erythritol kinase (IspE)                      |
| *ispF* | 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase (IspF)                 |
| *ispG* | 4-Hydroxyl-3-methyl-but-2-enyl-pyrophosphate synthase (IspG)                 |
**ispH** 4-Hydroxyl-3-methyl-but-2-enyl-pyrophosphate reductase (IspH)

**idi** Isopentenyl pyrophosphate isomerase (Idi)

**ispA** Farnesyl diphosphate synthase (IspA)

**ispB** Polyprenyl diphosphate synthase (IspB)

**ddsA** Decaprenyl diphosphate synthase (DdsA or DPS)

**ubiA** PHB-polyprenyltransferase (UbiA)

**ubiG** O-Methyltransferase (UbiG)

**ubiE** C-Methyltransferase (UbiE)

**ubiH** Monoxygenase (UbiH)

**ubiF** Monoxygenase (UbiF)

**ubiB** Monoxygenase (UbiB)

**ubiC** Chorismate lyase (UbiC)

**ubiD/ubiX** Decarboxylase (UbiD/UbiX)

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**Extraction of CoQ10**

1 mL of cells was harvested and ruptured at 75 °C in the presence of 200 μL of HCl (pH 2.0). Pellets were subsequently harvested and CoQ10 extracted using 5 mL of extraction solution (ethyl acetate/ethanol = 5:3, v/v). The mixture was vigorously vortexed for 10 min before incubating at room temperature for 15 min. Cell debris were removed, and 15 μL of supernatant sent for HPLC analysis.

**Purification of Coenzyme Q10 by LC-MS**

CoQ10 can be purified by liquid chromatography tandem mass spectrometry (LC-MS/MS) with multiple reaction monitoring (MRM). Electrospray ionization is used for ion generation, while positive ion mode helps accelerate the ions prior to separation by magnetic quadrupoles. CoQ10 can also be isolated and quantified from mitochondria by LC-MS/MS using the isotope dilution methodology. Addition of an antioxidant, butylhydroxytoluene results in a constant in vivo ratio between ubiquinol and ubiquinone in rat tissue for analysis by reversed phase LC-MS/MS; perhaps the same technique can be used in other cell types.

CoQ10 could be purified on a C18 reverse phase HPLC column (250 x 4.6 mm, Dikma) with methanol-hexane (80:20 v/v) as the mobile phase at flow rate of 1.5 mL/min. Injection volume is 15 μL with UV detection at 275 nm and column temperature of 30 °C.

Wondasil C18 column (5 μm x 4.6 mm x 150 mm, GL Sciences, Tokyo, Japan) at 40 °C. Mobile phase: methanol/isopropyl alcohol (3:1 v/v, HPLC grade, Tedia, OH), flowrate: 1.0
ml/min. Signal detection: UV detector at 275 nm. Calibration was performed via injecting serial dilution of CoQ10 (Sigma-Aldrich) into the HPLC system.\textsuperscript{7}

Unison UK-C8 75 x 3 mm column; Mobile phase: 0.1\% formic acid/acetonitrile/isopropanol $= 1/5/5$; 0.8 ml/min, Injection volume: 10 $\mu$L (50 fg/$\mu$L). Mass spectrometry detection: APCI (atmospheric pressure chemical ionization), acceleration of ions in positive ion mode, and separation of target ions via multiple reaction monitoring.

\textit{Cloning of genes/pathways}

Thiamine represses the \textit{nmt1} promoter. In general, rich media with yeast extract supplementation would contain sufficient thiamine to repress the promoter.\textsuperscript{16} \textit{LEU2} is a selectable marker for auxotrophic mutants.