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Isolation of improved free fatty acid overproducing strains of *Escherichia coli* via Nile red based high-throughput screening

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Abstract

Biological production of hydrocarbons is an attractive strategy to produce drop-in replacement transportation fuels. Several methods for converting microbially-produced fatty acids into reduced compounds compatible with petrodiesel have been reported. For these processes to become economically viable, microorganisms must be engineered to approach the theoretical yield of fatty acid products from renewable feedstocks such as glucose. Strains with increased titers can be obtained through both rational and random approaches. While powerful, random approaches require a genetic selection or facile screen that is amenable to high throughput platforms. Here, we present the use of a high throughput screen for fatty acids based on the hydrophobic dye Nile red. The method was applied to screening a transposon library of a free fatty acid overproducing strain of *Escherichia coli* in search of high producing mutants. Ten gene targets were identified via primary and secondary screening. A strain comprising a clean knockout of one of the identified genes led to a 20% increase in titer over the baseline strain. A selection strategy that combines these findings and can act in an iterative fashion has been developed and can be used for future strain optimization in hydrocarbon producing strains.

Introduction

Declining oil reserves and increasing concern over greenhouse gas emissions have motivated research to develop replacement technologies for existing petrochemical processes [1]. In particular, identifying alternative sources of energy dense fuels for use in the heavy transportation sector (i.e. diesel or jet fuel) is a major challenge because these needs are not likely to be met with ethanol, the leading biofuel. While biodiesel, the product of transesterification of waste oils with short chain alcohols [2], shows promise as a diesel fuel, the current supply of oils limits the volume that can be produced using existing technologies. Alternatively, microbial hydrocarbons generated from lignocellulosic feedstocks could bypass many of the challenges associated with conventional biodiesel [3].

Fatty acids are an attractive intermediate that can be produced in microorganisms and subsequently converted to hydrocarbon biofuels. Fatty acids are synthesized in nearly all

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organisms for incorporation into cell membranes, which means that any advantageous microbe could be engineered to produce hydrocarbon biofuels. Furthermore, processes for converting fatty acids to several reduced compounds have been reported. For example, fatty acids can be extracted from culture and catalytically reduced or decarboxylated to yield alkanes [4,5]. Alternatively, fatty acids can be enzymatically reduced to fatty alcohols [6], esterified to yield fatty acid methyl esters [6,7], or reduced to alkanes or olefins via decarboxylation[8,9]. The versatility of the fatty acid platform has led to a significant research aimed at increasing flux through the fatty acid biosynthetic pathway [10].

The challenge in engineering fatty acid biosynthesis is circumventing the native regulation that controls lipid synthesis and membrane homeostasis [11,12]. In *Escherichia coli*, fatty acid biosynthesis begins with the condensation of CO₂ with acetyl-CoA, a central metabolite generated by many sugar degradation pathways, to yield malonyl-CoA, the monomer used to synthesize acyl-chains. Iterative cycles of extension and reduction occurs with the acyl chain bound to an acyl-carrier protein (ACP). After seven or eight elongation cycles, long chain acyl-ACPs are used to synthesize membrane lipids. The long chain-acyl-ACP also acts as a key regulator in the cell by inhibiting enzymes involved in fatty acid initiation and the elongation cycle[11]. Prior engineering work in *E. coli* has focused on alleviating the feedback regulation of long-chain acyl-ACP by heterologous expression of an acyl-ACP thioesterase, an enzyme that catalyzes a hydrolysis reaction and generates a free fatty acid [13]. The thioesterase catalyzed reaction provides a product sink and decreases the amount of long-chain acyl-ACP in the cell, leading to upregulation of fatty acid biosynthesis. To further increase free fatty acid production, researchers have removed genes whose products catalyze steps of fatty acid degradation (i.e. β -oxidation) and altered central carbon metabolism to increase supply of fatty acid precursors [4,14,15]. These rational approaches have led to significant increases in fatty acid production, but titers remain below 50% of the theoretical yield from glucose, (e.g. 0.35 gram lauric acid per gram glucose) Further gains in productivity may require the alteration of factors indirectly involved in fatty acid biosynthesis, regulation, or precursor forming reactions. These targets may be difficult to predict using rational metabolic engineering approaches. Alternatively, implementation of a high throughput screen for fatty acid production would enable the use of combinatorial or random approaches to identify new targets linked with increased fatty acid production capabilities.

Random approaches such as mutagenesis have been used for improving characteristics of organisms with much success. For example, chemical mutagenesis has been used to increase the production of antibiotics and other medically important compounds, such as penicillin and nystatin [16]. In addition, several combinatorial methods for targeted strain improvement have been described such as genomic library complementation [17], transposon mutagenesis, engineering transcriptional machinery [18], altering ribosome binding sites [19], and altering post transcriptional processing [20]. However, to effectively apply combinatorial approaches, either a genetic selection or facile phenotypic screen must be available. Initial work describing these methods was done using easily accessible phenotypes, such as red pigmentation from lycopene overproduction in *E. coli* [21] or using reporter proteins (e.g. β -galactosidase, GFP). Indirect methods such as using the pigment melanin to detect tyrosine production [22] and the development of biosensors [23] have also

been demonstrated to be effective methods of screening combinatorial libraries. While these examples show the ability of high throughput screening to be a useful tool for strain improvement, most systems do not have the ability to use such a screen to allow for a combinatorial approach to strain improvement.

Here, we present the results of a high throughput screen of a transposon library of a free fatty acid overproducing strain of *E. coli* (Figure 1). The lipid soluble fluorescent dye, Nile red [24], was used to identify cultures containing high titers of fatty acids similar to prior studies which used the dye to evaluate intracellular lipid content of bacteria [25], microalgae [26,27], and yeast [28]. To test the ability of a Nile red assay to identify improved fatty acid producing strains, we implemented a robotic high throughput screen on a library of transposon mutants generated in a fatty acid overproducing strain of *E. coli* [4]. Despite a high false discovery rate, a strain producing 20% more free fatty acid was identified.

Materials and Methods

Bacterial strains and growth conditions

Escherichia coli RL08 (K-12 MG1655 *araBAD fadD*), described previously [4], was used as the base strain for this study. *E. coli* RL08 was transformed with pBAD33-BTE, a low copy plasmid harboring an arabinose-inducible expression cassette controlling expression of a gene encoding a medium chain acyl-ACP thioesterase from *Umbellularia californica* (BTE) [29]. The resulting strain overproduces C₁₂ and C₁₄ free fatty acids when induced with arabinose [4]. All strains were grown at 37°C in Luria Bertani (LB) broth supplemented with the appropriate antibiotics (chloramphenicol 34 µg/mL, kanamycin 25 µg/mL) unless specified otherwise.

Nile red assay

Nile red was used as a fluorescent proxy for free fatty acid content in cultures of *E. coli* RL08 pBAD33-BTE. The dye fluoresces in the presence of hydrophobic compounds (i.e. fatty acids, membrane lipids) and has been used to rapidly screen through the lipid content of various organisms up to concentrations of 5g/L [28]. However, as the optimal emission spectra varies with the hydrophobicity of the compound of interest [24], it was necessary to select excitation emission windows that gave the maximum fluorescence for C₁₂ free fatty acids. Nile red fluorescence was measured at 550nm excitation with emission at 630nm using a Tecan M1000 microplate reader. Five µL of an overnight culture grown from a single colony was added to an individual well of a black, clear-bottomed 96-well polystyrene plate (Costar, Corning, Inc, Corning, NY) containing 195µL of LB supplemented with 0.4% glycerol and 0.2 % arabinose. Plates were incubated for 24h with shaking at 37°C to allow for free fatty acid production. To each well, 5 µL of 0.025mg/mL Nile red in DMSO was added. The plate was incubated at room temperature for ten minutes.

Transposon library construction

To prepare a library of mutagenized fatty acid overproducing strains, an aliquot of electrocompetent *E. coli* RL08 harboring pBAD33-BTE was transposed with EZ-Tn5™ <R6Kγ_{ori}/KAN-2>Tnp Transposome™ Kit (Epicentre Biotechnologies, Madison, WI)

according to the manufacturer's instructions. Transformants were plated on LB-agar containing 25µg/mL kanamycin. Individual clones were arrayed into 96-well cultures by a RapidPick™ robotic colony picker (Hudson Robotics, Inc, Springfield, NJ) for archival storage and subsequent Nile red screening.

Nile red high throughput screening

The primary screen was performed using a Tecan Freedom EVO liquid handling robot. Microtiter plates containing overnight cultures of arrayed mutants were used to inoculate a corresponding plate containing 200µL of LB with 0.4% glycerol and 0.2% arabinose. Each plate was incubated for 24h with shaking at 37°C after which Nile red was added and the fluorescence of each well was determined as described above. Thirty plates, containing ~2800 mutants and ~100 controls (*E. coli* RL08 pBAD33-BTE) were screened. Sixty strains that demonstrated high fluorescence (>3 standard deviations above control) were struck out onto LB-agar plates from freezer stocks made prior to the primary screen. For secondary screening, individual colonies were used to inoculate test tubes containing 5mL of LB supplemented with 0.4% glycerol and 0.2% arabinose. Cultures were incubated for 24h at 37°C with shaking. The fatty acids produced by each culture were extracted, derivatized to yield the corresponding methyl ester (FAME), and quantified by GC/MS using commercial standards as described previously [4].

Transposon insertion site determination

The location of transposon insertion sites were determined by sequencing plasmids generated from fragments of genomic DNA isolated from interesting mutants. Genomic DNA was isolated from five mL of an overnight culture using a Wizard genomic DNA isolation Kit (Promega, Madison, WI). Genomic DNA was digested with a mixture of *Pvu* II, *Eco*R V, and *Sap* I (New England Biolabs, Ipswich, MA) for 30m at 37°C. Digested genomic DNA (250ng) was ligated overnight at 16°C using T4 DNA ligase (New England Biolabs). Ligations were transformed into ElectroMax EC100D pir+ *E. coli* cells (Epicentre Biotechnologies, Madison, WI) and the resulting cells were plated on LB-agar containing (25µg/mL kanamycin). For each transformation, plasmid DNA was isolated from two separate overnight cultures inoculated with a colony from the corresponding plate. Plasmid DNA was sequenced by the UW Biotechnology center using either an M13 Forward Primer (5' CTACCCTGTGGAACACCTACATCT 3') or an M13 Reverse Primer (5' ACCTACAACAAAGCTCTCATCAACC 3') whose target sequence is located within the transposon. The resulting sequences were aligned with the *E. coli* genome using the Basic Local Alignment Search Tool (BLAST) from NCBI to identify the location of the transposon insertion [30].

Construction and analysis of clean disruption mutants

Clean disruption mutants (i.e. no transposon) were generated for three of the top ten strains. The National BioResource Project (NIG, Japan) [31] maintains a collection of single gene disruption mutants harboring removable kanamycin or chloramphenicol resistance cassettes. The collection's mutant strain corresponding to the site of each transposon insertion was transferred to *E. coli* RL08 via P1 phage transduction as described by Miller [32]. The resistance marker was removed using the plasmid pcp20 which was then cured from the

strain as described by Yazdani [33]. Each disruption mutant was confirmed by polymerase chain reaction using primers flanking the gene disruption site.

Fatty acid production from engineered strains was confirmed in biological triplicate. Fifty milliliters of LB supplemented with 0.4% glycerol was inoculated to an OD₆₀₀ of 0.02 using starter cultures grown overnight in LB. At an OD₆₀₀ of 0.2 each culture was induced with 0.2% arabinose and incubated at 37°C with shaking. After 24 hours, 400 µL of a 1:1 ethanol:antifoam 204 (Sigma Aldrich, St. Louis, MO) antifoaming solution was added. After all foam had collapsed, fatty acids were extracted, derivatized, analyzed by GC/MS (2 technical replicates), and quantified as described above [4].

Results and Discussion

Determining Z-factor/significance of the screen

Nile red is a fluorescent dye whose intensity scales with its hydrophobic environment [24]. It has been used as a proxy for the production of hydrophobic compounds in microorganisms [25,28] and is applicable for high throughput screening [26]. Forty-eight wells of induced and uninduced cultures of *E. coli* RL08 pBAD33-BTE were used as positive and negative controls respectively to calculate a Z-factor [34] and optimize two parameters of the assay, detection from above or below the plate (a top read versus bottom read) and Nile red solvent (DMSO, ethanol, water). Z-factor is a metric used to assess the degree of separation that can be confidently detected among two populations of cells. We chose to use induced and uninduced samples to ensure that the assay could differentiate low and high levels of free fatty acid production. The best assay conditions (Figure 2) used dimethyl sulfoxide (DMSO) as a solvent for Nile red and a bottom read. The large difference in fluorescence intensity resulted in a Z-factor of 0.77 which was suitable for application of high throughput screening. Based on prior work by Zhang [34], a Z-factor of greater than 0.5 is indicative of an excellent assay as a value of 0.5 indicates there is at least twelve standard deviations between the means of the positive hit and negative hit.

Primary Screen

To test the proposed Nile red high throughput screen and to search for native genes that limit free fatty acid production in *E. coli*, a library of transposon mutants in *E. coli* RL08 pBAD33-BTE was created. Members of the library were arrayed using a robotic colony picker and used to inoculate cultures in 96-well microtiter plates. A robotic liquid handling system was used to screen the fatty acid production of ~2800 clones (subsaturating coverage). After 24 hours of incubation at 37°C, Nile red was added to each well and the fluorescence of each culture was recorded (Figure 3). The wide distribution included several hundred wells demonstrating increased fluorescence relative to the base strain.

A set of statistical criteria was used to determine which strains (“hits”) to pursue. One of the more common statistical scoring methods is the Z-score, which is computed on a plate by plate basis and is defined in equation (1),

$$Z = \frac{x_i - x}{SD} \quad (1)$$

where x_i is the raw value of an individual sample, x is the mean of all the sample values on each plate (control and variable), and SD is the standard deviation of those values. A Z-score of greater than 3 indicated the observed fluorescence did not fall within a 99% confidence interval (of a normal distribution) around the mean fluorescence of the baseline strain. A second metric, the B-score [35], which accounts for positional effects, was also used to select hits from the primary screen. The B-score was calculated using individual residuals according to equation (2).

$$r_{ijp} = x_{ijp} - (\mu_p + R_{ip} + C_{jp}) \quad (2)$$

The residual (r_{ijp}) for row i and column j on the p -th plate was obtained by the difference between the observed value (x_{ijp}) and the sum of the estimated average of the plate (μ_p), the estimated systematic measurement row offset for row i on plate p (R_{ip}), and the estimated systematic measurement column offset for column j on plate p (C_{jp}). The control plates were used to generate the systematic column and row offsets. The B-score was computed using these residuals as seen in equation (3),

$$B - score = \frac{r_{ijp}}{(1.4826 \times MAD_p)} \quad (3)$$

where 1.4826 is a multiplicative constant and MAD_p equals $\text{median}[|r_{ijp} - \text{median}(r_{ijp})|]$.

The B-score, which accounts for positional effects, was used as the primary source for selecting hits, with Z-score acting as a backup to ensure no hits were missed. The primary screen identified 48 transposon mutants with a B-score of greater than 3, indicating that these transposon mutants generated Nile red fluorescence at a level more than three standard deviations away from positionally corrected mean. Eleven additional mutants which generated a Z-score of greater than three were also selected, bringing the total number of transposon mutants selected up to 59.

Secondary Screen

As a secondary screen, the fatty acid titer from 5mL cultures of the 59 “hits” was assayed by GC/MS. After 24h of incubation, fatty acids were extracted and derivatized as methyl-esters. The resulting FAME peaks were identified by GC/MS and normalized by compiling ratios of sample peak area to the $C_{15:0}$ internal standard peak areas (Figure 4). In 57 of the 59 selected transposon mutants, the peak area ratio for the $C_{12:0}$ species, the predominant free fatty acid, was greater than the positive control (*E. coli* RL08 pBAD33-BTE). However, only 11 of the 57 colonies (Table 1) contained a peak area ratio more than three standard

deviations away from the mean of the positive control indicating that the Nile red assay has a significant false discovery rate.

Determining affected genes

For ten of the top eleven strains identified in the secondary screening, we identified the site of transposon integration. Taking advantage of the origin of replication located within the transposon, we isolated genomic DNA from each mutant, digested with a mixture of restriction enzymes, and circularized the resulting fragments by ligation. Ligations were transformed into *E. coli* and plated on kanamycin to select for strains harboring DNA fragments containing both an origin and the kan^R marker. Oligonucleotide primers were designed to prime DNA replication at either end of the transposable element to allow for sequencing of the region of the genomic DNA surrounding the transposon. Once sequences were obtained, they were used as probes to identify the region of insertion using the Basic Local Alignment Search Tool (BLAST); the results of which can be seen in Table 1. The loci disrupted by ten hits were all unique and included metabolic genes, transporters, and hypothetical proteins.

Tertiary screening of RL08 *fadB*

Of the ten genes identified, *fadB* was of interest because of its function within *E. coli* fatty acid catabolism. The *fadB* gene product catalyzes two reactions in the β -oxidation cycle (Figure 5), ultimately converting an enoyl-CoA into a 3-ketoacyl-CoA. Active β -oxidation would enable the breakdown of free fatty acids produced in the engineered strains. *E. coli* RL08 contains a deletion of *fadD*, encoding the first enzyme in fatty acid β -oxidation, and is unable to grow on oleic acid (C18:1). However, under anaerobic conditions FadK has been shown to carry out a similar reaction to that catalyzed by FadD (acyl-CoA synthetase) [46] and may enable limited flux into β -oxidation. To test the effect of β -oxidation genes on free fatty acid titers, we generated precise disruption mutants of *fadB* and *fadE* in *E. coli* RL08. The gene *fadE* was chosen as its gene product catalyzes the conversion of the acyl-CoA to the enoyl-acyl-CoA, the reaction immediately preceding the reactions catalyzed by FadB [47]. Three biological replicates of each clean mutant harboring the pBAD33-BTE plasmid were grown in 250-mL shake flasks with 50 mL LB medium supplemented with 0.4% glycerol as a carbon source. Total fatty acids were extracted and derivatized for analysis by GC/MS 24 hours after induction. Two technical replicates were taken from each biological replicate culture. The average titers of the major fatty acid species (C₁₂-C₁₈) for each strain are displayed in Figure 6. The baseline strain, *E. coli* RL08 pBad33-BTE produced a titer of 743 ± 33 mg/L C₁₂ fatty acids, while the corresponding titers from RL08 *fadE* pBad33-BTE and RL08 *fadB* pBad33-BTE strains were 766 ± 23 and 890 ± 29 mg/L respectively. The resulting data from Figure 6 shows a 20% increase in titer for RL08 *fadB* compared to a 3% increase for RL08 *fadE* over RL08. Amounts of other fatty acid species (C₁₄, C₁₆, and C₁₈) were quantified, but no statistically significant difference was observed.

Conclusions

Although the microbial production of fatty acids has been extensively studied and reviewed in recent years [10], application of high throughput screening to increasing free fatty acid

production in *E. coli* has not been reported. Here, we report that use of Nile red as a primary screen is capable of identifying mutants possessing superior free fatty acid production capabilities. Unfortunately, the Nile red assay has a high degree of variability which reduced the Z-factor for the high throughput screen and led to a high number of false discoveries. While the secondary screen correctly identified 11 out of 59 mutants from the primary screen as capable of producing free fatty acids at a level that was three standard deviations greater than the base strain, fatty acid titers of the majority of “hits” fell between the base strain and the mean plus three standard deviation level. In addition, two false positives produced significantly less free fatty acid than the base strain. Variation in fatty acid production may be due to the different environments found in a 96-well plate versus a 5ml culture tube versus a 250ml shake flask culture. Our data suggests that the screen will work well in situations where large differences in fatty acid production (i.e. fluorescence) can be generated, such as identifying novel thioesterase genes. The screen is not optimal for identifying marginal improvements over strains that already overproduce large amounts of hydrophobic compounds. In these situations, a Nile red primary screen must be coupled with significant secondary screening to be effective. Nevertheless, our screening efforts identified a transposon mutant capable of producing 20% more free fatty acid relative to the base strain. We hypothesize that deleting *fadB* in addition to *fadD* further reduces or completely eliminates β -oxidation and thereby prevents consumption of free fatty acids in stationary phase. Given that *fadD* mutants cannot grow on exogenous free fatty acids [4,46], it is surprising that β -oxidation needs to be further impaired to prevent consumption of the desired product. This discovery motivates the use of additional combinatorial approaches to identify improved strains with the ultimate goal of approaching the theoretical limit of free fatty acid production. Further work will be needed to explain how deletions in the other 9 hits contribute to increasing free fatty acid titer. Additionally, we anticipate that applying an iterative process to create multiple mutations through random approaches will increase free fatty acid production in *E. coli*.

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Figure 1.

Procedure for a high throughput screening strategy: A. Create variants (e.g. transposon library) of a baseline free fatty acid producing strain. B. Inoculate individual cultures of select mutant strains and screen for increased Nile red fluorescence to identify strains with improved fatty acid production capabilities. C. Confirm primary screen hits by directly measuring fatty acid titer from small 5mL cultures. D. Identify site of transposon insertion and create clean mutations of the affected gene (1 and 2 represent arbitrary genes). E. Confirm newly created strain's fatty acid titer. F. Use identified strain as baseline for iterative use of the screen.

Figure 2.

The average fluorescence observed for 48 wells of induced (filled) and uninduced (open) cultures of *E. coli* RL08 pBad33-BTE are plotted with respect to the Nile red solvent: dimethyl sulfoxide (DMSO), ethanol (EtOH), or water (H₂O) and detection orientation: (reading from above (top) or below the plate (bottom)). The corresponding Z-factor is listed below each tested condition.

Figure 3.

Nile red fluorescence values for each of ~2800 assays of *E. coli* RL08 pBad33-BTE grown in microtiter culture in the primary screen. Black bars represent the RL08 pBad33-BTE controls and gray bars represent strains isolated from a library of transposon mutants. The solid line represents the average fluorescence of 96 RL08 pBad33-BTE microtiter cultures, while the dashed lines represent fluorescence values three standard deviations away from the average.

Figure 4.

Bar height represents the peak area ratio of C_{12:0} fatty acid methyl ester to C_{15:0} internal standard of each transpositional mutant in the secondary screen. Final two positions represent the peak area ratios of the negative (0.15 ratio) and positive control. The solid line represents the average ratio of the positive control (RL08 pBad33-BTE in triplicate). The dashed lines represent ratios 3 standard deviations away from the positive control.

Figure 5.

Left: Fatty acid biosynthesis begins with acetyl-CoA, a product of many central metabolic pathways. Acetyl-CoA is converted to malonyl-CoA by the acetyl-CoA carboxylase complex (AccABCD). Malonyl-CoA is converted to malonyl-ACP by FabD and used as a monomer in a condensation reaction with growing fatty acyl chains. After each condensation reaction, a series of reductions and dehydrations are performed to generate a fully reduced β -carbon. A thioesterase (e.g. BTE) cleaves the ACP from a specific chain length acyl-ACP (C12 for BTE) and generates a free fatty acid. Alternatively, continued elongation and reduction cycles generate C16 and C18 acyl-ACPs that are used to generate membrane lipids. Right: free fatty acids can be broken down by the enzymes involved in β -oxidation. Each enzyme catalyzes an opposite reaction of fatty acid synthesis, but uses CoA substrates instead of ACP bound substrates. Boxed enzyme names represent heterologous enzymes expressed within *E. coli* RL08. Dotted line boxed enzyme names represent those whose encoding gene was disrupted during the study. Usage of cofactors such as ATP and NADH as well as the production of H_2O is not shown.

Figure 6.

Fatty acid production for precisely engineered β -oxidation mutants. Average fatty acid titers for *E. coli* strains RL08, RL08 *fadE*, and RL08 *fadB* all harboring pBad33-BTE. Combined titers are separated into C₁₂, C₁₄, C₁₆, and C₁₈ fatty acid species.

Table 1

Genes knocked out by transposon mediated mutagenesis and production values

Clone #	Location of insertion	Proposed gene function (reference)	C _{12:0} /C _{15:0} peak area ratio
52	<i>rhIE</i>	ATP-dependent RNA helicase[36]	220
51	<i>yhdW</i>	probable ATP-dependent amino acid transporter[37]	163
15	<i>livM</i>	leucine transport system [38]	157
53	<i>yhcA</i>	predicted outer membrane porin[39]	141
44	<i>yagR</i>	aldehyde ferredoxin oxidoreductase [40]	140
54	<i>fadB</i>	enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase[41]	132
23	<i>yhgF</i>	predicted transcriptional accessory protein ^a	128
41	<i>yhdP</i>	conserved membrane protein, predicted transporter ^b	128
33	<i>hyaA</i>	small subunit of hydrogenase I [42]	128
39	<i>gntX</i>	involved in utilization of DNA as a carbon source[43]	127

^aSequence similarity to Rnr [44]^bSequence similarity to Prc [45]