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High level recombinant protein expression in *Ralstonia eutropha* using T7 RNA polymerase based amplification

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Abstract

We report further development of a novel recombinant protein expression system based on the Gram-negative bacterium, *Ralstonia eutropha*. In this study, we were able to express soluble, active, organophosphohydrolase (OPH), a protein that is prone to inclusion body formation in *Escherichia coli*, at titers greater than 10 g/L in high cell density fermentation. This represents a titer that is approximately 100-fold greater than titers previously reported in *E. coli* for this enzyme. *R. eutropha* strains expressing OPH were generated in two cloning steps. First, the T7 RNA polymerase gene was placed under the control of the strong, inducible *phaP* promoter and integrated into the *phaP* locus of *R. eutropha* NCIMB 40124. Second, a single copy of the *oph* gene under control of the T7 promoter was randomly integrated into the chromosome using a transposon cloning vector.

Keywords: Recombinant protein; High cell density; Fermentation; T7 RNA polymerase; Organophosphate hydrolase; Phosphotriesterase; Parathion hydrolase

Improving recombinant protein expression technologies is one of the key focus areas of our research group [1–4]. A protein expression platform should yield high recombinant protein productivities (g/L h) and high titers (g/L) of soluble, active recombinant protein. We have previously reported the development of a novel high cell density protein expression platform based on the Gram-negative bacterium, *Ralstonia eutropha* [3,4]. This system has been developed in our laboratory to overcome some of the shortcomings associated with recombinant protein expression in other bacteria (e.g., poor fermentation performance, inclusion body formation, and proteolysis). High level expression of organophosphohydrolase (OPH), an enzyme originally isolated from *Pseudomonas diminuta* [5] and prone to inclusion body formation in *Escherichia coli* [6–8], was demonstrated with this new system.

Using a proteomics approach, we identified the *phaP* promoter as a strong, endogenous promoter that is induced by phosphate limitation during high cell density fermentation [3]. By placing a single copy of the *oph* gene under the control of the *phaP* promoter and integrating the *phaP*p::*oph* fusion into the chromosome, we were able to obtain a maximum specific activity of 145 U/mg soluble protein in high cell density fermentation, corresponding to an OPH titer of 1.2 g/L [3]. In a second study that examined the effect of gene dosage on OPH expression, multiple copies of the *phaP*p::*oph* fusion were integrated into the chromosome. We measured maximum specific activities of 170, 312, and 487 U/mg for single, double, and triple copies of the *phaP*p::*oph* fusion, respectively, in high cell density fermentation [4]. A specific activity of 487 U/mg represents 6% of total cellular protein and corresponds to an OPH titer of 4.3 g/L [4].

Because an increase in gene dosage led to a proportional increase in OPH expression, we speculated that OPH expression was transcriptionally limited. Introducing

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the *phaP*p::*oph* fusion into *R. eutropha* on a self-replicating plasmid vector resulted in high OPH specific activities in shake flasks, but not in high cell density fermentation [4]. We attributed the low OPH expression levels in high cell density fermentation to plasmid instability, despite continually feeding antibiotic. To further increase recombinant protein expression levels, other strong promoter systems, where constructs could be stably integrated into the chromosome, were sought.

The T7 RNA polymerase (T7 RNAP) system has been extensively studied in *E. coli* [9–11]. Very high recombinant protein expression levels have been achieved in *E. coli* using the bacteriophage T7 RNAP system. Up to half of all cellular protein can be comprised of a recombinant protein using the T7 system [10]. The T7 RNAP recognizes a highly conserved, 20 base pair sequence with great specificity, resulting in high levels of transcription [9–11].

We adopted a two step strategy of utilizing the T7 RNAP system to express high levels of OPH in *R. eutropha*. First, the T7 RNAP gene was placed under the control of the *phaP* promoter, which is a strong, inducible, endogenous promoter and integrated into the chromosome in the *phaP* locus. Second, the *oph* gene was placed under control of the T7 promoter and randomly integrated into the chromosome using a transposon vector.

Materials and methods

Strains, plasmids, and oligonucleotides

The strains and plasmids used in this study are listed in Table 1. The oligonucleotides used in this study are listed in Table 2.

Growth media, antibiotics, and cultivation conditions

Escherichia coli strains were grown in Luria–Bertani (LB) medium. *R. eutropha* strains were grown in one of the following media depending on the application: LB medium, PCT medium [3] (20 g/L glucose, 2.2 g/L $MgSO_4 \cdot 7H_2O$, 3 g/L K_2SO_4, 0.18 g/L Na_2SO_4, 0.18 g/L $FeSO_4 \cdot 7H_2O$, 14 mM H_3PO_4, and 2.4 ml/L trace element solution) or Lee medium [12] (20 g/L glucose, 3 g/L $Na_2HPO_4 \cdot 7H_2O$, 1 g/L KH_2PO_4, 2 g/L NH_4Cl, 0.2 g/L $MgSO_4 \cdot 7H_2O$, and 2.4 ml/L trace element solution). Antibiotics were added to the growth media to the following concentrations depending on the application: chloramphenicol (50 μg/ml), tetracycline (10 μg/ml),

Table 1
Strains and plasmids used in this study

Strain or plasmid	Description	References or source
R. eutropha strains		
NCIMB 40124	Wild type; gentamicin resistant	NCIMB
SS15	Wild type containing *phaP*p::T7 RNAP expression cassette; gentamicin and chloramphenicol resistant	This study
GH29.1 through GH29.20	SS15 containing T7p::*oph* expression cassette; gentamicin, chloramphenicol, and tetracycline resistant	This study
E. coli strains		
TOP10	Host strain for plasmids derived from pCR2.1-TOPO	Invitrogen
S-17	Host strain for plasmids derived from pKNOCK-Cm, pKNOCK-Tc, and pUTminiTn5gfp	[13,22,23]
Plasmids		
pCR2.1-TOPO	High copy number plasmid for cloning; conferring ampicillin and kanamycin resistance	Invitrogen
pKNOCK-Cm	Suicide plasmid used for homologous recombination into *R. eutropha*; conferring chloramphenicol resistance	[24]
pKNOCK-Tc	Suicide plasmid used for homologous recombination into *R. eutropha*; conferring tetracycline resistance	[24]
pUCPPCm	*phaP*p::*oph* transcriptional fusion cloned into pUC19; conferring chloramphenicol resistance	[3]
pTARA	Plasmid containing the T7 RNA polymerase gene; conferring chloramphenicol resistance	[25]
pUTminiTn5gfp	Transposon vector for integrating *gfp* gene randomly into host chromosome; conferring tetracycline resistance	ATCC
pGH1	pKNOCK-Tc containing 0.4 kb fragment of *cat* gene; conferring tetracycline resistance	This study
pGH2	Derivative of pGH1 containing multiple cloning site (MCS); conferring tetracycline resistance	This study
pGH3	Derivative of pGH2 containing a single copy of *oph* in the MCS; conferring tetracycline resistance	This study
pGH10	Multiple cloning site and transcriptional terminator in pCR2.1-TOPO	This study
pGH12	Transposon vector derived from pUTminiTn5gfp; *gfp* gene replaced with multiple cloning site; conferring tetracycline resistance	This study
pGH14	T7p::*oph* transcriptional fusion cloned into pGH12; conferring tetracycline resistance	This study

Table 2
Oligonucleotides used in this study

Oligonucleotide	Sequence[a]	Location and orientation[b]
PromUp1	GCAAACTAGTCGCGCATCGTTAACACGC	Region upstream of *phaP* promoter in *R. eutropha* chromosome (+)
T7intDn	GAATGGTATCTTCCAGCACT	3′ end of T7 RNAP ORF (−)
oGEH13	GAAGAAAGATCTGGCGCGCCATTTAAATGCGGCCGC GTTTAAACGCCCGGGCGGATCTCGATCCCG	First oligonucleotide for constructing multiple cloning site and transcriptional terminator by overlap PCR (+)
oGEH14	ACAGGGAAACCGTTGTGGTGTCCCTATAGTGAGTCGT ATTAATTTCGCGGGATCGAGATCCGCCCGGG	Second oligo for overlap PCR product (−)
oGEH15	AGGGACACCACAACGGTTTCCCTGTAGAAATAATTTT GTTTAACTTTAAGAAGGAGATGAGCTCATGG	Third oligo for overlap PCR product (+)
oGEH16	GACGTCGAATCCCGACCCATTTGCTGTCCACCAGTCA TGCTAGCCATGAGCTCATCTCCTTCTT	Fourth oligo for overlap PCR product (−)
oGEH17	GGGTCGGGATTCGACGTCACCGGTGGGCCCGGATCC GTTAACCTGCAGCGATCGACTAGTCTCGA	Fifth oligo for overlap PCR product (+)
oGEH18	CAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAG CCATGGCTCGAGACTAGTCGATCGCTGC	Sixth oligo for overlap PCR product (−)
oGEH19	GGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGTCTA GACGCGCGAGACGGGCCAGAGACCCTTAAGCGTAC	Seventh oligo for overlap PCR product (+)
oGEH20	GAAGAACCGCGGCATGCTTAATTAAATGCATCAATTG CGTACGCTTAAGGGTCTCTGGCCCGT	Eighth oligo for overlap PCR product (−)
oGEH21	GAAGAAGGTACCCACTGGATATACCAC	5′ end of *cat* ORF (+)
oGEH22	GAAGAAGGATCCCTCAATAAACCCTTTAG	*cat* internal primer (−)
oGEH29	GAAGAATGGCCAGGCGCGCCATTTAAATG	5′ end of overlap PCR product (+)
oGEH30	GAAGAAATCGATCCGCGGCATGCTTAAT	3′ end of overlap PCR product (−)

[a] Restriction sites engineered into the sequences are underlined.
[b] Forward (+) or reverse (−) orientation relative to ORF is indicated.

kanamycin (50 μg/ml), and gentamicin (10 μg/ml). *R. eutropha* and *E. coli* strains were cultivated at 30 and 37 °C, respectively.

DNA preparation and manipulation

Standard procedures were used for the preparation and manipulation of DNA and for PCR. All constructs containing PCR products were confirmed by sequencing at the Molecular Biology Core Facility at Dartmouth College.

Construction of R. eutropha strain harboring T7 RNAP under the control of phaP promoter (SS15)

The 2.7 kb *Sac*I/*Xba*I fragment of pTARA containing the T7 RNAP gene was inserted into the vector pUCPPCm digested with *Sac*I and *Xba*I. Electrocompetent *R. eutropha* NCIMB 40124 was electroporated with the ligation mix (13 V/cm, 200 Ω, 25 μF). Colonies were selected on LB plates containing gentamicin (*R. eutropha* NCIMB 40124 being gentamicin resistant) and chloramphenicol. Site-directed integration of the suicide plasmid into the *phaP* locus of *R. eutropha* NCIMB 40124 was confirmed by colony PCR using primers PromUp1 and T7intDn. In addition to the colony PCR, a Southern blot confirmed integration of the *phaP*p::T7 RNAP transcriptional fusion into the *phaP* locus of the strain, which was designated SS15 (data not shown).

Construction of overlap PCR product containing T7p, MCS, and transcriptional terminator

A DNA fragment containing the T7 promoter, a multiple cloning site compatible with our existing vectors and a T7 transcriptional terminator (identical to transcriptional terminator in Novagen pET-3a cloning vector), was assembled by overlap PCR using eight oligonucleotides (oGEH13 to oGEH20). Initially oligonucleotides were subjected to PCR in pairs (oGEH13/14, oGEH15/16, oGEH17/18, and oGEH19/20, see Fig. 1). The derivatives of oGEH13/14 and oGEH15/16 were mixed together and amplified using oGEH13 and oGEH16. Similarly, the derivatives of oGEH17/18 and oGEH19/20 were mixed together and amplified using oGEH17 and oGEH20. The two resulting PCR fragments were mixed together and amplified with oGEH13 and oGEH20. The 0.4 kb product was cloned into pCR2.1-TOPO (see Fig. 2).

Construction of transposon vector pGH12, designed for integrating genes under the control of the T7 promoter into the R. eutropha chromosome

A 0.4 kb fragment of the chloramphenicol acetyl transferase (*cat*) gene from pUCPPCm was PCR-amplified using primers oGEH21 and oGEH22 and cloned into pCR2.1-TOPO. The 0.4 kb *Kpn*I/*Bam*HI fragment was excised from the resulting pCR2.1-TOPO derivative and cloned into pKNOCK-Tc digested with *Kpn*I and

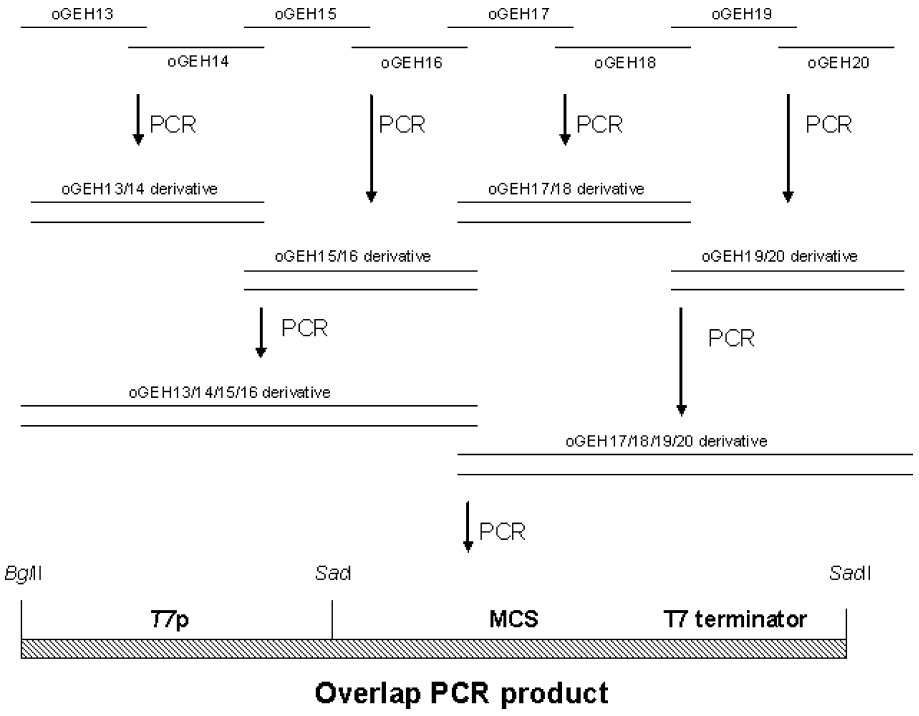

Fig. 1. Construction of overlap PCR product containing T7 promoter (*T7*p), multiple cloning site (MCS), and T7 transcriptional terminator (T7 terminator identical to sequence in Novagen pET-3a cloning vector). Multiple PCR steps were required for complete assembly (see Materials and methods for details).

*Bam*HI yielding plasmid pGH1. The 0.4 kb overlap PCR product shown in Fig. 1, cloned into pCR2.1-TOPO, was digested with *Bgl*II and *Sac*II and inserted into pGH1 digested with *Bam*HI and *Sac*II to create plasmid pGH2. To add the required restriction sites for cloning into plasmid pUTminiTn5gfp, the overlap PCR product cloned into pGH2 was amplified by PCR from pGH2 using primers oGEH29 and oGEH30. The 0.4 kb fragment was cloned into pCR2.1-TOPO, generating plasmid pGH10. The 0.4 kb *Cla*I/*Msc*I fragment of plasmid pGH10 was excised and cloned into pUTminiTn5gfp digested with *Cla*I and *Eco*ICRI, generating plasmid pGH12.

Construction of integration plasmid pGH14, designed to introduce T7p::oph randomly into the chromosome of R. eutropha strain SS15

The 1.0 kb *Sac*I/*Bam*HI fragment of plasmid pUC-PPCm containing the *oph* gene was cloned into pGH2 digested with *Sac*I and *Bam*HI yielding plasmid pGH3. The 1.3 kb *Asc*I/*Pac*I fragment of plasmid pGH3 containing the *T7p::oph* fusion and transcription terminator was cloned into pGH12 digested with *Asc*I *and Pac*I, yielding plasmid pGH14.

Construction of recombinant R. eutropha strains expressing the oph gene under control of the T7 promoter

Plasmids harbored in *E. coli* S17 strains were transferred into *R. eutropha* strains using standard biparental mating protocols [3,13]. Strains GH29-1 through GH29-20 were generated by introducing the suicide plasmid pGH14 into strain SS15.

Screening R. eutropha strains for OPH expression

Ralstonia eutropha strains were inoculated into 5 ml of LB medium containing chloramphenicol and incubated overnight. To induce OPH expression, 0.5 ml of the overnight culture was transferred into 5 ml of Lee medium containing chloramphenicol. Lee medium is a phosphate deficient growth medium and induces the *phaP* promoter during batch growth conditions. After incubation for 20 h, cultures were assayed for OPH activity.

Enzyme activity assay

The OPH enzyme activity determination method used in this study has previously been described [3,4]. Cell pellets were resuspended in 150 mM CHES buffer, pH 9.0, to an approximate OD_{600} ranging between 1 and 3. The samples were sonicated in a Fisher Scientific Sonic dismembrator 550 in two pulsed cycles (2 s ON, 0.5 s OFF, 30 s duration, and 5 min cooling on ice between cycles). One milliliter of this cell free extract was centrifuged at 16,000*g* for 5 min and the supernatant was analyzed for enzyme activity using paraoxon (Sigma, St. Louis, MO) as described previously [5,14]. An extinction coefficient of $17{,}000\ M^{-1}\,cm^{-1}$ for *p*-nitrophenol was used to calculate the activity. The total protein concentration

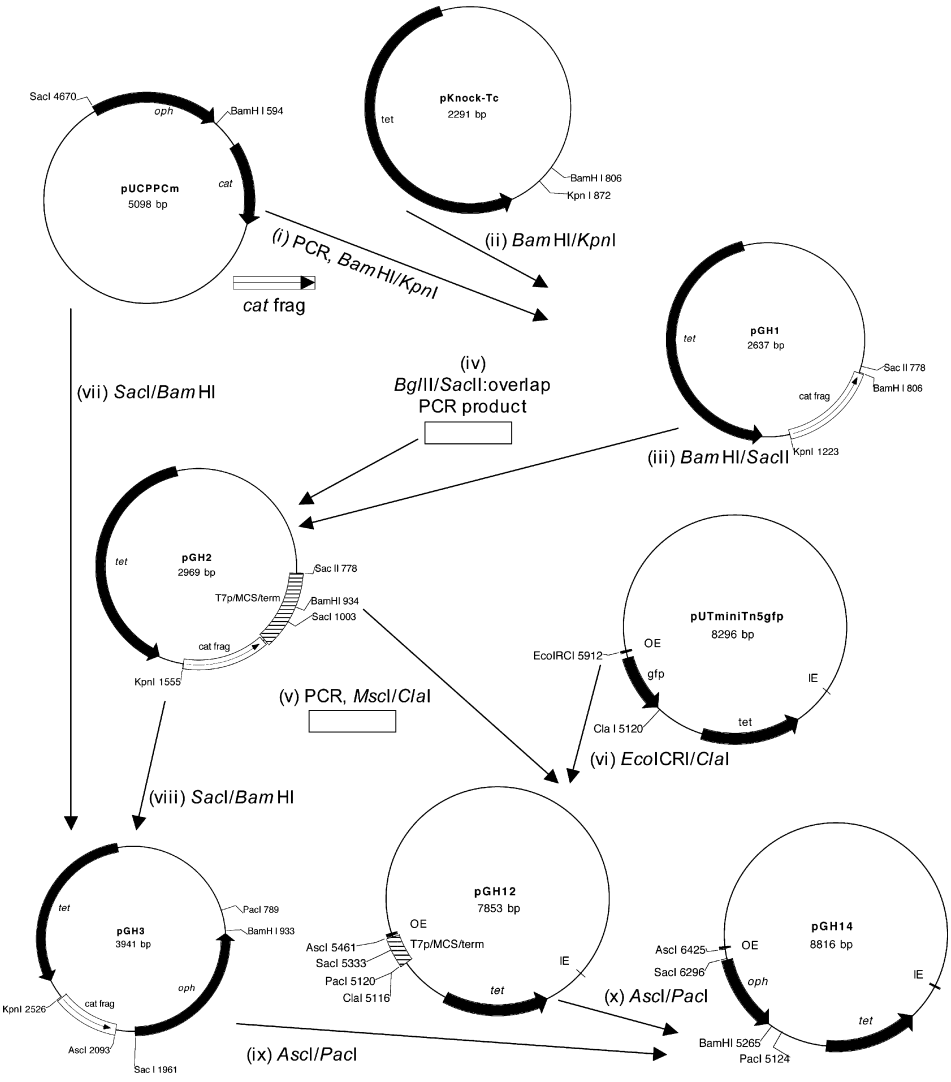

Fig. 2. Plasmid vector construction diagram (see Materials and methods for details). A 0.4 kb fragment of the *cat* gene was amplified from pUCPPCm, digested with *Bam*HI and *Kpn*I (i) and cloned into pKNOCK-Tc digested with *Bam*HI and *Kpn*I (ii) yielding pGH1. The overlap PCR product (shown in Fig. 1) was digested with *Bgl*II and *Sac*II (iv) and cloned into pGH1 digested with *Bam*HI and *Kpn*I (iii), generating pGH2. The overlap PCR product cloned into pGH2 was PCR-amplified with new restriction sites, namely *Msc*I and *Cla*I allowing digestion (v) and cloning into pUTminiTn5gfp digested with *Eco*ICRI and *Cla*I (vi) generating plasmid pGH12. The *oph* gene from pUCPPCm was cloned into pGH2 as a *Sac*I/*Bam*HI fragment (vii and viii) generating pGH3. The *T7*p::*oph*::term fusion from pGH3 was cloned into pGH12 as a *Asc*I/*Pac*I fragment (ix and x) yielding pGH14. The 19 bp end recognition sequences of the Tn5 transposase are designated as OE (outer end) and IE (inner end).

was measured using a Bradford protein assay kit (Bio-Rad, Hercules, CA).

Fermentation procedure

Fermentation procedures used in this study have previously been described [3,4]. Fed batch fermentations were carried out in a 3 L fermentor (Applikon, Foster City, CA) at 30 °C, 1.5 L/min airflow, and 630 rpm stirrer speed with a initial working volume of 1 L (1× PCT medium with 5% [v/v] inoculum). An Applikon programmable logic controller (ADI1030) was used for maintaining the temperature at 30 °C and pH at 6.8. The pH was maintained by the addition of aqueous ammonia (29%). Dissolved oxygen concentration was maintained at 30% by controlling the stirrer speed up to a maximum speed of 1250 rpm at which point the dissolved oxygen concentration dropped below 30% and became a function of the glucose feed rate. The fermentation was carried out in a batch mode until the initial glucose (20 g/L) was consumed. Following the batch phase a feed containing glucose, phosphoric acid, $CoCl_2 \cdot 7H_2O$, and $MgSO_4$ (600 g/L, 220, 0.15, and 19 mM, respectively) was used to promote linear growth until a desired biomass concentration was achieved. At that point, induction was initiated by changing the feed solution to glucose (600 g/L) containing 15 mM $CoCl_2$. The second glucose feed solution contains no phosphate,

inducing the expression of OPH. Offgas was analyzed in real time for O_2 and CO_2 concentrations with an Illinois Instruments Model 3750 carbon dioxide/oxygen analyzer (Illinois Instruments, Ingleside, IL).

Quantification of polyhydroxybutyrate

The concentration of polyhydroxybutyrate (PHB) was quantified by the sulfuric acid-HPLC method of Karr et al. [15] with modifications [16].

Western blot

One milliliter samples of culture were collected, centrifuged, and frozen. The entire frozen pellet was resuspended in 50 µl of 2× SDS cracking buffer (100 mM Tris–HCl, pH 6.8, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) and boiled for 10 min. Samples (15 µl) were then loaded on a 12% Tris–HCl SDS–polyacrylamide gel (Bio-Rad, Hercules, CA) and resolved at 100 V. The proteins were then transferred from the gel to a nitrocellulose membrane (0.2 µm, Schleicher-Schuell, Keene, NH) for 1 h at 100 V/350 mA. The membrane was then blocked in TBST buffer (50 mM Tris base, 188 mM NaCl, and 0.05% Tween 20, pH 7.5) containing 3% BSA (Sigma, St. Louis, MO) and incubated for 2 h at room temperature. The membrane was then exposed to an anti-T7 RNAP antibody (T7 RNA Polymerase Monoclonal Antibody, EMB Biosciences, Novagen brand, Madison, WI) diluted 1:10,000 in TBST buffer. After repeated washing with TBST buffer, the membrane was exposed to a secondary goat anti-mouse antibody conjugated to HRP (Pierce, Rockford, IL), diluted 1:5000 in TBST buffer. After repeated washing with TBST buffer, the blot was developed in TBS buffer containing 500 mg/L diaminobenzine (DAB) (Pierce, Rockford, IL) and 100 µl/L of a 30% H_2O_2 solution (Fisher, Fair Lawn, NJ).

Results and discussion

Verification of T7 RNAP expression

A *R. eutropha* strain expressing T7 RNAP under the control of the *phaP* promoter was constructed and named SS15 (see Materials and methods). To verify T7 RNAP expression, strain SS15 was grown in Lee medium in the presence of chloramphenicol. A Western blot confirmed phosphate responsive expression of T7 RNAP (data not shown).

Screening R. eutropha strains for OPH expression (strains GH29-1 to GH29-20)

High transformation efficiency was found when introducing plasmid pGH14 into strain SS15. OPH expression levels of *R. eutropha* strains were measured using a simple screening protocol (see Materials and methods) to confirm OPH expression and to investigate the effect of random chromosomal integration of the T7p::*oph* transcriptional fusion. The specific enzyme activity measurements of the 20 *R. eutropha* strains are shown in Fig. 3. Great variability in OPH expression levels was observed. Based on expression levels, four strains were selected for high cell density fermentations, namely GH29-7, GH29-8, GH29-15, and GH29-16.

High cell density fermentation

High cell density fermentations were performed using protocols and equipment previously described [3,4]. A typical fermentation profile is summarized in Fig. 4, illustrating salient trends generated in a high cell density fermentation inoculated with strain GH29-8. The batch growth phase spanned the first 18 h. During the fed batch phase (hours 18–40), the feed solution (600 g/L glucose; 4.5 g/L $MgSO_4 \cdot 7H_2O$; 220 mM H_3PO_4; and 0.15 mM $CoCl_2 \cdot 6H_2O$) feed rate was initially set at 23.1 g/h, which corresponds to a glucose addition rate of 11.2 g/h. Between hours 25 and 40, the feed solution feed rate was set at 30.3 g/h, which corresponds to 14.7 g/h glucose. After reaching a dry cell weight of 86 g/L at 40 h, OPH expression was induced by switching feed solutions. The glucose feed solution was substituted with the induction feed solution (600 g/L glucose; 15 mM $CoCl_2 \cdot 6H_2O$). Cobalt was added as several studies have shown that OPH requires a divalent cation such as cobalt to be active [18,19,21]. The feed rate of the induction feed solution during this phase (hours 40–66) remained unchanged at 29.6 g/h, which corresponds to a glucose addition rate of 14.3 g/h. The maximum oxygen uptake rate (OUR) mea-

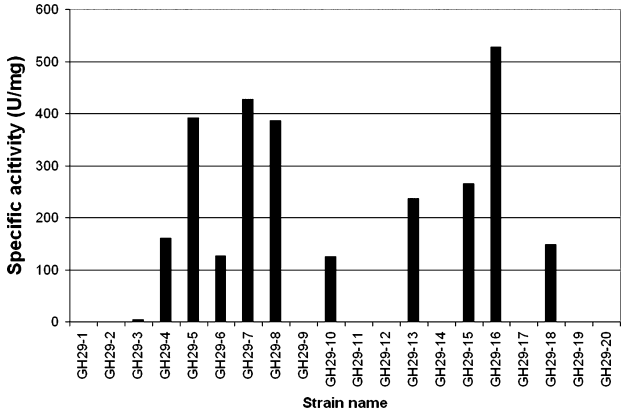

Fig. 3. OPH expression levels of 20 independent *R. eutropha* recombinant strains measured by a simple screening protocol (see text). These strains were isolated following random chromosomal integration of a T7p::*oph* transcriptional fusion (see text) into strain SS15 (see Table 1). The genetic locus of the T7p::*oph* transcriptional fusion is unknown in each recombinant strain. The data represent a single measurement for each strain.

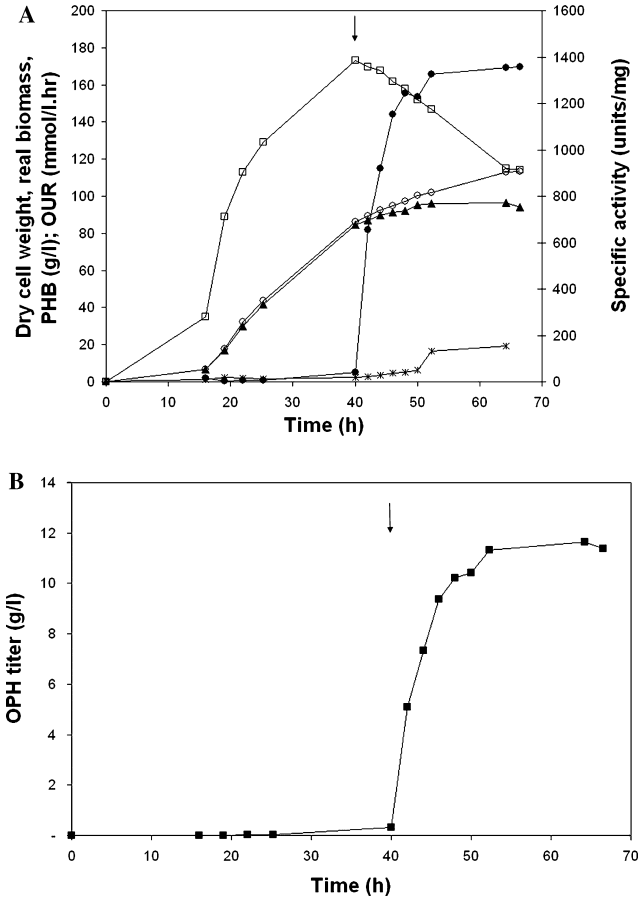

Fig. 4. Typical high cell density fermentation profile for strain GH29-8 (see Tables 1 and 3). (A) Dry cell weight (open circles), real biomass (closed triangles), PHB (crosses), O_2 uptake rate (OUR) (open squares), and OPH activity (closed circles). (B) OPH titer (closed squares). The arrow in both panels indicates the time of induction.

sured was 173 mmol/L h, which is within the constraints of large industrial bioreactors [17].

A maximum specific activity of 1353 U/mg was measured in this fermentation at 64 h. The specific activity of purified OPH in the presence of cobalt has been reported as 7250 [18] and 8020 U/mg [19]. A specific activity of 1354 U/mg therefore corresponds to 18% of total soluble cellular protein. The OPH titer was calculated using a method previously described [3,4] using the specific enzyme activity, dry cell weight, polyhydroxybutyrate (PHB) concentration, a specific activity of 7635 U/mg for the pure enzyme and assuming 68% of real biomass represents total cellular protein. In the absence of PHB, the protein content of *R. eutropha* has been measured at 68% [3,20]. Due to the synthesis of PHB during induction, the PHB concentration was measured during the course of the fermentation. Real biomass refers to the difference between dry cell weight and PHB. From the figure, a maximum OPH titer of 11.6 g/L was measured at 64 h (1353 U/mg, 113 g/L dry cell weight, 16 g/L PHB, and 97 g/L real biomass).

Results of high cell density fermentations in this study are summarized in Table 3. The high cell density fermentation represented by the profiles in Fig. 4 is designated Ferm. #2 in Table 3 and depicted in bold face. The high level expression of OPH in strain GH29-8 was reproduced in a subsequent high cell density fermentation and designated Ferm. #5. High cell density fermentations were also performed on the other *R. eutropha* strains identified from the OPH screening assay (GH29-7, GH29-15, and GH29-16).

Therefore, using several *R. eutropha* strains we were able to express soluble, active OPH at levels in excess of 10 g/L. Previous studies report OPH expression at titer levels below 100 mg/L. Omburo et al. [19] reported an OPH specific activity of 69 U/mg in *E. coli* cell lysate. However, only 160 g of wet cell paste was isolated from 7 liters of culture volume grown at 30 °C. A total of 3.7×10^6 U of OPH were measured in the lysate. This corresponds to an OPH titer of 69 mg/L (3.7×10^6 U ÷ 7635 U/mg OPH ÷ 7 L). Serdar and Murdock [21] reported a maximum OPH specific activity of 59.0 U/mg in recombinant *E. coli* cultivated at 30 °C. However, cells were harvested at an OD_{600} of 30 which corresponds to a dry cell weight of approximately 10 g/L. This corresponds to an OPH titer of approximately 39 mg/L (59 U/mg protein ÷ 7635 U/mg OPH × 10 g/L DCW * 0.5 mg protein/mg DCW). Therefore the high OPH titer in our system is due to higher specific activity and higher cell density. A titer of 10 g/L is therefore approximately 100 times greater than expression levels previously reported in *E. coli*.

This study further demonstrates the potential of *R. eutropha* as an alternative recombinant protein expression platform. Due to an ever increasing demand for the production of commodity enzymes, therapeutic proteins, vaccines, and peptides, we are currently adapting this system for the expression of other heterologous proteins.

Table 3
Summary of high cell density fermentations in this study

Ferm. #	Strain	Maximum OPH activity (U/mg)	Corresponding dry cell weight (g/L)	Corresponding PHB concentration (g/L)	Corresponding "real" biomass (g/L)	OPH titer (g/L)
1	GH29-7	1037	104	9.1	95	8.4
2	**GH29-8**	**1353**	**113**	**16.0**	**97**	**11.6**
3	GH29-15	1197	84	5.5	78	8.4
4	GH29-16	301	114	20.0	94	2.4
5	GH29-8	1214	99	2.4	96	10.4

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References

[1] S.R. Hamilton, P. Bobrowicz, B. Bobrowicz, R.C. Davidson, H. Li, T. Mitchell, J.H. Nett, S. Rausch, T.A. Stadheim, H. Wischnewski, S. Wildt, T.U. Gerngross, Production of complex human glycoproteins in yeast, Science 301 (2003) 1244–1246.

[2] B.K. Choi, P. Bobrowicz, R.C. Davidson, S.R. Hamilton, D.H. Kung, H. Li, R.G. Miele, J.H. Nett, S. Wildt, T.U. Gerngross, Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast *Pichia pastoris*, Proc. Natl. Acad. Sci. USA 100 (2003) 5022–5027.

[3] S. Srinivasan, G.C. Barnard, T.U. Gerngross, A novel high cell density protein expression system based on *Ralstonia eutropha*, Appl. Environ. Microbiol. 68 (2002) 5925–5932.

[4] S. Srinivasan, G.C. Barnard, T.U. Gerngross, Production of recombinant proteins using multiple copy gene integration in high cell density fermentations of *Ralstonia eutropha*, Biotechnol. Bioeng. 84 (2003) 114–120.

[5] C.M. Serdar, D.T. Gibson, Enzymatic-hydrolysis of organophosphates—cloning and expression of a parathion hydrolase gene from *Pseudomonas diminuta*, Biotechnology 3 (1985) 567–571.

[6] H.J. Cha, C.F. Wu, J.J. Valdes, G. Rao, W.E. Bentley, Observations of green fluorescent protein as a fusion partner in genetically engineered *Escherichia coli*: monitoring protein expression and solubility, Biotechnol. Bioeng. 67 (2000) 565–574.

[7] C.F. Wu, H.J. Cha, G. Rao, J.J. Valdes, W.E. Bentley, A green fluorescent protein fusion strategy for monitoring the expression, cellular location, and separation of biologically active organophosphorus hydrolase, Appl. Microbiol. Biotechnol. 54 (2000) 78–83.

[8] C.F. Wu, H.J. Cha, G. Rao, J.J. Valdes, W.E. Bentley, Enhancement of organophosphorus hydrolase yield in *Escherichia coli* using multiple gene fusions, Biotechnol. Bioeng. 75 (2001) 100–103.

[9] F.W. Studier, B.A. Moffatt, Use Of bacteriophage T7 RNA polymerase to direct selective high level expression of cloned genes, J. Mol. Biol. 189 (1986) 113–130.

[10] F.W. Studier, A.H. Rosenberg, J.J. Dunn, J.W. Dubendorff, Use of T7 RNA-polymerase to direction expression of cloned genes, Methods Enzymol. 185 (1990) 60–89.

[11] A.H. Rosenberg, B.N. Lade, D.S. Chui, S.W. Lin, J.J. Dunn, F.W. Studier, Vectors for selective expression of cloned DNAs by T7 RNA-polymerase, Gene 56 (1987) 125–135.

[12] J.S. Park, H.C. Park, T.L. Huh, Y.H. Lee, Production of poly-β-hydroxybutyrate by *Alcaligenes eutrophus* transformants harboring cloned phaCAB genes, Biotechnol. Lett. 17 (1995) 735–740.

[13] J. Quandt, M.F. Hynes, Versatile suicide vectors which allow direct selection for gene replacement in Gram-negative bacteria, Gene 127 (1993) 15–21.

[14] K.I. Wild, C.E. Miller, J.R. Wild, Characterization of organophosphorous hydrolases and the genetic manipulation of the phosphotriesterase from *Pseudomonas diminuta*, Chem. Biol. Interact. 87 (1993) 55–68.

[15] D.B. Karr, J.K. Waters, D.W. Emerich, Analysis of poly-β-hydroxybutyrate in *Rhizobium japonicum* bacteroids by ion-exclusion high-pressure liquid chromatography and UV detections, Appl. Environ. Microbiol. 60 (1983) 3952–3958.

[16] G.M. York, B.H. Junker, J. Stubbe, A.J. Sinskey, Accumulation of the PhaP phasin of *Ralstonia eutropha* is dependent on production of polyhydroxybutyrate in cells, J. Bacteriol. 183 (2001) 4217–4226.

[17] M. Charles, J. Wilson, Fermentor design, in: B.K. Lydersen, N.A. D'Elia, K.L. Nelson (Eds.), Bioprocess Engineering: Systems, Equipment and Facilities, Wiley, New York, 1994, pp. 5–67.

[18] K.R. Rogers, A. Wang, A. Mulchandani, P. Mulchandani, W. Chen, Organophosphorous hydrolase-based assay for organophosphate pesticides, Biotechnol. Prog. 15 (1999) 517–521.

[19] G. Omburo, J.M. Kuo, L.S. Mullins, F.M. Raushel, Characterization of the Zn binding site of bacterial phosphotriesterase, J. Biol. Chem. 267 (1992) 13278–13283.

[20] R.A. Henderson, C.W. Jones, Physiology of poly-3-hydroxybutyrate (PHB) production by *Alcaligenes eutrophus* growing in continuous culture, Microbiology—UK 143 (1997) 2361–2371.

[21] C.M. Serdar, D.C. Murdock, Parathion hydrolase analogs and methods for production and purification, US patent 5,484,728, to Amgen Inc., Patent and Trademark Office, 1996.

[22] R. Simon, U. Priefer, A. Pfihler, A broad host-range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram-negative bacteria, Bio-Technology 1 (1983) 784–791.

[23] V. de Lorenzo, M. Herrero, U. Jakubzik, K.N. Timmis, Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in Gram-negative eubacteria, J. Bacteriol. 172 (1990) 6568–6572.

[24] M.F. Alexeyev, The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of Gram-negative bacteria, BioTechniques 26 (1999) 824–826.

[25] D.R. Wycuff, K.S. Matthews, Generation of an AraC-araBAD promoter-regulated T7 expression system, Anal. Biochem. 277 (2000) 67–73.