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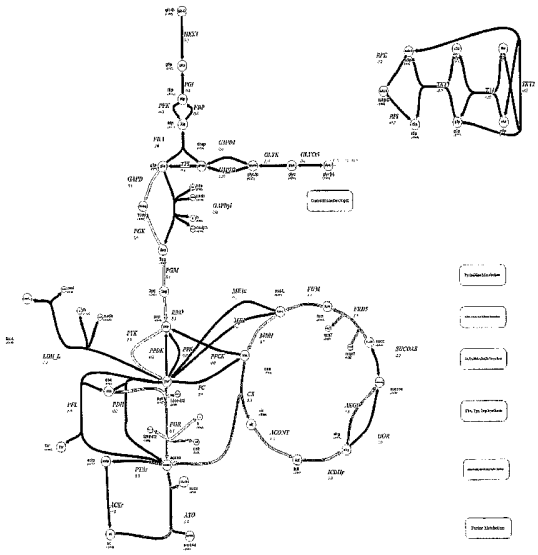
(54) Title: COMPOSITIONS AND METHODS FOR BIOELECTRICITY PRODUCTION



•The analysis of the metabolic network of *G. sulfurreducens* using the Simpheny™ platform allows us to identify potential substrates with high electron/mol ratio.
•**Predicted flux** Acetate: 71 mmol/10mM Acetate
Glycerol:65 mmol/10 mM Glycerol

Genome based *in silico* model

(57) **Abstract:** The invention provides a microbial fuel cell having a dissimilatory metal-reducing microbe expressing exogenous or native ATPase subunits, the ATPase subunits assembling into an active ATP synthase and consuming ATP in a futile cycle. The dissimilatory metal-reducing microbe can include an organism selected from the organisms set forth in Table 1. The one or more exogenous ATPase subunits can include a subunit selected from the ATPase subunits set forth in Tables 2 or 3. Also provided is a microbial fuel cell having a dissimilatory metal-reducing microbe expressing one or more exogenous genes encoding a gene product that promotes ATP consumption, the gene products of the one or more exogenous genes having an activity that reduces ATP synthesis, increases ATP consumption or both. The one or more gene products can increase ATP consumption through a futile cycle or through altering a metabolic reaction directly involved in ATP synthesis. Further provided is a microbial fuel cell having a dissimilatory metal-reducing microbe expressing one or more exogenous genes encoding a gene products that increases the electron/mole ratio compared to an unmodified microbe, wherein the increased ratio enhances electron transfer to an electrode. A method of producing electricity from an microbial organism is further provided. The method includes: (a) culturing a microbial fuel cell under anaerobic conditions sufficient for growth, the microbial fuel cell comprising a dissimilatory metal-reducing microbe expressing

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exogenous ATPase subunits, the ATPase subunits assembling into an active ATP synthase and consuming ATP in a futile cycle when grown under anaerobic conditions, and (b) capturing electrons produced by an increased ATP demand with an electron acceptor.

COMPOSITIONS AND METHODS FOR BIOELECTRICITY PRODUCTION

BACKGROUND OF THE INVENTION

There is a pressing need to reduce our reliance on energy derived from fossil fuels, and develop alternative strategies for the generation of energy from renewable resources. One such strategy aims to directly convert carbohydrates into electrical energy by using the reducing potential inherent in biological systems whereby introducing the concept of microbially-driven fuel cells.

A microbial fuel cell is basically a system that harvests electrons produced during microbial metabolism and channels them for electric current generation. These type of fuel cells allow compounds such as simple carbohydrates or waste organic matter to be converted into electricity[1]. One form of a microbial fuel cell uses artificial redox mediators that are capable of penetrating bacterial cells. When added to a culture solution within an anodic fuel cell compartment, these mediators enable electrons produced during fermentation or other metabolic processes to be shuttled to the anode. A drawback associated with these microbial fuels cells is that the microbes oxidize only a part of the substrates and also require soluble mediators to facilitate electron transfer, which can be costly. In some cases, these mediators are even toxic and cannot be used for electricity generation in open environments.

Another concept in the construction of microbial fuel cells resulted from the observation[2] that if graphite or platinum electrodes were placed into anoxic marine sediments, and connected to similar electrodes in the overlying oxic water, sustained electrical power could be harvested (on the order of 0.01 Watts/m^2 of electrode). This finding has led to the discovery that specific groups of microorganisms, most notably the *Geobacteraceae*, are capable of directly transferring electrons to electrodes, without the need for mediators[3-5]. Recently, organisms from the species *Rhodoferax ferrireducens* were shown to oxidize glucose to CO_2 and quantitatively transfer electrons to graphite electrodes without the need for an electron-shuttling mediator[6]. Furthermore, the recovery of electrons from glucose oxidation was over 80% of that theoretically available from glucose oxidation.

SUMMARY OF THE INVENTION

The invention provides a microbial fuel cell having a dissimilatory metal-reducing microbe expressing exogenous or native ATPase subunits, the ATPase subunits assembling into an active ATP synthase and consuming ATP in a futile cycle. The dissimilatory metal-reducing microbe can include an organism selected from the organisms set forth in Table 1. The one or more exogenous ATPase subunits can include a subunit selected from the ATPase subunits set forth in Tables 2 or 3. Also provided is a microbial fuel cell having a dissimilatory metal-reducing microbe expressing one or more exogenous genes encoding a gene product that promotes ATP consumption, the gene products of the one or more exogenous genes having an activity that reduces ATP synthesis, increases ATP consumption or both. The one or more gene products can increase ATP consumption through a futile cycle or through altering a metabolic reaction directly involved in ATP synthesis. Further provided is a microbial fuel cell having a dissimilatory metal-reducing microbe expressing one or more exogenous genes encoding a gene products that increases the electron/mole ratio compared to an unmodified microbe, wherein the increased ratio enhances electron transfer to an electrode. A method of producing electricity from an microbial organism is further provided. The method includes: (a) culturing a microbial fuel cell under anaerobic conditions sufficient for growth, the microbial fuel cell comprising a dissimilatory metal-reducing microbe expressing exogenous ATPase subunits, the ATPase subunits assembling into an active ATP synthase and consuming ATP in a futile cycle when grown under anaerobic conditions, and (b) capturing electrons produced by an increased ATP demand with an electron acceptor.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a genome based *in silico* model. The analysis of the metabolic network of *G. sulfurreducens* using the SimphenyTM platform allowed identification of potential substrates with high electron/mol ratio. The predicted flux for acetate was 71 mmol/10mM acetate. The predicted flux for glycerol was 65 mmol/10 mM glycerol.

Figure 2 shows the effect on bioelectricity production when alternate substrates are utilized. The glycerol processing operon from *D. vulgaris* was cloned in pRG plasmid (Dglyop) and expressed in *G. sulfurreducens*. The engineered strain containing the glycerol transporter was able to grow in NBAF with glycerol (Gly) while the wild type was comparable to growth with a minimum concentration of Acetate (Ac). The engineered strain also was able to grow with Glycerol as the only carbon and electron source on iron oxide.

Figure 3 shows the effect on bioelectricity production when respiration rate is increased. The left panel shows growth of the modified ATPase expressing *G. sulfurreducens* strain under increasing ATPase induction. The right panel shows the level of respiration in the presence or absence of increased respiration caused by ATPase induction.

Figure 4 shows bioelectricity production and direct transfer of electrons to an electrode using engineered *Geobacter* cells. A two-chambered microbial fuel cell is shown in the left panel of Figure 4. The right panel of Figure 4 shows current generation following ATPase induction.

DETAILED DESCRIPTION OF THE INVENTION

Direct transfer of electrons to electrodes can be harnessed for the production of electricity by biological organisms. For example, microbial cells can be attached to electrodes as catalysts for harvesting electricity from sources such as organic wastes, carbohydrate, feedstocks, and contaminated groundwaters. Thus, in this alternative form of a fuel cell, metal-reducing bacteria are incorporated that partly exhibit special membrane-bound cytochromes capable of transferring electrons directly to the electrodes rather than having to use a redox mediator to shuttle electrons to the anode. Bioelectricity production can be augmented to increase amounts sufficient for commercial purposes employing the genetic modifications described below. Further, because the bioelectrical enhancements described herein rest on genetic compositions and gene product expression levels or activity, the bioelectrical organisms of the invention can be

genetically modified to modulate the expression or activity of one, some or all of the molecular components of the bioelectricity machinery in order to increase or decrease bioelectricity production.

The invention is directed to the metabolic engineering of dissimilatory metal reducing microbes so as to channel more electrons through the respiratory machinery of a cell for transfer to an electrode. Increasing respiratory electron flow can be accomplished by, for example, increasing the ATP/energy demand that is placed on the cells whereby forcing the cells to generate more ATP. Increasing ATP production will in turn increase bioelectricity production by transferring more electrons to an external electrode.

Bioelectricity production can be generated in a variety of organisms. A particularly useful organism is *Geobacter sulfurreducens*. However, metabolic engineering to increase ATP production with a concomitant increase in electron transfer and electrical production is applicable, for example, to all dissimilatory metal-reducing microbe for use in a microbial fuel cell. *G. sulfurreducens*, is a particular member of the class of dissimilatory metal reducing bacteria, with applications in bioremediation and bioelectricity generation. This microorganism belongs to the *Geobacteraceae* family, that have been shown to be a dominant member of the communities of bacteria associated with uranium bioremediation[7,8], and in bioelectricity generation[5] in microbial fuel cells.

Previous rates of transfer of electrons in *G. sulfurreducens* is quite slow and can support, if at all, only very low powered devices. Hence, there is a critical need to genetically engineer the metabolism of these and other organisms to enhance the rate of electron transport, so that these microbial fuel cells become commercially practical.

The application of metabolic engineering has been used to synthesize bulk commodity chemicals such as 1,3 propanediol, acetate, lactate, and other metabolites. The invention is directed to the engineering of microorganisms to enhance the rate of electron transfer to electrodes, through the introduction of heterologous genes into the genome of such microorganisms. For example, *G. sulfurreducens*, for which current rates of electron transfer are low and a genetic system has been identified to facilitate the

insertion of novel genes [9], can be engineered to increase bioelectrical production over previously obtained electron transfer rates. By modulating heterologous gene expression substantial increases can be observed over that previously obtained.

As described previously, initial metabolic engineering attempts have primarily focused on increasing the supply of metabolic enzymes. However, merely increasing the supply of metabolic enzymes in a pathway often fails to increase the product synthesis rate, as the interactions between the different subsets of metabolism are not considered in this simple strategy. Recently, metabolic engineering through demand management has been proposed[10], where the demand of key intermediates such as ATP is engineered. This concept has been attempted for increasing the flux through the glycolytic enzymes[11] and for the production of acetate[12] in *Escherichia coli.* However, engineering of important intermediates has never been contemplated for enhancing the transfer of electrons to an electrode for electricity generation.

In the first instance described above, where the glycolytic flux was desired to be increased, an ATPase consisting of the genes encoding the alpha, beta, and the gamma subunits of the ATP synthase was introduced into *E. coli.* These subunits of the ATP synthase act as a cytoplasmic ATPase. The ATPase created a futile cycle that increased ATP consumption and increased the glycolytic flux as the demand for ATP increased. In the second instance described above, the genes corresponding to the F_0 part of the $(F_1F_0)H^+$ ATP synthase was deleted, creating a cytoplasmic ATPase that lead to a futile cycle consuming ATP. Since, the only fermentation pathway available was the acetate production pathway that regenerated ATP, the acetate production of up to 75% of the maximum theoretical yield was obtained.

In *Geobacter sulfurreducens*, the rate of electron transfer through the electron transport chain depends on the efficiency of the chain. For example, for growth on Fe(III), the yield on acetate is three times lower than for growth on fumarate, and the rates of electron transport is higher for growth on Fe(III).

The invention provides organisms having a gene operatively inserted for an ATPase that when expressed will cause consumption of ATP. This metabolic result in turn will increase the demand for the production of ATP by the cell's metabolic

machinery. In dissimilatory metal-reducing microbes this increased demand can be met, for example, by channeling more protons out of the cell to produce more ATP via the proton-gradient. This result comes with the concomitant channeling of more electrons through the respiratory chain ending with the transfer of these electrons to an electron acceptor such as a graphite electrode.

An alternative possibility is to decrease the efficiency of the electron transport chain, so that more electrons flow through the chain to generate equivalent amounts of ATP. In both of the above bioelectricity modes, the activity of the operatively inserted ATPase and the degree of the efficiency can be controlled so that the cell maintains homeostasis. In this regard, controlling the efficiency ensures that the cell is not overwhelmed by the increased energy demand as these organisms could be potentially energetically limited for growth. The inserted ATPase genes can be placed, for example, under the control of a promoter so that the expression of the ATPase can be initiated once there is sufficient build-up of the organism's biomass.

The invention also provides a microbial fuel cell having a dissimilatory metal-reducing microbe expressing one or more exogenous or native genes encoding a gene products that promote ATP consumption, the gene products of the one or more exogenous or native genes having an activity that reduces ATP synthesis, increases ATP consumption or both.

The invention has been exemplified by reference to an embodiment that causes ATP consumption through the expression of an ATPase. Given the teachings and guidance provided herein, those skilled in the art will understand that essentially any gene or gene modification that promotes ATP consumption will similarly increase the demand for ATP production and concomitant increase of electron flux through the respiratory chain. This result can be accomplished by, for example, genetically modifying a microbe to increases ATP consumption through a futile cycle resulting in reduced ATP synthesis and/or increased ATP consumption.

The genetic modifications can include metabolic reactions or pathways directly involved in ATP synthesis. Such modifications include, for example, inactivating an ATP synthesis gene. Inactivation can be accomplished by, for example, introducing

one or more mutations, substituting one or more genes with a non-functional exogenous gene by site specific recombination or by expressing a regulator or inhibitor of a gene directly involved in ATP synthesis. Specific examples of such gene products include the genes for phosphofructokinase, and pyruvate kinase. By coupling the expression of phosphofructokinase with a fructose-bisphoshotase, a futile cycle that dissipates ATP can increase the consumption of ATP. Similarly a futile cycle can be created by simultaneous use of pyruvate kinase and phosphoenolpyruvate synthase, or any kinase enzyme and it's reciprocal phosphatase enzyme.

Alternatively, ATP consumption can be accomplished by, for example, genetic modifications of metabolic reactions or pathways indirectly involved in ATP synthesis. Genes indirectly involved in ATP synthesis include gene products that act a distal point such as at a precursor pathway or it blocks the coupling of ATP synthesis to electron transport. Such modifications include, for example, introducing one or more mutations, substituting one or more genes with a non-functional exogenous gene by site specific recombination or by expressing a regulator or inhibitory of a gene indirectly involved in ATP synthesis.

The above described metabolic engineering for bioelectricity production also can be applied, for example, to any organism, natural or engineered, that transfers electrons to an electrode, to enhance the generation rate of electrical current. The operable introduction of ATPases also can be successfully applied to all dissimilatory metal reducing microbes where, for example, the metal reduction is coupled to growth or coupled to other microbes, including fermentative or sulfate reducing microbes such as *Clostridium beijerinkii*[13] or *Desulfotomaculum reducens*[14], where the metal reduction can be coupled to growth, for example. Exemplary dissimilatory metal reducing microbes that can be metabolically engineered to produce practical quantities of bioelectricity are set forth below in Table 1.

Table 1: Exemplary Dissimilatory Metal Reducing Microbes

Kingdom	Intermediate Rank	Genus	Species
Bacteria	delta subdivision proteobacteria	Geobacter	bremensis, chapelleii, grbiciae, hydrogenophilus, metallireducens, pelophilus, sulfurreducens
Bacteria	delta subdivision proteobacteria	Geothermobacter	ehrlichii
Bacteria	Acidobacteria	Geothrix	fermentans
Bacteria	beta subdivision proteobacteria	Rhodoferax	ferrireducens
Bacteria	gamma subdivision proteobacteria	Shewanella	amazonensis, frigidimarina, gelidimarina, oneidensis, olleyana, *livingstonensis*
Bacteria	Thermodesulfobacteria	Geothermobacterium	ferrireducens
Bacteria	Thermotogae	Thermotoga	maritima
Archae	Thermoprotei crearchaeota	Pyrobaculum	islandicum
Archae	Arcaeoglobi euryachaeota	Geoglobus	ahangari

For the production of bioelectricity producing microbes, genes encoding an ATPase can be introduced in operable form for expression and functional assembly of the encoded gene products. Briefly, the genes encoding the F_1 part of the ATPase can come from essentially any organism including, for example, any of the several organisms shown below in Table 2. The genes coding for the corresponding subunits in eukaryotic species such as *Saccharomyces cerevisiae* can also be incorporated into the dissmilatory metal reducing bacteria. In these cases, codon optimization to eliminate rare codons in the eukaryotic genes could be necessary to increase the expression of the gene products.

In addition to the F-type ATPase, the genes coding for the V_1 subunit of the V type ATPase shown in Table 3 or the A-type ATPase[15] can also be inserted into the dissimilatory metal reducing bacteria for creating an extra ATP demand.

In the specific instance of *Geobacter sulfurreducens*, the gene coding for the F_1 part of the ATPase from, for example, *Escherichia coli* can be introduced into a microbe of the invention and expressed for bioelectricity production. An exemplary vector useful for introduction and expression is the plasmid pCM66, a high copy-number

plasmid that is stable in *G. sulfurreducens* even in the absence of antibiotic pressure. The genes coding for the F_1ATPase (atpAGD coding for the alpha, beta, gamma subunits in *E. coli*) can be, for example, cloned into this plasmid under the control of either a constitutive or inducible promoter. Constitutive promoters can be chosen that exhibit different expression strengths to achieve a desired level of exogenous ATPase expression. These genes can be obtained from the source organism or organisms or from source plasmids using restriction enzymes followed by amplification with sequence specific primers or other recombinant techniques well known to those skilled in the art. The gene can then be cloned into the host plasmid and the cells cultured for polypeptide expression and self-assembly of the ATPase subunits. The expression of these genes can be verified by subsequent analysis including, for example, RNA expression, polypeptide expression or activity measurements. These analysis as well as other means for determining the level or activity of an exogenously expressed polypeptide are well known to those skilled in the art.

In addition, all of the above designs and methods for expressing ATPase encoding nucleic acids for the consumption of ATP also can be applied to the expression of non-ATPase genes or metabolic regulators, for example, that similarly increase the consumption of ATP which can be harnessed for the production of bioelectricity. For example, a futile cycle can be created by coordinated expression of genes for phosphofructokinase and fructose-bisphosphotase that will result in a net reaction that consumes ATP.

The invention also provides a microbial fuel cell having a dissimilatory metal-reducing microbe expressing one or more exogenous genes encoding a gene products that increases the electron/mole ratio compared to an unmodified microbe, wherein the increased ratio enhances electron transfer to an electrode. A specific example of altering the carbon or substrate utilization to increase electron transfer is described further below in Example I where the one or more gene products confers glycerol processing activity. Other carbon or substrate utilization sources that can provide increased electron/mole ratio are well known in the art. These include carbohydrates such as glucose, fructose, arabinose, and xylose, as well as benzene.

Once the foreign genes are expressed in the host organism in a stable manner, consisting of, for example, two or more generations, the bioelectricity producing strains can be evaluated for enhanced electricity production in, for example, an electrode-containing chamber. Briefly, *G. sulfurreducens* can be grown in temperature-controlled, anaerobic, two-chambered electrode cells, under control of a potentiostat. The more tightly regulated the anaerobic conditions can be maintained, the greater the ATP consumption and the more efficient production of bioelectricity can be achieved. A graphite electrode can be poised at a fixed potential and serves as a consistent electron acceptor for the dissimilatory metal reducing bacteria. Output from multiple potentiostats can be continuously logged via a computerized data logging system, allowing multiple strains or conditions to be assessed simultaneously.

Using this system, for example, the rate of electron transport to electrodes can be directly measured under controlled conditions, and following measurement of the amount of biomass attached to electrodes, the rates can be expressed per unit cell mass for comparisons. To examine the abilities of unengineered and engineered strains, cells can be grown on electrodes using similar concentrations of a common electron donor, such as acetate. Following this establishment phase, for example, the medium surrounding the electrodes can be removed and replaced with fresh, anaerobic medium. The biofilms which remain attached to the electrodes can be measured, for example, for their ability to transfer electrons and the rate of electrical current generation could be measured to demonstrate the improved power generation capabilities. The improvement in the electrical current generation will enable the creation of microbial fuel cells that can generate higher power, thereby making the biological fuel cells of the invention commercially viable.

Table 2: Representative orthologs coding for the alpha, beta, and gamma subunits of the F_1 ATPase

Organism	alpha subunit		beta subunit		gamma subunit	
	Locus	Gene	Locus	Gene	Locus	Gene
Agrobacterium tumefaciens C58	Atu2624	atpA	Atu2622	atpD	Atu2623	atpG
Anabaena sp. PCC7120 (Nostoc sp. PCC7120)	all0005	atpA	all5039	atpB	all0004	atpC
Aquifex aeolicus	aq_679	atpA	aq_2038	atpD	aq_203	atpG2
Bacillus anthracis	BA5549	atpA	BA5547	atpD	BA5548	atpG
Bacillus halodurans	BH3756	atpA	BH3754	atpD	BH3755	atpG
Bacillus subtilis	BG10819	atpA	BG10821	atpD	BG10820	atpG
Bifidobacterium longum	BL0359	atpA	BL0357	atpD	BL0358	atpG
Blochmannia floridanus	Bfl006	atpA	Bfl008	atpD	Bfl007	atpG
Bordetella bronchiseptica	BB4607	atpA	BB4605	atpD	BB4606	atpG
Bordetella parapertussis	BPP4137	atpA	BPP4135	atpD	BPP4136	atpG
Bordetella pertussis	BP3286	atpA	BP3288	atpD	BP3287	atpG
Bradyrhizobium japonicum	bll0442	atpA	bll0440	atpD	bll0441	atpG
Brucella suis	BR1801	atpA	BR1799	atpD	BR1800	atpG
Buchnera aphidicola Bp	bbp006	atpA	bbp008	atpD	bbp007	atpG
Buchnera aphidicola Sg	BUsg006	atpA	BUsg008	atpD	BUsg007	atpG
Buchnera sp. APS	BU006	atpA	BU008	atpD	BU007	atpG
Campylobacter jejuni	Cj0105	atpA	Cj0107	atpD	Cj0106	atpG
Chromobacterium violaceum	CV0670	atpA	CV0672	atpD	CV0671	atpG
Clostridium acetobutylicum	CAC2867	atpA	CAC2865	atpD	CAC2866	atpG
Clostridium perfringens	CPE2189	atpA	CPE2187	atpB	CPE2188	atpG
Corynebacterium diphtheriae	DIP1050	atpA	DIP1052	atpD	DIP1051	atpG
Corynebacterium efficiens	CE1313	atpA	CE1315	atpB	CE1314	atpG
Coxiella burnetii	CBU1943	atpA	CBU1945	atpD	CBU1944	atpG
Enterococcus faecalis	EF2610	atpA	EF2608	atpD	EF2609	atpG
Escherichia coli CFT073	c4660	atpA	c4658	atpD	c4659	atpG
Escherichia coli K-12 MG1655	b3734	atpA	b3732	atpD	b3733	atpG
Escherichia coli K-12 W3110	JW3712	atpA	JW3710	atpD	JW3711	atpG
Escherichia coli O157 EDL933	Z5232	atpA	Z5230	atpD	Z5231	atpG
Geobacter sulfurreducens	GSU0111	atpA	GSU0113	atpD	GSU0112	atpG
Gloeobacter violaceus	gll2905	atpA	gll2570	atpB	glr4315	atpC
Haemophilus ducreyi	HD0008	atpA	HD0010	atpD	HD0009	atpG
Haemophilus influenzae	HI0481	atpA	HI0479	atpD	HI0480	atpG
Helicobacter hepaticus	HH0427	atpA	HH0429	atpD	HH0428	atpG
Helicobacter pylori 26695	HP1134	atpA	HP1132	atpD	HP1133	atpG
Lactobacillus plantarum	lp_2366	atpA	lp_2364	atpD	lp_2365	atpG
Lactococcus lactis	L8990	atpA	L6563	atpD	L8105	atpG
Leptospira interrogans	LA2779	atpA	LA2776	atpD	LA2778	atpG
Mycobacterium bovis	Mb1340	atpA	Mb1342	atpD	Mb1341	atpG
Mycobacterium leprae	ML1143	atpA	ML1145	atpD	ML1144	atpG
Mycobacterium tuberculosis H37Rv	Rv1308	atpA	Rv1310	atpD	Rv1309	atpG
Mycoplasma genitalium	MG401	atpA	MG399	atpD	MG400	atpG
Mycoplasma penetrans	MYPE600	atpA	MYPE620	atpD	MYPE610	atpG
Mycoplasma pneumoniae	D02_orf518	atpA	D02_orf475	atpD	D02_orf279	atpG
Neisseria meningitidis Z2491	NMA0517	atpA	NMA0519	atpD	NMA0518	atpG
Nitrosomonas europaea	NE0204	atpA	NE0206	atpD	NE0205	atpG
Oceanobacillus iheyensis	OB2977	atpA	OB2975	atpD	OB2976	atpG
Pasteurella multocida	PM1492	atpA	PM1494	atpD	PM1493	atpG
Photorhabdus luminescens	plu0042	atpA	plu0040	atpD	plu0041	atpG
Prochlorococcus marinus MED4	PMM1451	atpA	PMM1438	atpB	PMM1450	atpC
Prochlorococcus marinus MIT9313	PMT1467	atpA	PMT1451	atpB	PMT1466	atpC
Prochlorococcus marinus SS120	Pro1604	atpA	Pro1591	atpD	Pro1603	atpG
Pseudomonas aeruginosa	PA5556	at	PA5554	atpD	PA5555	atpG
Pseudomonas putida	PP5415	atpA	PP5413	atpD	PP5414	atpG
Pseudomonas syringae pv. tomato	PSPTO5601	atpA	PSPTO5599	atpD	PSPTO5600	atpG
Ralstonia solanacearum	RS02549	atpA	RS02547	atpD	RS02548	atpG
Rhodopseudomonas palustris	RPA0178	atpA	RPA0176	atpD	RPA0177	atpG
Rickettsia conorii	RC1237	atpA	RC1235	atpD	RC1236	atpG
Rickettsia prowazekii	RP803	atpA	RP801	atpD	RP802	atpG
Salmonella typhi CT18	STY3911	atpA	STY3913	atpD	STY3912	atpG
Salmonella typhi Ty2	t3652	atpA	t3654	atpD	t3653	atpG
Salmonella typhimurium	STM3867	atpA	STM3865	atpD	STM3866	atpG
Shewanella oneidensis	SO4749	atpA	SO4747	atpD	SO4748	atpG
Shigella flexneri 2457T	S3954	atpA	S3956	atpD	S3955	atpG
Shigella flexneri 301	SF3814	atpA	SF3812	atpD	SF3813	atpG
Sinorhizobium meliloti	SMc02499	atpA	SMc02501	atpD	SMc02500	atpG
Staphylococcus aureus Mu50 (VRSA)	SAV2105	atpA	SAV2103	atpD	SAV2104	atpG
Staphylococcus aureus MW2	MW2029	atpA	MW2027	atpD	MW2028	atpG
Staphylococcus aureus N315 (MRSA)	SA1907	atpA	SA1905	atpD	SA1906	atpG
Streptococcus agalactiae 2603	SAG0861	atpA	SAG0863	atpD	SAG0862	atpG
Streptococcus agalactiae NEM316	gbs0879	atpA	gbs0881	atpD	gbs0880	atpG
Streptococcus mutans	SMU.1530	atpD	SMU.1528	atpB	SMU.1529	atpC
Streptococcus pneumoniae R6	spr1362	atpA	spr1360	atpD	spr1361	atpG
Streptococcus pyogenes MGAS8232	spyM18_0816	atpA	spyM18_0818	atpD	spyM18_0817	atpG
Streptococcus pyogenes SF370	SPy0758	atpA	SPy0760	atpD	SPy0759	atpG
Streptomyces avermitilis	SAV2883	atpA	SAV2881	atpD	SAV2882	atpG
Streptomyces coelicolor	SCO5371	2SC6G5.15	SCO5373	2SC6G5.17	SCO5372	2SC6G5.16
Synechococcus sp. WH8102	SYNW0494	atpA	SYNW0512	atpB	SYNW0495	atpC
Synechocystis sp. PCC6803	sll1326	atpA	slr1329	atpB	sll1327	atpC
Thermoanaerobacter tengcongensis	TTE0635	atpA	TTE0637	atpD	TTE0636	atpG
Thermosynechococcus elongatus	tlr0435	atpA	tlr0525	atpB	tll0385	atpC
Tropheryma whipplei TW08/27	TW342	atpA	TW344	atpD	TW343	atpG
Tropheryma whipplei Twist	TW426	atpA	TW424	atpD	TW425	atpG
Wigglesworthia brevipalpis	Wbr0132	atpA	Wbr0130	atpD	Wbr0131	atpG
Wolinella succinogenes	WS0514	atpA	WS0516	atpD	WS0515	atpG
Xanthomonas axonopodis	XAC3651	atpA	XAC3649	atpD	XAC3650	atpG
Xanthomonas campestris	XCC0552	atpA	XCC0554	atpD	XCC0553	atpG
Xylella fastidiosa Temecula1	PD0430	atpA	PD0428	atpD	PD0429	atpG
Yersinia pestis CO92	YPO4123	atpA	YPO4121	atpD	YPO4122	atpG
Yersinia pestis KIM	y4137	atpA	y4135	atpD	y4136	atpG
Saccharomyces cerevisiae	YBL099W	atp1	YJR121W	atp2	YBR039W	atp3

Table 3: Representative orthologs coding for the A, B, and D subunits of the V_1 ATPase[16]

Organism Name	A subunit Locus	Gene	B subunit Locus	Gene	D subunit Locus	Gene
Archaeoglobus fulgidus	AF1166	atpA	AF1167	atpB	AF1168	atpD
Borrelia burgdorferi	BB0094	atpA	BB0093	atpB	BB0092	atpD
Chlamydia trachomatis	CT308	atpA	CT307	atpB	CT306	atpD
Clostridium perfringens	CPE1638	ntpA	CPE1637	ntpB	CPE1636	ntpD
Halobacterium sp. NRC-1	VNG2139G	atpA	VNG2138G	atpB	VNG2135G	atpD
Methanococcus jannaschii	MJ0217	atpA	MJ0216	atpB	MJ0615	atpD
Methanopyrus kandleri	MK1017	ntpA	MK1673	ntpB	MK1674	ntpD
Methanosarcina acetivorans	MA4158	atpA	MA4159	atpB	MA4160	atpD
Porphyromonas gingivalis	PG1803	atpA	PG1804	atpB	PG1805	atpD
Pyrobaculum aerophilum	PAE0663	atpA	PAE1146	atpB	PAE0758	atpD
Pyrococcus abyssi	PAB2378	atpA	PAB1186	atpB	PAB2379	atpD
Streptococcus pyogenes MGAS315 (serotype M3)	SpyM3_0120	ntpA	SpyM3_0121	ntpB	SpyM3_0122	ntpD
Streptococcus pyogenes SF370 (serotype M1)	SPy0154	ntpA	SPy0155	ntpB	SPy0157	ntpD
Sulfolobus solfataricus	SSO0563	atpA	SSO0564	atpB	SSO0566	atpD

Throughout this application various publications have been referenced within parentheses. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains.

It is understood that modifications which do not substantially affect the activity of the various embodiments of this invention are also included within the definition of the invention provided herein. Those skilled in the art will readily appreciate that the specific examples and studies detailed above are only illustrative of the invention. Accordingly, specific examples disclosed herein are intended to illustrate but not limit the present invention. It also should be understood that, although the invention has been described with reference to the disclosed embodiments, various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

Example I

Engineering *Geobacter sulfurreducens* for Enhanced Electricity Production

Previous studies have reported that *Geobacteraceae* can harvest electricity from waste organic matter by oxidizing organic compounds to carbon dioxide coupled to electron transfer onto electrode surfaces. Although the conversion of organic matter to electricity in this manner can be efficient, the process is slow. Furthermore, *Geobacter* species have a selective number of electron donors they can utilize and thus fermentative

organisms are required in order to convert complex organic substrates to the organic acids that *Geobacter* species can oxidize. This Example describes the engineered expansion of *Geobacter* species substrate range to accelerate their rate of electron transfer in order to enhance electricity production.

The developmental design for engineered expansion of substrate range employed a genome-based *in silico* model of the physiology of *Geobacter sulfurreducens*. For example, glycerol has a relatively high electron per mole ratio, and the model predicted that glycerol could be used as an electron donor if the appropriate transporter was present. This prediction was confirmed by cloning the glycerol uptake and processing operon from *Desulfovibrio vulgaris*, another δ-proteobacterium. As predicted by the *in silico* model, the engineered strain of *G. sulfurreducens* had the ability to grow with glycerol as the sole electron donor. Furthermore, a hierarchical optimization strategy was used to identify specific *in silico* gene deletions that could enhance the rate of electron transport during growth on glycerol or acetate. The *in silico* prediction that deletions in ATP synthesizing reactions will lead to increased activity of the ATP synthase and an enhanced rate of electron transfer was confirmed. These studies further corroborate bioelectricity using the engineered organisms and methods of the invention and also demonstrate that genome-based *in silico* modeling of microbial physiology can significantly augment the design and implementation process for bioelectricity improvement and optimization.

Briefly, generation and analysis of an *in silico* metabolic network of *G. sulfurreducens* was performed using the system and methods described in U.S. Patent Application 10/173,547, filed June 14, 2002, entitled Systems and Methods for Constructing Genomic-Based Phenotypic Models, which is incorporated herein by reference in its entirety. These *in silico* systems and methods allow for the identification of potential substrates having a high electron/mole ration. As shown in Figure 1, *G. sulfurreducens* was predicted to have a flux on acetate of 71 mmol/10mM acetate. When grown on glycerol, the *in silico G. sulfurreducens* model also predicted a flux of 65 mmol/10 mM glycerol.

A modified *G. sulfurreducens* was constructed to enable it to utilize the alternative substrate glycerol by recombinantly incorporating genes encoding glycerol processing functions operably linked for expression. In this regard, a glycerol processing

operon from *D. vulgaris* was cloned in pRG plasmid (Dglyop) and expressed in *G. sulfurreducens* using methods well known to those skilled in the art. As shown in Figure 2 (top), the engineered strain containing the glycerol transporter was able to grow in NBAF with glycerol (Gly) while the wild type was comparable to growth with a minimum concentration of Acetate (Ac). The engineered strain also was able to grow with glycerol as the only carbon and electron source on iron oxide (Figure 2 (bottom)).

The modified glycerol-utilizing *G. sulfurreducens* strain was engineered to increase the respiration rate for efficient bioelectricity production. Briefly, the optknock framework of the *in silico* strain was used to identify potential gene knock-out that would increase the rate of electron transport. All predicted knockouts were identified as directly contributing to ATP synthesis. One means of increasing the respiration rate can be by deleting one or more of the identified genes. Alternatively, the modified glycerol-utilizing *G. sulfurreducens* strain was engineered to contain an inducible ATPase. To do this, the hydrolytic portion of the F_1 domain of the membrane-bound $(F_1F_0)H^+$ ATPase was cloned and expressed under the control of an IPTG inducible promoter. The inducible promoter utilized was the lac Z promoter and the ATPase subunits α, β and γ were expressing as an operon as illustrated in the construct shown in Figure 3. Further, the left panel shows growth of the modified ATPase expressing *G. sulfurreducens* strain under increasing ATPase induction. The right panel shows the level of respiration in the presence or absence of increased respiration caused by ATPase induction. The results indicate that high respiration rate induced by the ATP drain reduced cell yield. However, high IPTG induction levels of ATP consumption also increased the yield of iron reduction by more than threefold.

To demonstrate the ability of the modified glycerol-utilizing *G. sulfurreducens* strains expressing ATPase can generate electricity and directly transfer of electrons to an electrode, these engineered *Geobacter* cells were grown in an anode chamber containing acetate as the electron donor and a graphite electrode as the electron acceptor. The anode was connected to the cathode via a 560-ohm fixed resistor. This two-chambered microbial fuel cell is shown in the left panel of Figure 4. The right panel of Figure 4 shows current generation following ATPase induction. The results indicate that following IPTG addition to the anode side of the microbial fuel cell, the current increase is observed only in the engineered *Geobacter* strain having an inducible F1-

ATPase activities. These results corroborate that bioelectricity can be produced by modifying a cell or organism to increase ATP consumption. These results further exemplify that numerous genetic designs other than ATPase expression can be implemented to increase the level of ATP consumption for enhanced production of bioelectricity. Identification, design and implementation can be particularly efficient using *in silico* models to identified reactions and pathways that can be modified to confer physiological properties beneficial to enhancing ATP consumption. Thus, the results further confirm that microbial fuel cells converting renewable biomass to electricity can be generated with high efficiency.

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What is claimed is:

1. A microbial fuel cell, comprising a dissimilatory metal-reducing microbe expressing exogenous or native ATPase subunits, said ATPase subunits assembling into an active ATP synthase and consuming ATP in a futile cycle.

2. The microbial fuel cell of claim 1, wherein said dissimilatory metal-reducing microbe comprises an organism selected from the organisms set forth in Table 1.

3. The microbial fuel cell of claim 1, wherein one or more exogenous ATPase subunits comprise a subunit selected from the ATPase subunits set forth in Tables 2 or 3.

4. The microbial fuel cell of claim 1, wherein said futile cycle of ATP consumption produces a flow of electrons through the respiratory machinery at a rate higher than endogenous electron flow of about 2-fold, preferable about 5-fold, more preferably about 10-fold or more.

5. The microbial fuel cell of claim 1, wherein said futile cycle of ATP consumption produces a flow of electrons through the respiratory machinery at a rate higher than endogenous electron flow of about 20-fold, preferably about 25-fold, more preferably about 50-fold or more.

6. The microbial fuel cell of claim 1, wherein said futile cycle of ATP consumption produces a flow of electrons through the respiratory machinery at a rate higher than endogenous electron flow of about 100-fold or more.

7. A microbial fuel cell, comprising a dissimilatory metal-reducing microbe expressing one or more exogenous genes encoding a gene product that promotes ATP consumption, said gene products of said one or more exogenous genes having an activity that reduces ATP synthesis, increases ATP consumption or both.

8. The microbial fuel cell of claim 7, wherein said one or more gene products increase ATP consumption through a futile cycle.

9. The microbial fuel cell of claim 7, wherein said one or more gene products increase ATP consumption through altering a metabolic reaction directly involved in ATP synthesis.

10. The microbial fuel cell of claim 7, wherein said one or more gene products increase ATP consumption through altering a metabolic reaction indirectly involved in ATP synthesis.

11. A microbial fuel cell, comprising a dissimilatory metal-reducing microbe expressing one or more exogenous genes encoding a gene products that increases the electron/mole ratio compared to an unmodified microbe, wherein the increased ratio enhances electron transfer to an electrode.

12. The microbial fuel cell of claim 11, wherein said one or more gene products comprise a glycerol processing operon.

13. The microbial fuel cell of claim 11, wherein said one or more gene products confers the ability of the microbe to use a substrate that is not possible to metabolize without the exogenous genes.

14. A method of producing electricity from an microbial organism, comprising:

(a) culturing a microbial fuel cell under anaerobic conditions sufficient for growth, said microbial fuel cell comprising a dissimilatory metal-reducing microbe expressing exogenous ATPase subunits, said ATPase subunits assembling into an active ATP synthase and consuming ATP in a futile cycle when grown under anaerobic conditions, and

(b) capturing electrons produced by an increased ATP demand with an electron acceptor.

15. The method of claim 14, wherein said dissimilatory metal-reducing microbe comprises an organism selected from the organisms set forth in Table 1.

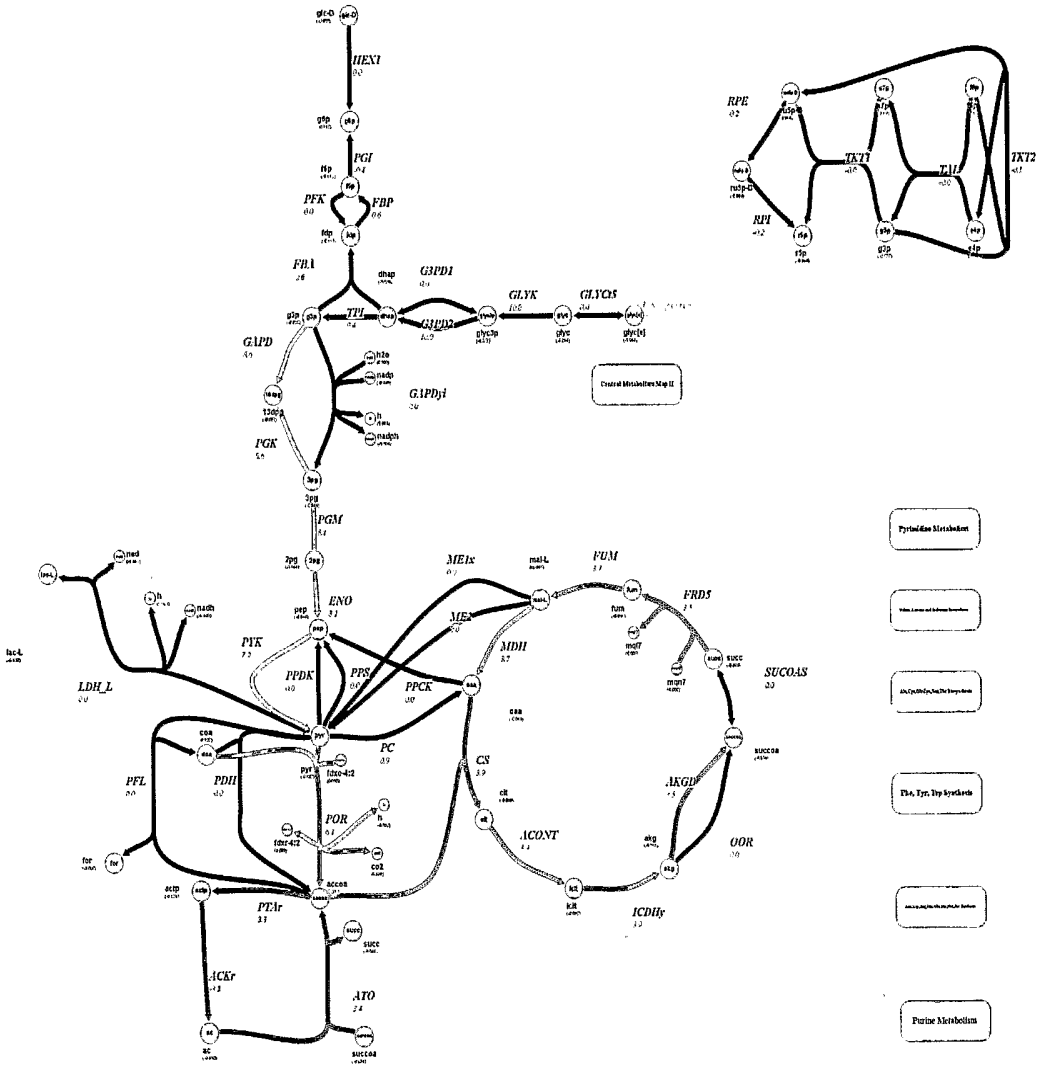
16. The method of claim 14, wherein one or more exogenous ATPase subunits comprise a subunit selected from the ATPase subunits set forth in Tables 2 or 3.

17. The method of claim 14, wherein said futile cycle of ATP consumption produces a flow of electrons through the respiratory machinery at a rate higher than endogenous electron flow of about 2-fold, preferable about 5-fold, more preferably about 10-fold or more.

18. The method of claim 14, wherein said futile cycle of ATP consumption produces a flow of electrons through the respiratory machinery at a rate higher than endogenous electron flow of about 20-fold, preferably about 25-fold, more preferably about 50-fold or more.

19. The method of claim 14, wherein said futile cycle of ATP consumption produces a flow of electrons through the respiratory machinery at a rate higher than endogenous electron flow of about 100-fold or more.

20. The method of claim 14, wherein said electron acceptor comprises a graphite electrode.

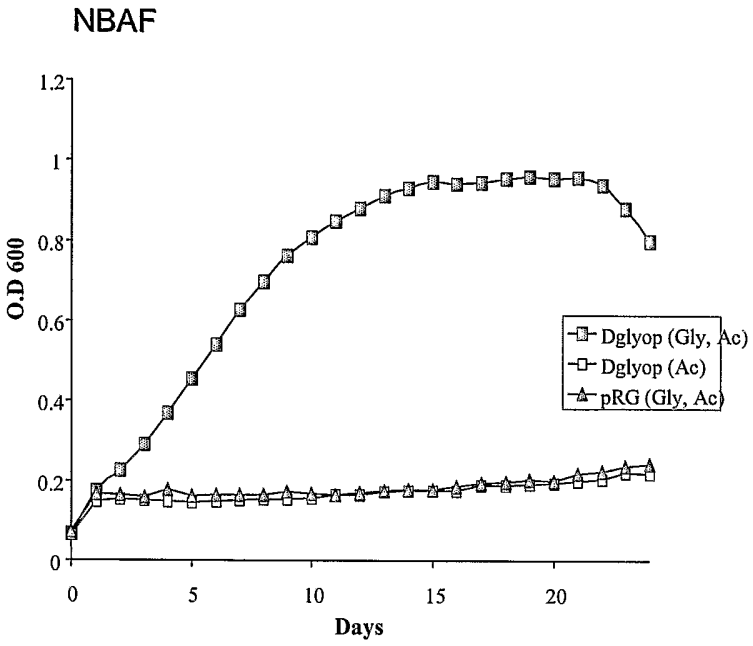


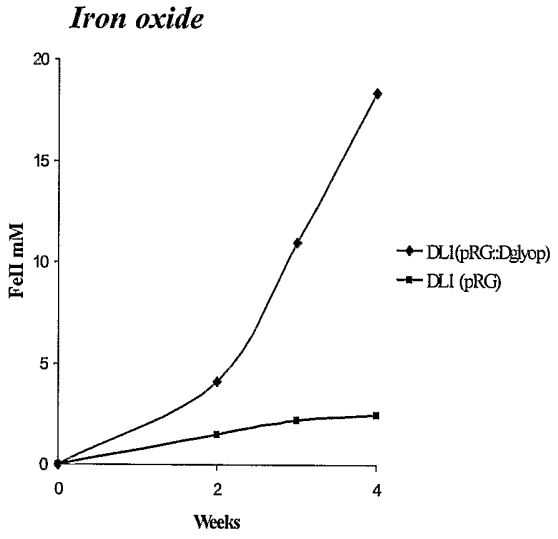
•The analysis of the metabolic network of *G. sulfurreducens* using the Simpheny™ platform allows us to identify potential substrates with high electron/mol ratio.

•**Predicted flux** Acetate: 71 mmol/10mM Acetate

Glycerol:65 mmol/10 mM Glycerol

Figure 1. Genome based *in silico* model





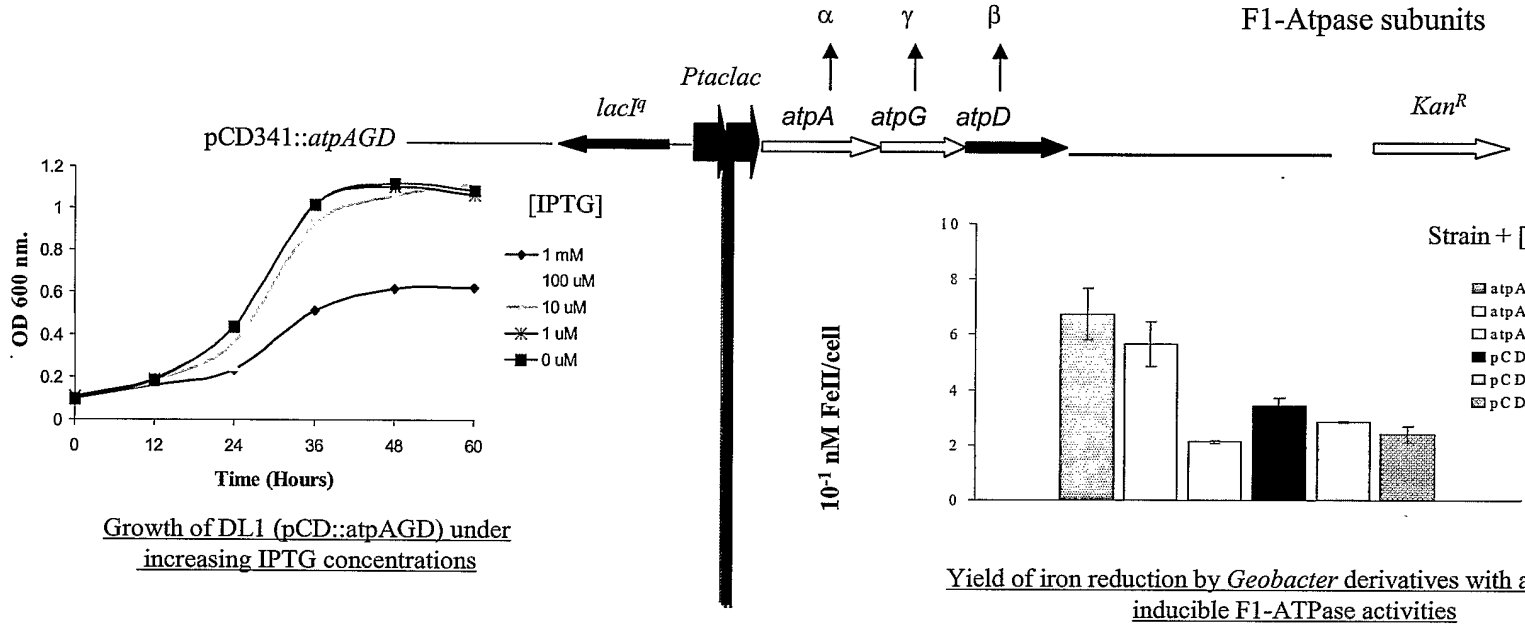
- The glycerol processing operon from *D. vulgaris* was cloned in pRG plasmid (Dglyop) and expressed in *G. sulfurreducens*.
- The engineered strain containing the glycerol transporter was able to grow in NBAF with glycerol (Gly) while the wild type was comparable to growth with a minimum concentration of Acetate (Ac).
- The engineered strain was also able to grow with Glycerol as the only carbon and electron source on iron oxide.

Figure 2. Increasing Substrate Range

Figure 3. Increasing Respiration Rate

We used the optknock framework to identify potential gene knock-out that would increase the rate of electron transport.

- All the predicted knockouts directly contributed to ATP synthesis.
- Instead of deleting these metabolic pathways we constructed an inducible Atpase.
- The hydrolytic part of the F1 domain of the membrane-bound $(F_1F_0)H^+$ Atpase was cloned and expressed under the control of an IPTG inducible promoter.



Growth of DL1 (pCD::atpAGD) under increasing IPTG concentrations

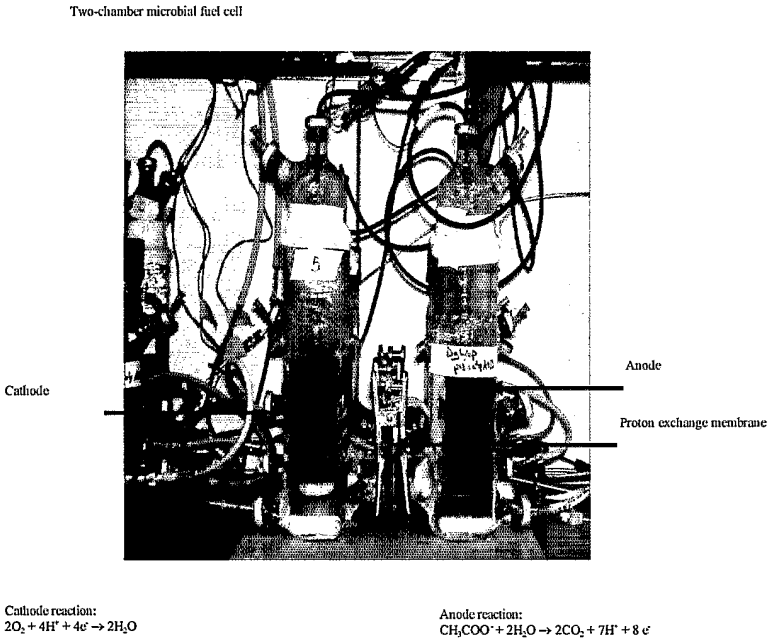
Yield of iron reduction by *Geobacter* derivatives with and without inducible F1-ATPase activities

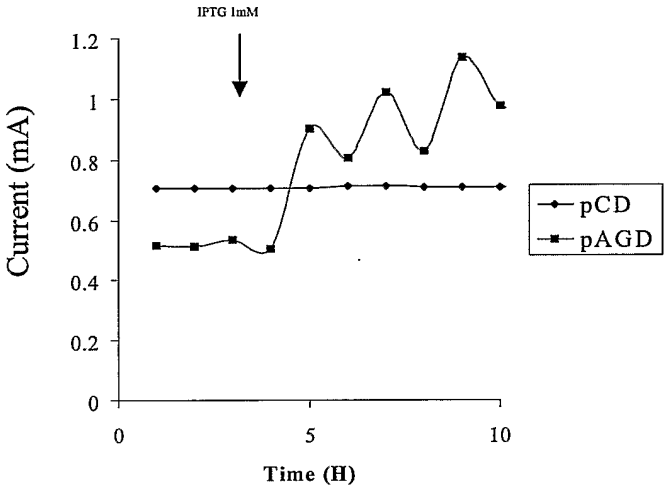
Respiration Rate :No induction: 0.88 mM Succinate/OD/h 1mM IPTG : 1.13 mM Succinate/OD/h

➢The high respiration rate induced by the ATP drain causes low cell yield.

➢High IPTG induction levels of ATP drain increases the yield of iron reduction more than threefold.

Figure 4. Microbial fuel cell studies





- Geobacter cells were grown in the anode chamber containing acetate as the electron donor and a graphite electrode as the sole electron acceptor. The anode was connected to the cathode via a 560-ohm fixed resistor.

- Following IPTG addition to the anode side of the microbial fuel cell, the current increase is observed only in *Geobacter* derivative with the inducible F1-ATPase activities.