Red/ET recombineering enhances extracellular lipase production by *Burkholderia glumae*

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Abstract

Background: Microbial lipases are utilized widely in industrial fields, and the LipA lipase produced by *Burkholderia glumae* PG1 has been used for the production of enantiopure pharmaceuticals. However, efficient lipase expression and secretion are still problematic. LipA is encoded in an operon that also contains the lipase-specific foldase gene *lipB*. The purpose of this study was to enhance the production of active lipase by overexpressing *lipAB* in *B. glumae* PG1 using a host-vector system.

Results: The *lipAB* operon, isolated from the genomic DNA of *B. glumae* PG1, was directly cloned into broad-host-range vectors, with pBBR1 or pRk2 backbones, using the Red/ET recombination system and then transformed into the original host strain. Lipase activity of the derivative strains, including PG1/BI and PG1/RI, were evaluated. When 1% olive oil was included in the growth medium, the maximum activities of the resultant strains were approximately 39.5- and 15.3-fold higher, respectively, than that of the wild-type PG1, indicating that increased lipase gene dosage resulted in high expression yields of lipase. To further enhance lipase expression, two recombinant strains, PG1/Ba and PG1/Ra, were generated with the plasmid-borne *lipAB* operon driven by the strong constitutive promoter P_{apra}. PG1/Ba and PG1/Ra displayed 15.0- and 4.3-fold improvements in lipase production in 0.5% glucose-containing medium compared to PG1/BI and PG1/RI, respectively. However, glucose repressed lipase gene expression in strain PG1 when the native *lipAB* promoter was used.

Conclusions: Homologous expression of lipase genes in *B. glumae* PG1 was achieved by the use of high-copy expression vectors, and the resulting presence of multiple copies of *lipAB* significantly improved extracellular lipase expression. Our results demonstrate that modification of *B. glumae* PG1 can improve this host system for lipase production.

Background

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) belonging to the family of α/β hydrolases are widely produced by organisms from animals to bacteria. Microbial lipases are important biocatalysts in the synthesis of detergents, food ingredients, agrochemicals and pharmaceuticals [1–4]. Many biotechnologically relevant lipases are relatively stable in organic solvents, do not require a cofactor, show broad substrate specificity and are readily available at low production costs [5]. Since lipases can catalyze a variety of synthetic and hydrolytic reactions under non-aqueous reaction conditions, they have received increasing attention over the past few years for their multiple potential applications in industry [6, 7].

In recent years, the nucleotide and amino acid sequences of several microbial lipases have become available, and many bacterial lipases have been cloned and characterized, particularly those from the genera *Pseudomonas* and *Burkholderia* [8–12]. One of the most frequently used lipases, lipase A, is secreted by the Gram-negative bacterium *Burkholderia glumae* PG1 [5, 13]. The regulation of lipase expression and secretion in *B. glumae* is complex and requires effective cooperation of a variety of

proteins. The *Burkholderia* lipase structural gene *lipA* is located in a single operon together with a secondary gene, *lipB* [13–15]. The LipA is synthesized as a precursor with an N-terminal signal peptide. This precursor is first transported across the inner membrane via the Sec secretion system and is then folded into an enzymatically active conformation by the lipase-specific foldase, encoded by *lipB*, in the periplasm [14, 16–18]. The mature lipase is then secreted into the extracellular medium via the type II secretion pathway [19].

Pseudomonas lipases have been studied extensively, but *Pseudomonas* is an opportunistic human pathogen and so is less suitable for bulk lipase production [2]. To enhance the production of extracellular lipase, many biotechnologically interesting lipases have been cloned and expressed in *Escherichia coli* [20–23]. Unfortunately, the efficient production of active lipases in heterologous hosts and secretion into the culture medium under normal laboratory growth conditions can be difficult. Less than 3% of an expressed lipase may be active, with the remainder of the protein misfolding and accumulating as insoluble inclusion bodies in the *E. coli* cell [24]. In order to overcome the problem of insoluble protein arising with a heterologous host, we set out to use *B. glumae* as a homologous expression host. Compared with heterogeneous expression, homologous expression can take full advantage of the transcription and translation systems of the original host without any pretreatment and make the enzyme isolation and purification easier [25].

The lipase produced by *B. glumae* was found to perform well in several industrial applications, and several classical mutagenesis techniques have been applied to *B. glumae* PG1 to construct lipase-overproducing strains [2, 5, 9]. The aim of this study was to construct a host-vector system that would generate higher levels of active lipase production, in which PG1 was used as the host with multicopy expression vectors containing *lipAB* genes. The complete *lipAB* operon from PG1 was directly cloned into the expression vector p15A using homologous recombination (HR) in *E. coli*. We also utilized the full-length λ prophage proteins RecE and RecT, which can mediate highly efficient HR between two linear DNA segments carrying homology arms at both ends [26–28]. HR mediated by Red $\alpha\beta$ from λ phage between a linear and circular DNA molecule in *E. coli* was used for retrofitting the expression vector with a broad-host-range replication origin and a strong constitutive promoter [29–31]. The final constructs were transformed into the PG1 wild-type strain for homologous expression of lipase. Our findings indicate that the secretion of active lipase can be enhanced via overexpression of *lipAB* genes on a multicopy vector in *B. glumae* PG1.

Results And Discussion

Vector construction for plasmid-borne lipase gene expression in *B. glumae* PG1

Nucleotide sequencing revealed that the *lipAB* genes are organized as an operon of 2139 bp in the *B. glumae* PG1 chromosome (GenBank accession number: CP002581), with an internal promoter upstream of *lipA* and a transcription terminator downstream of *lipB* [15]. Analysis of the sequence indicates that *lipA* encode a protein of 358 amino acid residues and LipB is a 353 amino acid protein. In previous

studies, lipase genes were inserted into vectors, resulting in high-level expression of active lipase in the heterologous hosts [32–34]. However, there are few examples of lipase genes being cloned and overexpressed in *B. glumae* PG1. To achieve higher levels of lipase secretion, the full lipAB operon was cloned from purified PG1 genomic DNA directly into multicopy plasmids. The methods for the direct cloning and engineering of the lipAB gene constructs are illustrated in Fig. 1.

Following digestion with appropriate restriction enzymes, genomic DNA from *B. glumae* PG1 was co-electroporated with the linear cloning vector p15A-cm, flanked by 80-nucleotide (nt) homology arms (ha), into arabinose-induced, RecET-expressing GB05-dir cells for direct cloning of the lipAB operon. The final construct was modified using the Red $\alpha\beta$ recombination system. Since p15A origin is not capable of replication in *B. glumae* PG1, we subcloned the lipAB operon into broad-host-range vectors to establish plasmids pBI and pRI, which contain a pBRR1 and pRK2 replication origin, respectively, and the gene cluster encoding LipA and LipB regulated under the native promoter P_{lipAB} . As promoter exchange can be an important strategy for achieving high expression yields of lipase, the vectors pBa and pRa were generated by exchanging the P_{lipAB} promoter with the strong constitutive promoter P_{apra} . DNA analyses of the constructs are shown in Supplementary Fig S1. The final constructs were transferred into the homologous host *B. glumae* PG1, and the four resulting recombinant strains, PG1/BI, PG1/RI, PG1/Ba and PG1/Ra, were used to examine lipase expression.

Effect of copy number of the lipAB gene on lipase production

One of the common strategies to increase the expression level of a specific gene is to increase the copy number of the expression cassette [32]. *B. glumae* PG1 wild-type and two derivative strains, PG1/BI and PG1/RI, were cultivated in PG medium in the presence of 1% olive oil as a carbon source at 30 °C [8]. Culture supernatant and the whole-cell extract of each *B. glumae* strain were obtained after 72 h of cultivation and subjected to SDS-PAGE to investigate lipase expression. The mature lipase of PG1 has a calculated molecular mass of 33.1 kDa [9], and a protein of that size was visible in the extracts of all three strains and in the supernatants of PG1/BI and PG1/RI (Fig. 2A, arrow). The secreted lipase was produced almost as a single band on SDS-PAGE, but the amount of it in the culture supernatant of wild-type *B. glumae* PG1 was markedly lower than in the supernatants from the recombinant strains, consistent with the fact that the wild-type strain harbors just one chromosomal copy of the lipAB operon. Additionally, PG1 harboring pBI (lane 5) secreted much more lipase than the host harboring pRI did (lane 3), which can be attributed to the lower copy number of pRK2 than of pBRR1. Real-time qPCR assays were performed to precisely quantify the transcription levels of lipA after 24 h of cultivation. As seen in Fig. 2B, the expression levels of lipA in the strains PG1/RI and PG1/BI were 12.9 times higher and 6.4 times higher than that of the PG1 wild-type strain, respectively.

The pBRR1 origin (about 30–40 copies per cell in *E. coli*) is functional in a wide range of Gram-negative bacterial genera, such as *Burkholderia*, *Escherichia*, *Pseudomonas* and *Agrobacterium* [35–37]. The copy number of the RK2 replicon is estimated to be 5 to 7 per cell in *E. coli*, and the origin delivers stable replication in more than 30 species of Gram-negative bacteria [38, 39]. However, to the best of our

knowledge, this is the first report of the use of the RK2 origin in *B. glumae*. The recombinant strains produced more lipase than did the wild-type strain, and no lipase was observed in the PG1 wild-type culture supernatant, which indicates that the increased dosage of the lipase gene enabled *B. glumae* PG1 to secrete much more lipase. Therefore, overexpression of a lipase gene from a multicopy plasmid is a feasible strategy for enhancing lipase production, which is in accordance with a previous report [32].

Comparison of lipase activity and cell growth

B. glumae PG1 variants secreted a considerable amount of lipase, but the lipase production and activity might have been influenced not only by the high-level expression of lipAB, but also by bacterial cell growth and density. Fang et al. demonstrated that cell density largely correlates with the yield of secreted protein and that optimized cultivation conditions are essential to obtain maximum cell growth and lipase activity [32]. The extracellular lipase activities of *B. glumae* PG1 and its variants were monitored during different growth stages in PG medium containing 1% olive oil as a carbon source at 30 °C (Fig. 3A, B). We did not observe any growth defects in the strains harboring the plasmids with the lipAB operon when compared to the wild-type strain (Fig. 3B). However, there were marked strain differences in lipase activity as well as growth-associated changes in activity, with significantly increased lipase activity detected during the log phase (Fig. 3A). The lipase activities reached a maximum at the end of the stationary phase and then decreased, but did not disappear completely as the cells entered the cell death phase. After 54 hours (h) of induction, the lipase activities in the culture supernatants of PG1/BI and PG1/RI were 3.63 U/ml and 1.41 U/ml, corresponding to 39.5 and 15.3 times the level in the wild-type PG1 strain (0.092 U/ml), respectively.

Effect of the lipAB gene promoter on lipase production and activity

Next, to analyze the influence of the lipAB promoter on lipase production and activity, the native promoter P_{lipAB} in the vectors was replaced with the strong constitutive promoter P_{apra} , generating the two transformants PG1/Ba and PG1/Ra. Previous work has indicated that promoter optimization is an effective strategy for improving the expression levels of intracellular and secreted proteins by increasing transcription levels [40]. Wang et al. confirmed that the P_{apra} promoter remarkably enhanced yields of target products in *Burkholderia* [41].

B. glumae PG1 strains carrying the four plasmids constructed in this study were grown in PG medium containing 1% olive oil as a carbon source at 30 °C. Secreted lipase was detected by the lipase activity assay (Fig. 4A) and SDS-PAGE in culture supernatants (Fig. 4B) after 24 h of growth. Interestingly, the results revealed that strains PG1/BI and PG1/RI secreted much more lipase when compared to the two variants with the P_{apra} promoter. In our test system, olive oil likely functions as an inducer when the lipase operon is under the control of the native promoter, consistent with the findings of Boekema et al. [2].

To increase the production of lipase, we attempted to optimize the culture conditions. Previous studies on the synthesis of natural products had emphasized that most constitutive promoters were active in a glucose-containing medium [42]. Therefore, 0.5% glucose was used as a carbon source in the growth

medium in subsequent experiments. The results revealed that the addition of 0.5% glucose in the PG medium stimulated the expression of the lipAB operon under the control of the P_{apra} promoter. Lipase activity (Fig. 4C) and SDS-PAGE (Fig. 4D) analyses of culture supernatants were performed after 24 h of growth. The lipase activities of PG1/Ba (0.629 U/ml) and PG1/Ra (0.060 U/ml) were increased by 15.0- and 4.3-fold in comparison with those of PG1/BI (0.148 U/ml) and PG1/RI (0.004 U/ml), respectively. These results indicate that the P_{apra} promoter is more active than the native promoter in a glucose-containing medium.

In summary, the lipAB operon under the P_{apra} promoter was effective in enhancing active lipase production when 0.5% glucose was used as a carbon source, and the production of lipase using the P_{lipAB} promoter was optimal when 1% olive oil was added. Olive oil exerted a negative effect on the expression levels of lipAB under the control of P_{apra} , most probably by repressing transcriptional efficiency. The transcription levels of lipA were notably higher in strains PG1/RI and PG1/BI compared to PG1/Ra and PG1/Ba in the PG medium containing 1% olive oil (Fig S2). The effect of different carbon sources on promoters still need more exploration.

Conclusions

To enhance active lipase production, the lipase gene lipA and its chaperone gene, lipB, were cloned from the genomic DNA of *B. glumae* PG1 into high-copy vectors using the Red/ET systems followed by overexpressing in the homologous host. When the plasmid-borne lipase gene was expressed under native promoter control, the derivative strains, PG1/BI and PG1/RI, yielded 39- and 15-fold higher production of the active lipase, respectively, in comparison with the PG1 wild-type strain in the presence of 1% olive oil. This finding indicates that use of a multicopy expression vector had a positive effect on the expression level of lipase. To achieve higher lipase production, the lipase operon was expressed under the control of a strong constitutive promoter, yielding two transformants PG1/Ba and PG1/Ra. The lipase activities of PG1/Ba and PG1/Ra were 15- and 4-fold higher in the presence of 0.5% glucose as a carbon source, respectively, than that of PG1/BI and PG1/RI. Overall, the lipase produced by PG1/Ba in the medium containing glucose was less than that of the PG1/BI in the medium using olive oil as substrate, but lipase gene expression driven by a native promoter in PG1 was repressed by glucose.

Materials And Methods

Strains, plasmids and reagents

E. coli GB05-dir was derived from strain GB2005 (DH10B, *fhuA::IS2*, $\Delta ybcC$, $\Delta recET$) by integrating an arabinose-inducible ETyA operon (full-length *recE*, *recT*, *redY* and *recA*) at the *ybcC* locus [26, 31]. GB08-red was derived from GB2005 by integrating an arabinose-inducible $\gamma\beta\alpha$ A operon (*red α* , *red β* , *redY* and *recA*) at the *ybcC* locus [43]. GB05-dir and GB08-red were used for direct cloning and recombineering experiments and grown in LB medium (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter, pH 7.0) at 37 °C or 30 °C. *B. glumae* PG1 wild-type was cultivated in LB medium at 30 °C and used as a host for

homologous expression of the lipAB genes. For production of lipase, *B. glumae* strains were cultivated in PG medium [2], which contains 6 g $(\text{NH}_4)_2\text{SO}_4$, 3.5 g KH_2PO_4 , 3.5 g K_2HPO_4 , 0.02 g CaCl_2 , 1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g yeast extract per liter (pH 6.5) with 1% (vol/vol) olive oil or 0.5% (wt/vol) glucose as a carbon source.

Plasmid p15A-cm-tetO-tetR-hyg-ccdB contains a p15A origin, a hyg-ccdB cassette, a tetracycline-inducible promoter P_{tetO} , and conveys chloramphenicol resistance [36]. Plasmid pRk2-km-apra carries the broad-host-range Rk2 origin and conveys kanamycin and apramycin resistance. Plasmid pBBR1-km has the broad-host-range pBBR1 origin and conveys kanamycin resistance. Appropriate antibiotics were added when necessary at the following concentrations: for *E. coli* strains, kanamycin, chloramphenicol and apramycin were each used at 15 $\mu\text{g/ml}$; for *B. glumae* strains, kanamycin and apramycin were each used at 30 $\mu\text{g/ml}$. The concentration of L-arabinose used for induction was 1.4 mg/ml. Plasmids used in this work are listed in Table S1.

Direct cloning of the lipAB operon

The p15A-cm vector was amplified from p15A-cm-tetO-tetR-hyg-ccdB with PCR using the PAGE-purified oligonucleotides PG1-lipAB-A and PG1-lipAB-S, which contain 80 nt homology arms to the target DNA sequence, and 20 nt standard PCR primers at the 3' end. DNA oligonucleotides are listed in Table S2. The template plasmid and PCR products were purified with the Qiagen plasmid mini kit and eluted from the column with autoclaved ddH_2O . The genomic DNA of *B. glumae* PG1 was prepared from the lysate by phenol-chloroform-isoamyl alcohol extraction (25:24:1, pH 8.0) and ethanol precipitation, and digested with EcoR V and EcoR I to expose the terminal homology arms [36]. Ten micrograms of digested genomic DNA and 200 ng of p15A-cm linear vectors were co-electroporated into arabinose-induced, RecET-expressing GB05-dir cells for direct cloning of the lipAB operon, which was expressed under the control of its native promoter, P_{lipAB} . Transformants were selected after growth on LB agar plates containing chloramphenicol at 37 °C overnight, and correct recombinants were identified by restriction analysis. The final construct, p15A-cm- P_{lipAB} -lipAB, was electroporated into GB08-red cells for further engineering.

Subcloning the lipAB operon into a broad-host-range vector

Subcloning vectors pBBR1-km and pRk2-km were prepared by PCR amplification from plasmids pBBR1-km and pRk2-km-apra with the pRlmer pairs RK2-lipAB-A/S and BBR1-lipAB-A/S, respectively. PCR products were purified using the Qiagen plasmid mini kit. The pBBR1-km and pRk2-km cassettes, flanked by the 80 nt homology arms, contain a broad-host-range origin and a kanamycin-selection marker and were electroporated into arabinose-induced, $\text{red}\alpha\beta$ -expressing GB08-red cells harboring p15A-cm- P_{lipAB} -lipAB to subclone the lipAB genes. Transformants were selected with kanamycin, and correct recombinants were identified by restriction digestion. The plasmid-borne lipase was expressed under an native promoter. The final constructs, pBl and pRl, were electroporated into *B. glumae* PG1 for further studies.

Promoter exchange of the lipAB genes

The promoter of the lipAB was exchanged with the constitutive promoter P_{apra} via Red $\alpha\beta$ recombineering. P_{apra} segments, flanked by homology arms, were amplified with PCR from the template plasmid pRK2-km-apra using the 100 nt primers P_{apra} -A and P_{apra} -S. Purified PCR products were recombined with plasmids pBl or pRl in arabinose-induced, Red $\alpha\beta$ -expressing GB08-red cells to exchange the P_{lipAB} promoter. Following confirmation by restriction analysis, the final constructs, pBa and pRa, were electroporated into *B. glumae* PG1 to examine lipase activity.

Electroporation of the expression plasmid into *B. glumae* PG1

For *B. glumae* PG1, overnight cultures were diluted into 1.3 ml fresh LB medium without any antibiotics ($OD_{600} = 0.1$) and incubated at 30 °C, 950 rpm, until the OD_{600} was around 0.6. Cells were pelleted by centrifugation at 9500 rpm for 1 min at room temperature, washed, and then 10 μg of resuspended plasmid was added to the electrocompetent cells. Electroporation was conducted using an Eppendorf 2510 electroporator at 1250 V with a 1 mm electroporation cuvette. The cells were resuspended in 1 ml LB medium and transferred into a 1.5 ml punctured micro-centrifuge tube. The culture was incubated at 30 °C, 950 rpm, for 2 h before plating on LB plates containing kanamycin (30 $\mu\text{g}/\text{ml}$) followed by incubation at 30 °C for 48 h. LB agar plates contained 1.2% (wt/vol) of agar. Recombineering in *E. coli* was performed as previously described [41].

Lipase activity assay

Lipase activity in culture supernatants was assayed with para-nitrophenylpalmitate (pNPP) as the substrate [44]. First, 100 μl acetonitrile containing 0.9 mg pNPP was mixed with 900 μl of 50 mM Tris-HCl buffer (pH 8.0). After 5 min of prewarming at 37 °C, freshly prepared substrate solution was mixed with 50 μl of 5000-fold dilutions of supernatant, and then incubated at 37 °C for 5 min. Next, 200 μl of the complex was taken to measure the OD_{410} against an enzyme-free control using a spectrophotometer. Relative lipase activity was calculated as units/ml. One unit (U) was defined as the amount of lipase that liberates 1 mmol of para-nitrophenol per minute.

SDS-PAGE

Lipase proteins in whole-cell extracts and supernatants were analyzed with SDS-PAGE [20]. Culture supernatants obtained by centrifugation (4 °C, 8000 rpm, 1 min) were mixed with SDS sample buffer (5 $\times$), and then 20 μl was subjected to SDS-PAGE. Cell pellets were resuspended by sonication, and 20 μl cell suspension was mixed with SDS sample buffer for analysis. SDS-PAGE was carried out using 12% polyacrylamide gels, and proteins were stained with Coomassie brilliant blue R-250.

Quantitative real-time PCR

The analysis of transcriptional levels of lipA was performed with real-time qPCR in an ABI PRISM 7500 System (Applied Biosystems, USA) using SYBR green. Primers used for qPCR are listed in Table S2. The

constitutively expressed gene *rpoD* was used as the internal reference. The amount of PCR product was calculated using the $\Delta\Delta CT$ -method, as previously described [45].

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Availability of data and materials

(1) The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

(2) All data generated or analyzed during this study are included in this published article.

Competing interests

A Chinese patent application (ZL 201810571727.3) has been filed and granted for the technology/invention disclosed in the present work.

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Authors' contributions

Z.B., Q.F. and H.C. planned and performed cloning experiments. Z.B., Q.F. and B. Y. performed genetic transfers, cultivation experiments and data analysis. Z.X., D.P. and L.X. performed lipase activity experiment and data analysis. Y.Z., Y.G. and Q.T. designed the study and wrote the paper. All authors discussed the results and commented on the manuscript.

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Figures

Fig 1

Figure 1

Schematic diagram of the direct cloning and engineering of the *lipAB* operon for homologous expression. (A) Direct cloning of the *lipAB* operon using the RecET recombination system. Genomic DNA isolated from *B. glumae* PG1 was digested with restriction enzymes to release the target genes. The linear vector p15A-cm used for direct cloning and the target genomic segment share matching sequences at both ends, named homology arms (ha). (B) Retrofitting the cloned genes with a broad-host-range vector. The broad-host-range pBBR1 and pRk2 origins flanked by homology arms, were used for retrofitting of the p15A vectors using the Redαβ recombination system, and then a strong constitutive promoter (Papra)

was exchanged with the native promoter of lipAB to regulate expression of lipAB. A kanamycin resistance gene was used as a selectable marker.

Fig 2

Figure 2

Lipase production of wild-type *B. glumae* PG1 (PG1wt) and PG1 transformants in PG medium supplemented with 1% olive oil. (A) SDS-PAGE analysis of the culture supernatants and cell extracts after 72 h of cultivation. PG1/RI and PG1/BI contain pRI and pBI, respectively. Lane M, molecular weight standards; lanes 1, 3 and 5, culture supernatants; lanes 2, 4 and 6, cell extracts. The position of the lipase protein is indicated by an arrow. (B) Comparison of the expression levels of lipA detected by qPCR after 24 h of cultivation. Error bars show standard deviations derived from three experimental replications.

Fig 3

Figure 3

Lipase activity and cell growth of *B. glumae* PG1 (PG1wt) and PG1 transformants. (A) Lipase activity in the supernatants at different growth phases. Activity was measured after cell removal. PG1/RI and PG1/BI contain pRI and pBI, respectively. (B) Cell growth curve. Wild-type *B. glumae* PG1, PG1/RI and PG1/BI were grown at 30°C in PG medium with 1% olive oil. Error bars indicate standard deviations from three experimental replications.

Fig 4

Figure 4

Lipase production of *B. glumae* PG1 transformants harboring different plasmids. (A, C) Relative lipase activity and (B, D) SDS-PAGE analysis of the supernatants of cultures grown with (A, B) 1% olive oil and (C, D) 0.5% glucose. Cells were grown for 24 h in PG medium at 30°C. PG1/RI and PG1/BI contain pRI and pBI, respectively, and PG1/Ra and PG1/Ba contain pRa and pBa, respectively. Error bars show standard deviations derived from three experimental replications.

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