Evaluating PHA Productivity of Bioengineered Rhodosprillum rubrum

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Abstract
This study explored the potential of using Rhodosprillum rubrum as the biological vehicle to convert chemically simple carbon precursors to a value-added bio-based product, the biopolymer PHA. R. rubrum strains were bioengineered to overexpress individually or in various combinations, six PHA biosynthetic genes (phaC1, phaA, phaB, phaC2, phaC3, and phaJ), and the resulting nine over-expressing strains were evaluated to assess the effect on PHA content, and the effect on growth. These experiments were designed to genetically evaluate: 1) the role of each apparently redundant PHA polymerase in determining PHA productivity; 2) identify the key gene(s) within the pha biosynthetic operon that determines PHA productivity; and 3) the role of phaJ to support PHA productivity. The result of overexpressing each PHA polymerase-encoding gene indicates that phaC1 and phaC2 are significant contributors to PHA productivity, whereas phaC3 has little effect. Similarly, over-expressing individually or in combination the three PHA biosynthesis genes located in the pha operon indicates that phaB is the key determinant of PHA productivity. Finally, analogous experiments indicate that phaJ does not contribute significantly to PHA productivity. These bioengineering strains achieved PHA productivity of up to 30% of dry biomass, which is approximately 2.5-fold higher than the non-engineered control strain, indicating the feasibility of using this approach to produce value added bio-based products.

Introduction
Since the industrial revolution humanity has increasingly become dependent on fossil carbon as a source of energy and chemicals. Most recently a number of factors have coalesced to provide the impetus to explore the use of biomass as an alternative, renewable source of carbon [1,2]. The recalcitrant nature of biomass, resisting degradation to useful intermediates that can be used as feedstocks in either chemical or biological conversion processes is impeding this development. One possible route through this multifaceted conversion process is to thermochemically depolymerize the biomass and deconstruct it to chemically simple carbon precursors, which could be utilized by microbial fermentation to produce value-added products. Fast pyrolysis is a non-enzymatic, thermal depolymerization of biomass in the absence or low levels of oxygen, which can produce gaseous (syngas), liquid (bio-oil), or solid (biochar) energy-rich materials [3,4]. Converting these materials to useful value-added products could be achieved via a fermentation-based method, but is hindered by the fact that these pyrolysis materials are relatively complex chemical mixtures, that also includes microbial growth inhibitors. The potential of using these deconstructed biomass materials as a carbon source for fermentation not only overcomes the expense associated with using a chemically more complex, recalcitrant carbon source (e.g., lignocelluloses), but also bypasses the high cost and poor yields of enzymatic hydrolysis of waste biomass into simple sugars that are suitable for fermentation.

Rhodosprillum rubrum is an attractive microbial fermentation organism for converting such deconstructed biomass-products to value-added biochemicals because it can utilize a variety of different carbon and energy source under anaerobic conditions [5,6]. Because of its flexible capabilities to grow aerobically, anaerobically or as an autotroph, R. rubrum is particularly attractive for fermenting syngas feedstocks (particularly biomass-derived syngas), which are primarily a mixture of carbon monoxide, hydrogen, carbon dioxide and methane. In this study we explored the potential of using this Gram negative, photosynthetic purple non-sulfur bacterium for the production of valuable biochemicals from simple carbon feedstocks, using a transcription regulatory system that is inducible with carbon monoxide, which would therefore be applicable in developing a syngas fermentation platform.

The biochemicals we targeted for these bioengineering efforts are polyhydroxyalkanoates (PHAs), which are biodegradable polyester polymers that are deposited within inclusion bodies, and many microbes use them as a means of storing carbon and energy [7–9]. Because PHAs can be used as biodegradable plastics there has been a great deal of interest in generating a PHA...
production system based upon microbial fermentation [10]. R. rubrum has the potential of accumulating up to 50% of dry weight as PHA, using an optimal carbon source such as butyrate [11]. Recent studies have evaluated the technical and economic feasibility of using such a microbial platform for the conversion of biomass feedstocks to biofuels or biochemicals [12,13], and one of the conclusions from these studies is that additional "metabolic engineering could be employed to increase yield and broaden the variety of available products" [14]. In this study we specifically targeted the bioengineering of R. rubrum PHA biosynthetic genes, and explored the effect of systematically overexpressing them on the production of PHA, when R. rubrum is grown in chemically simple carbon feedstocks in anaerobic conditions, and controlling the expression of the bioengineered genes with a carbon monoxide inducible promoter.

Previous studies of the R. rubrum genome have revealed the presence of three PHA polymerases that are designated phaC1 (Rru_A0275), phaC2 (Rru_A2413) and phaC3 (Rru_A1816) [15–17], respectively, and one (R-specific 2-ensoyl-CoA hydratase [phaJ (Rru_A2964)]) [18]. PhaJ converts trans-2-ensoylacyl-CoA to (R)-3-hydroxyacyl-CoA, the substrate of PhaC [9]. The sequence identities shared between PhaC1, PhaC2 and PhaC3 are 14.3, 18.4 and 50.2%, respectively. The phaC1 gene resides in the PHA biosynthetic operon that also includes the phaD (Rru_A0274) and phaB (Rru_A0273) genes. The products of this phaC1-phaA-phaB operon, are enzymes that sequentially catalyze condensation of two acetyl-CoA molecules to form acetoacetyl-CoA, the substrate of PhaC [9]. In contrast, the phaC2 and phaC3 gene are located in other discrete locations in the genome.

Materials and Methods

Bacterial Strains, Plasmids and Culture Conditions

The strains of R. rubrum and E. coli, and the plasmids used in this study were listed in Table S1 in File S1. The Table S2 in File S1 lists DNA primers used in this study. E. coli was grown at 37°C in LB medium. R. rubrum was grown photo-heterotrophically under 5000 Lux light intensity, at 25°C in supplemented malate-ammonium medium (SMN medium; Table S3 in File S1) [19]. To assess PHA production, cells from a 0.5 ml aliquot of a normalized SMN culture (5 O. D.) were collected by centrifugation at 13,000 g for 2 min; the cells were washed once with nitrogen-limiting RNCEO medium (i.e., without ammonium chloride; Table S4 in File S1) [20]. To assess PHA biosynthesis, one phaC gene was PCR amplified from the translational start and stop codons, using the DNA primers listed in Table S2 in File S1.

DNA Isolation and Manipulation

Genomic DNA was isolated from R. rubrum as described by Kerby et al. [19]. Plasmids were isolated from E. coli cells grown in LB medium by using QIAprep Spin Miniprep Kit (Qiagen Inc., Valencia, CA). Agarose gel electrophoresis and transformation of E. coli were carried out as described by Sambrook and Russell [21]. PCR products were cloned into pCR2.1-TOPO using a TOPO TA cloning kit (Invitrogen Corporation, Carlsbad, CA).

Construction of Plasmids

The plasmids for expressing protein in E. coli strain BL21-AI were constructed as recommended by Invitrogen Gateway technology. The plasmids pUX19-PT carrying different pha genes, and pUX19-PT-GUS, which were used to generate strains for over-expressing phaC genes and the gusC gene, respectively, were constructed as follows. Using PCR, XbaI and NdeI sites were first introduced into the upstream and downstream regions of an 1818-bp fragment that contains the promoter of the carbon monoxide inducible R. rubrum CooFSCTJ operon [22]. The CooFSCTJ promoter has been previously characterized as the 120-bp fragment that is immediately upstream of the translational start codon of the CooF gene. We targeted an 1818-bp fragment immediately upstream of the translational start codon in order to encompass the promoter (Pcox) and ensure subsequent recombinational integration of bioengineered transgenes at the CooF locus (Fig. 1). The PCR product was purified and ligated into the TA cloning vector, pCR2.1. The resulting vector was digested with NdeI and SacI and the Pcox promoter fragment was purified and cloned into pUX19 [23] digested with XbaI and NdeI, forming vector pUX19-P1. The 110-bp terminator of CooFSCTJ operon [15], and each PHA biosynthetic gene or the GUS gene were prepared and ligated sequentially into the vector pUX19-P1 with the same method, forming vectors pUX19-PT-pha genes. In these constructs the ORFs of each PHA biosynthetic gene and the GUS gene were PCR amplified from the translational start and stop codons, using the DNA primers listed in Table S2 in File S1.

Conjugation

The pUX19-PT-pha-target gene) plasmids were mobilized from E. coli 17-1 into R. rubrum by conjugation conducted as described by Liang [24]. R. rubrum conjugants were selected on MN medium, supplemented with kanamycin. Repeated transfer of inoculum to kanamycin containing MN media, selecting a single colony after each transfer was used as a means of isolating R. rubrum conjugants. Two types of recombinational outcomes were isolated (Fig. 1 and Table 1): one occurs at the Pcox promoter locus, and the second occurs at the structural pha gene that was included in the pUX19-PT-pha genes vector. In the second case, the expression of any
other pha structural gene down-stream of the recombination site (i.e., pha gene 2, Figure 1) also became inducible via the CO-activation. All recombination integration sites were confirmed by PCR amplification, and DNA sequencing.

GUS Activity Assay

Cells expressing the GUS reporter were collected and resuspended in GUS assay buffer (1 mM EDTA, 50 mM NaHPO₄- Na₂PO₄, 0.1% Triton X-100, pH 7.0) and were disrupted ultrasonically. GUS activity was determined as described previously [25]. One unit of GUS activity is defined as the formation of 1 mM p-nitrophenol formed per minute at 37°C.

Protein Purification and Preparation of Antibodies

Recombinant His-tagged or GST-tagged fusion proteins were affinity-purified via their tags from extracts of E. coli BL21-AI strains harboring pDEST15-(pha-target gene) or pDEST17-(pha-target gene), grown in the presence of 0.2% L-arabinose. Recovered proteins were further purified via preparative scale SDS-PAGE. The recombinant protein bands was excised from gels, crushed in PBS solution and used to immunize mice and generate antibodies against each protein.

SDS-PAGE and Western Immunoblot Analysis

Proteins were separated by SDS-PAGE in 12.5% polyacrylamide gels. Gels were loaded with aliquots containing of equal amounts of protein; protein concentrations were determined using the Bio-Rad (Hercules, CA) Dc Protein Assay Kit, using BSA to generate the standard curve. Proteins were electrophoretically transferred to a nitrocellulose membrane [21]. PHA biosynthetic proteins were immunologically detected with a combination of primary mice antibodies directed against the PHA proteins, and secondary HRP-conjugated anti-mouse IgG antibody (Bio-Rad) (Hercules, CA). The blot was developed with Amersham (Little Chalfont, Buckinghamshire, UK) ECL western blot detection reagents. For quantitative analysis, an Epson Perfection 2400 scanner (Shiojiri-shi, Nagano-ken, Japan) was used to scan the x-ray film. The signal intensities were quantified by ImageJ software (http://rsbweb.nih.gov/ij/index.html).

Results

Recombinant Strains Carrying CO-inducible-Pha Genes

In this study we investigated the metabolic regulation of PHA biosynthesis by determining the in vivo effect of over-expressing the PHA biosynthetic genes on PHA productivity and culture-growth.

Table 1. Recombinant R. rubrum strains.

| Gene | R. rubrum strains | Genetic location targeted for integration | Expected phenotypic modification |
|------|-------------------|------------------------------------------|----------------------------------|
|      |                   | cooFSC7J Promoter | Structural Gene |               |
| Vector control | oxC0ntrol | + | none |
| phaC | oxBHA(C1AB) | + | Entire pha operon induced |
|      | oxBHA(C1) | + | phaC1 only induced |
| phaA | oxBHA(AB) | + | Both phaA and phaB induced |
|      | oxBHA(A) | + | phaA only induced |
| phaB | oxBHA(B) | + | phaB only induced |
| phaC2 | oxBHA(C2) | + | phaC2 only induced |
| phaC3 | oxBHA(C3) | + | phaC3 only induced |
| phaJ | oxBHA(U) | + | phaJ only induced |
| GUS  | oxBGUS | + | Expression of GUS; positive control |

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In the resulting bioengineered *R. rubrum* strains the PHA biosynthetic genes were overexpressed individually or in combination using the strong, carbon monoxide inducible *cooF* promoter [22]. To conduct these over-expression experiments we initially tested the efficacy of the carbon monoxide inducible system, by constructing the P*cooF*-GUS vector and generated a strain to evaluate GUS expression. The results establish that *cooF* promoter activity (determined as GUS activity) is undetectable in the absence of carbon monoxide, and reaches maximum activity (>100-fold induction) within 24-h of exposure to carbon monoxide, reaching 50% of the maximum within the initial 5-hours (Fig. 2).

**Metabolic Engineering of *R. rubrum* by Over-expressing PHA Biosynthetic Genes**

Bioengineered strains that over-express select PHA biosynthetic genes were constructed by introducing into *R. rubrum* each P*cooF*-PHA construct on a PUX19-based plasmid, and selecting for resistance to the antibiotic carried by that base-plasmid (KanR). Because this plasmid cannot replicate in this host, two types of homologous recombination events will confer KanR strains, recombination at the *cooF* promoter locus or recombination at the PHA structural gene locus. For example, conducting such an experiment with the vector pUX19-P*cooF*-phaC1 can result in: 1) recombination at the *cooF* promoter locus, generating a strain that has the *phaC1* gene under the control of *cooF* promoter and only this new, recombinant *phaC1* locus can be induced by carbon monoxide; or 2) recombination at the endogenous *phaC1* locus (Rru_A0275), generating a recombinant locus that is composed of a *cooF* promoter-driven transcription unit encoding *phaC1*, and the downstream located PHA structural genes within that operon (*phaB* and *phaA*). This recombinant locus would therefore become inducible with carbon monoxide. Using this strategy, ten different strains were constructed (Table 1). In Table 1, and in subsequent text herein we use the prefix “oxPHA” as the shorthand nomenclature to indicate which PHA biosynthetic genes have been overexpressed in the resulting recombinant strains.

These strains (along with the negative control strain generated with the plasmid pUX19-P*cooF*, which does not carry any PHA gene sequences) were cultivated anaerobically in a CO2/carbon monoxide atmosphere with nitrogen-limiting RRNCO medium containing acetate as a carbon-source. For each strain we determined its growth by monitoring A680 (Fig. 3A, 3C and 3E), the accumulation of dry weight biomass (data not shown), and in parallel, at the last six time points of the culture, we determined PHA accumulation by GC-MS analyses (Fig. 3B, 3D and 3F), and

**Figure 2.** Induction of GUS-expression by carbon monoxide from an integrated P*cooF*-GUS-TC*cooF* locus. Data represent average from triplicate biological samples, and error bars indicate the standard error.
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**Figure 3.** Growth curves (A, C and E, with doubling time indicated) and PHA content (B, D and F) of *R. rubrum* strains. R.
The Effect of Individually Over-expressing PhaC Paralogs and PhaJ on PHA Production

To investigate the potential role of each PHA polymerase isozymes to affect PHA production, three strains that individually over-express phaC1, phaC2 and phaC3, (i.e., oxPHA(C1), oxPHA(C2) and oxPHA(C3), respectively) were investigated regarding PHA content and growth. Western blot analysis of protein extracts from these strains show that PhaC1 and PhaC3 levels were at least 50-fold higher than in the control strain, whereas PhaC2 was overexpressed by 25-fold (Fig. 4). All three of these strains accumulate higher levels of PHA than the control strain, by between 1.25-fold and 1.8-fold. However, the PhaC2 overexpressing strain (oxPHA(C2)), which shows the smallest level of over-expression (25-fold increase), results in highest increase of PHA production (increased 1.8-fold); whereas, the other two overexpressing strains (oxPHA(C1) and oxPHA(C3)), increase PHA production by 1.4- and 1.25-fold, respectively (Fig. 3B). These in vivo alterations of PHA production by altering the expression of phaC paralogs have a differential effect on the growth of the cultures. Namely, the PhaC2 overexpressing strain, which accumulates more PHA than the other two PhaC-overexpressing strains grew at a significantly faster rate than the control strain, whereas the other two strains demonstrate growth rates similar the control strain (Fig. 3A).

Similar studies were conducted to assess whether altering the expression of the phaJ gene would affect the production of PHA. The phaJ gene encodes the enzyme 2-enoyl-CoA hydratase, which catalyzes a reaction that is thought to metabolically link intermediates of fatty acid catabolism to intermediates of PHA biosynthesis [26]. Even though PhaJ protein was over-expressed by a factor of at least 5-fold, this modification had relatively small effect on PHA production (increased 1.3-fold), and did not significantly affect the initial growth rate of the strain (Fig. 3C and 3D). Presupposing that the phaJ gene is involved in metabolically connecting fatty acid catabolism with PHA biosynthesis, the finding that phaJ overexpression does not affect PHA accumulation is consistent with the probable low rate of fatty acid catabolism when R. rubrum is grown on the acetate-carbon source.

The Effect of Over-expressing Individually or in Combination the PHA-operon Located Genes on PHA Production

The R. rubrum genome contains a single three-gene operon (phaC1-phaA-phaB) that is recognizable as the PHA biosynthetic operon based upon sequence homology with other orthologs from many microbial genomes. The effect of individually over-expressing each of these PHA operon-located biosynthetic genes on PHA accumulation was investigated by using the strains oxPHA(C1), oxPHA(A), and oxPHA(B). In addition, using the strains resulting from recombination at each of the PHA operon-
located genes we investigated the effect of overexpressing a combination of these genes, i.e., oxPHA(AB) and oxPHA(C1AB) (Table 1). Western blot analysis of extracts from strain oxPHA(A), oxPHA(B) and oxPHA(C1) shows that the expression level of PhaA, PhaB, PhaC1 proteins increased by 5-, 50- and >50-fold, over the control strain, respectively (Fig. 4). In the strain oxPHA(AB), PhaA and PhaB levels are 5- and 50-fold higher than in the control strains, and in the strain oxPHA(ABC1), PhaA, PhaB and PhaC1 levels are increased 5-, 7- and >50-fold, respectively.

All five recombinant strains produce significantly more PHA than the control strain (Fig. 3F). However, the strain oxPHA(AB) and oxPHA(AB) expresses the highest level of PHA production (increased by 2.2-fold); the other three recombinant strains (oxPHA(C1), oxPHA(A) and oxPHA(C1AB)), increased PHA levels by about 1.4-, 1.4- and 1.7-fold, respectively (Fig. 3F). These results indicate that the expression level of phaB is a major limiting determinant of PHA production.

This conclusion is based on the following comparisons. First, overexpressing phaB by 50-fold (either individually or in combination with phaC) provides the maximal increase in PHA content (from 13% to 28% and 29% of CDW, respectively). Hence, there is little additive effect in co-overexpressing both phaB and phaC on PHA levels over individually overexpressing phaB. Second, when phaC1 overexpression is added to this strain (i.e., strain oxPHA(C1AB)), PHA level is at 22% of CDW, and this is due primarily to the fact that phaB expression level is only 7-fold higher than the control strain. Finally, when phaC1 or phaC is individually overexpressed, it has the minimal effect on enhancing PHA levels.

Growth Rates of PHA Over-accumulating Strains

The results indicate that the growth rates of cultures (measured as the initial doubling time) are correlated with the maximal PHA accumulating capacity of the strain (Fig. 5). Specifically, the wild-type control strain that accumulates the lowest levels of PHA, showed the slowest growth rate (longest doubling time), and all recombinant strains, which accumulate higher levels of PHA showed increased growth rates. Among all the strains evaluated, the strains oxPHA(AB) and oxPHA(AB), accumulate the highest levels of PHA (about 30% of CDW), and they show the fastest growth rates. Figure 5 illustrates that in fact there’s a linear relationship between PHA levels and the initial growth rates of the cultures. The data therefore imply that PHA accumulation contributes to R. rubrum growth under anaerobic conditions, in nitrogen-limiting RRRNC0 medium.

Discussion

The fermentation-based chemical reduction of biomass carbon, via thermolytic deconstruction of the biomass through a syngas intermediate has been previously explored [12,13]. Elucidating the metabolic controlling mechanisms that influence the production of PHA by R. rubrum cells grown in anaerobic conditions, on chemically simple carbon precursors should further facilitate the use of this organism as a model for syngas fermentation to produce valuable bio-products. As a model, in this study we evaluated the effect of over-expressing R. rubrum-encoded PHA biosynthetic genes on PHA production and cellular growth. The strains investigated in these experiments explored the metabolic regulatory points of PHA biosynthesis when R. rubrum was grown on nitrogen-limiting medium, with a carbon source composed of CO2, CO and acetate. Our results show that R. rubrum strains can accumulate up to 30% PHA of CDW, which is composed of 99.4% polyhydroxybutyrate and 0.6% polyhydroxyvalerate. These evaluations demonstrate the potential of using R. rubrum for producing a value-added bio-based product from chemically simple carbon sources. Our bioengineering experiments addressed three questions: 1) which of the three phaC paralogs in the R. rubrum genome is most significant in PHA production? 2) Which of the enzymes in the three-reaction pathway from acetyl-CoA to PHA is limiting PHA production? And 3) Is phaJ limiting PHA production in syngas fermentation?

The data presented herein establish that over-expressing phaC2 results in increased production of PHA, while the impact of overexpressing phaC1 is less, and over-expressing phaC3 has little, to no effect. The conclusion that of the three phaC paralogs, phaC2 is the most significant in determining PHA accumulation, is based on the characterization of individual over-expressing alleles, and is corollary to our prior characterizations of individual phaC deletion-mutants, which also indicated that the PhaC2 paralog is the major polymerase isoform for producing PHA [16]. Although prior studies with Aeromonas hydrophila, Aeromonas caviae [27,28], and Pseudomonas oleovorans [29] have examined the importance of over-expressing phaC on increasing PHA production, the genomes of, each of these organisms harbor only a single-copy of the phaC gene, which are located within the PHA biosynthetic operon. Moreover, whereas the former two studies enhanced PHA productivity by overexpressing the polymerase, the latter did not significantly increase PHA production under nitrogen limiting conditions.

Characterizations of the three genes that constitute the PHA biosynthetic operon establish that phaB is the key genetic determinant of PHA productivity. This is consistent with the prior kinetic study of PHA biosynthesis from pentanoic acid by Alcanivorax borkumensis [30], but contrasts with the studies that suggest that PhaA is the key enzyme determining PHA production [31,32]. PhaB encodes the NADPH-dependent enzyme that reduces acetacetyl-CoA to 3-hydroxybutyryl-CoA. Therefore, there is a thermodynamic rationale for the energy requiring reaction to be limiting the generation of PHA, a product whose major physiological role is to store reduced carbon as a means of storing energy.

Over-expressing phaJ had little effect on PHA production, in these conditions. This is partly predictable based on prior characterization of this gene in Aeromonas hydrophila and Aeromonas caviae [27,28], and the fact that the reaction catalyzed by PhaJ, links fatty acid catabolism and PHA biosynthesis. Specifically, it’s energetically unfeasible of an organism to set up a futile cycle between fatty acid biosynthesis (from CO2, CO and acetate as carbon sources) and cycle the resulting fatty acids through

![Figure 5. Correlation between culture doubling time and the maximum PHA content of R. rubrum strains.](image-url)
catabolism in order to produce PHA from the intermediates of fatty acid catabolism.

Additional effectors that have to be considered to enhance the productivity of PHA are processes that stabilize the polymer, which includes eliminating or reducing the rate of degradation. The PHA polymer is encapsulated in “carbonosomes,” which include some “carbonosomes” [33], and these are considered to be analogous to oil droplets or oleosomes, which occur in eukaryotic organisms [34]. As with oleosomes that are stabilized by a coating of a phospholipid monolayer and the protein oleosin [35], carbonosomes are coated with a similar lipid monolayer and the protein, phasin encoded by the phaP gene [33]. Indeed, there are experimental outcomes that indicate a regulatory role for phasin [36] and oleosin [37] in determining the levels of PHA and oil accumulation, respectively.

The other metabolic function that can affect PHA levels in cells is the rate of polymer turnover, which is catalyzed by PHA depolymerase, encoded by phaZ genes [38]. These are difficult enzymes to assay because of the polymeric nature of their substrates, but evidence suggests that they are associated with the carbonosome granules, and cells express a set of paralogs that display different substrate specificities [39].

Finally, our results indicate that PHA content enhances growth rate of R. rubrum (Fig. 5). Though the underlying mechanism of this phenomenon is still unclear, previous studies have shown that PHA enhances resistance to stresses (radiation, desiccation and osmotic pressure) [39], and this resistance may be mediated by increasing of the levels of the growth regulator guanosine-triphosphate (ppGpp), which enhances PHA degradation during the stress of Pseudomonas oleovorans [40].

Supporting Information

File S1 This file contains: Tables S1–S4, (DOC)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: HJ BJN. Performed the experiments: HJ BJN. Analyzed the data: HJ BJN. Contributed reagents/materials/analysis tools: HJ BJN. Wrote the paper: HJ BJN.

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