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Construction and use of a *Cupriavidus necator* H16 soluble hydrogenase promoter (P_{SH}) fusion to *gfp* (green fluorescent protein)

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Hydrogenases are metalloenzymes that reversibly catalyse the oxidation or production of molecular hydrogen (H_2). Amongst a number of promising candidates for application in the oxidation of H_2 is a soluble [Ni-Fe] uptake hydrogenase (SH) produced by *Cupriavidus necator* H16. In the present study, molecular characterisation of the SH operon, responsible for functional SH synthesis, was investigated by developing a green fluorescent protein (GFP) reporter system to characterise PSH promoter activity using several gene cloning approaches. A PSH promoter-gfp fusion was successfully constructed and inducible GFP expression driven by the PSH promoter under de-repressing conditions in heterotrophic growth media was demonstrated in the recombinant *C. necator* H16 cells. Here we report the first successful fluorescent reporter system to study PSH promoter activity in *C. necator* H16. The fusion construct allowed for the design of a simple screening assay to evaluate PSH activity. Furthermore, the constructed reporter system can serve as a model to develop a rapid fluorescent based reporter for subsequent small-scale process optimisation experiments for SH expression.

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ABSTRACT

Hydrogenases are metalloenzymes that reversibly catalyse the oxidation or production of molecular hydrogen (H_2). Amongst a number of promising candidates for application in the oxidation of H_2 is a soluble [Ni-Fe] uptake hydrogenase (SH) produced by *Cupriavidus necator* H16. In the present study, molecular characterisation of the SH operon, responsible for functional SH synthesis, was investigated by developing a green fluorescent protein (GFP) reporter system to characterise P_{SH} promoter activity using several gene cloning approaches. A P_{SH} promoter-gfp fusion was successfully constructed and inducible GFP expression driven by the P_{SH} promoter under de-repressing conditions in heterotrophic growth media was demonstrated in the recombinant *C. necator* H16 cells. Here we report the first successful fluorescent reporter system to study P_{SH} promoter activity in *C. necator* H16. The fusion construct allowed for the design of a simple screening assay to evaluate P_{SH} activity. Furthermore, the constructed reporter system can serve as a model to develop a rapid fluorescent based reporter for subsequent small-scale process optimisation experiments for SH expression.

INTRODUCTION

Hydrogenases are ubiquitous enzymes with reversible hydrogen oxidation or production activity, showing tremendous promise as a bioelectrocatalyst in hydrogen fuel cells (Schlegel et al., 1961, Friedrich et al., 1981, Lamle et al., 2004, Jugder et al., 2013). A wide variety of H_2 oxidizing organisms including; aerobes, anaerobes, autolithotrophs, heterotrophs, fermentative, photosynthetic and thermophilic microorganisms, have been described and are capable of producing and utilising endogenous uptake hydrogenases. The Knallgas bacteria *C. necator* H16 (formerly *Ralstonia eutropha*) is a chemolitho-autotrophic proteobacterium that is capable of

growing both autotrophically using molecular hydrogen as the sole energy source and heterotrophically using organic compounds as the energy source (Pohlmann et al., 2006). *C. necator* H16 hosts three distinct O_2-tolerant hydrogenases (Burgdorf et al., 2005); a membrane-bound hydrogenase (MBH), a soluble hydrogenase (SH) and a regulatory hydrogenase (RH). Under heterotrophic growth conditions, the expression of [Ni-Fe] uptake hydrogenases in *C. necator* H16 is induced on poorly utilised carbon sources (e.g., glycerol). Culture of this organism in minimal medium FGN (fructose-glycerol-nitrogen) is characterized by initial growth on the preferred fructose carbon source with hydrogenase expression repressed, followed by de-repression of hydrogenase expression as the organism switches to growth on the less-preferred substrate glycerol upon fructose exhaustion (Jugder et al., 2015, Jugder et al., 2016).

The gene clusters for the three hydrogenases of *C. necator* H16 occupy a region of approximately 90 kbp of the megaplasmid pHG1. The SH is one of the most promising candidates for application in H_2-based technologies owing to its H_2 oxidation activity, oxygen tolerance, relatively favourable purification process and high expression under heterotrophic growth conditions (Burgdorf et al., 2005). The structural and accessory *hox* genes and maturation *hyp* genes of the SH reside on the large SH operon (10kb) (Schwartz et al., 2003, Schwartz, 2009). A strong promoter, P_{SH}, for these genes was identified in an upstream region of *hoxF* by primer extension analysis. The P_{SH} promoter is recognised by the sigma factor σ^{54} (RpoN) of the RNA polymerase and its sequence was proposed as 5'-TTGGCGCACATCCTGC-3' (Schwartz et al., 1998). It has been well reported that one of two physiological conditions must be met to induce/de-repress the P_{SH} promoter and subsequently express the hydrogenase genes in *C. necator* H16. Under the first condition, H_2 must be available in the growth media and typically a mixture of H_2, CO_2 and O_2 with a volume ratio of 8:1:1 (autotrophic growth) has been employed

widely to achieve induction in defined media. The second alternate inducing condition is achieved by the absence of preferentially utilized carbon and energy sources, such as fructose, in the medium which is conveniently achieved by using FGN medium whereby substrate shift occurs from fructose to glycerol under heterotrophic conditions (Friedrich et al., 1981, Schlegel et al., 1961, Jugder et al., 2015).

The use of reporter genes fused to a gene of interest has been widely reported for studying gene expression and promoter activity in a diverse array of living organisms. These reporters can be classified into conditional and non-conditional genes based on their need of an external substrate for detection (Xiong et al., 2012). Green fluorescent protein (*gfp)* has been amongst the most commonly used reporter genes since its first use as a reporter for gene expression in 1994 (Chalfie et al., 1994). As a reporter, *gfp* has great advantages over other reporters such as; direct real-time visualisation in living systems, little or no cytotoxicity on host cells, small size, and the availability of different mutants with modified spectral wavelengths (Xiong et al., 2012, Carroll and James, 2008). The use of *gfp* as a reporter gene was studied in *C. necator* cells with regard to polyhydroxyalkanoate (PHA) production (York et al., 2001, Fuchslin et al., 2003, Barnard et al., 2005). It has been reported that the expression of the *gfp* gene that is fused to the phaP promoter of phasin proteins, which are directly related to PHA synthesis, can be driven by the phaP promoter and thereby used as a tool to monitor PHA production. Nevertheless, P_{SH} promoter-driven GFP expression has not been reported in *C. necator*, to our knowledge.

We herein designed a recombinant reporter system to analyse the P_{SH} promoter activity in *C. necator* H16 utilising a mutant recombined operon comprising of a *gfp* gene assembled in a suicide vector, which is integrated within the megaplasmid pGH1. This tool allows for the analysis of potential growth conditions that de-repress promoter activity by monitoring the

induction of the *gfp* gene expression. Employing qRT-PCR methodologies is time consuming and cannot effectively be applied to broad screening strategies to determine conditions associated with elevated SH production. The use of a simple visual reporter, such as GFP, would potentially be a time-saving and robust screening tool to investigate alternative growth conditions for potentially obtaining a higher yield of the SH from *C. necator* H16 by measuring GFP fluorescence emitted by the generated mutant strains. Increased SH specific productivity would also potentially improve recovery of active soluble hydrogenase.

MATERIALS AND METHODS

Bacterial strains, growth conditions, plasmids and oligonucleotides

C. necator H16 (*Cupriavidus necator*, DSM 428) was routinely cultivated heterotrophically in minimal medium FGN as described in our previous work (Jugder et al., 2015, Jugder et al., 2016). The transconjugants were also grown under a hydrogenase-repressing condition in FN medium (FGN medium without glycerol) and hydrogenase de-repressing condition in GN medium (FGN medium without Fructose). *E. coli* strains were grown in Luria–Bertani medium (LB) except for conjugation processes where low-salt LB supplemented with 5% sucrose was used. *E. coli* DH10B containing the pJQ200mp18 suicide vector (ATCC 77485) was cultivated on LB media supplemented with gentamicin (15 μg/mL). *E. coli* S17-1 was maintained in LB media supplemented with trimethoprim (10 μg/mL). For blue/white screening, 100 μg/mL of ampicillin, 80 μg/mL of X-gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) and 0.5 mM IPTG (isopropylthio-β-galactoside) were added to the LB agar media. SOC medium was used for transformation of *E. coli* JM109 High Efficiency Competent Cells. *C. necator* and *E. coli* strains

were cultivated at 30 and 37 °C, respectively. The strains, plasmids and primers used in this study are listed in Table 1.

DNA isolation, manipulation and amplification

The UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, USA) was used for genomic DNA preparation from *C. necator* H16. After separation of PCR amplified products by agarose gel electrophoresis, DNA fragments were excised from the gel and extracted using the Wizard SV Gel and PCR Clean-up system kit (Promega, USA) following the manufacturer's protocol. The same kit was also used for clean-up of the pJQ200mp18 vector following the restriction enzyme digestion. The Wizard® Plus SV Minipreps DNA Purification System (Promega, USA) was used to isolate plasmids from microorganisms according to the manufacturer's instruction. For DNA amplification, 2X PCR Master Mix (Promega, USA) was used. For proof-reading PCR, Phusion™ High-Fidelity DNA Polymerase (Finnzymes, Finland) was used with 5x Phusion HF Buffer supplied. The cycling conditions vary depending on the purpose. The ABI 3730 Capillary Sequencer with BigDye™ Terminator Cycle Sequencing Ready Reaction kit v.3.1 (Applied Biosystems) was used for sequencing PCR of cloned insert DNA according to the manufacturer's instructions.

Hydrogenase activity assay

Soluble hydrogenase assays were performed as described previously in 50 mM H_2-saturated Tris/HCl buffer at pH 8.0 (Jugder et al., 2015, Jugder et al., 2016). NAD^+ was used as artificial electron acceptor and its reduction to NADH was measured spectrophotometrically at 340 nm.

Construction of a P_{SH} promoter-*gfp* fusion element

An overview of the amplification of the P_{SH} promoter-*gfp* fusion elements is shown in Fig. 1. A 353 bp fragment, phosphorylated at the 5' end, containing the region upstream of *hoxF* (nucleotides 79365 and 79382, sequence numbering according to GenBank entry AY305378.1) up to the translational stop codon of the previous ORF (nucleotides 79685 and 79711, sequence numbering according to GenBank entry AY305378.1) was amplified in the PCR by using primers *F-upstream* and *R-upstream* (Primers #1 and #2 in Fig. 1), and *C. necator* H16 chromosomal DNA as template. Similarly, a 784 bp fragment, containing a *gfp* plus the portion of the region post *hypF2* amplicon (nucleotides 89228 and 89285, sequence numbering according to GenBank entry AY305378.1) and phosphorylated at the beginning, was generated from pGLO template, which harbours the *gfp* gene, by using primers *F-gfp* and *R-gfp* (Primers #3 and #4 in Fig. 1). Thus, all transcriptional control and stop elements of the SH are located in the regions amplified by these primer pairs. These fragments were, after gel-extraction, ligated using T4 DNA Ligase (Promega, USA) and subjected to a further PCR by using primers *F-upstream* and *R-gfp-truncated* to amplify the ligation product of the expected size of 1137 bp (Fig. S1). The gel-purified ligated fragment was subcloned into the pGEM-T Easy vector and the resultant recombinant vector was designated as pGEM-SH*::gfp* (Fig. 1B), which was used for transformation of *E. coli* JM109 High Efficiency Competent Cells. The transformation culture was plated in duplicate on LB/ampicillin/IPTG/X-Gal plates for further blue-white screening. Sequencing PCR was performed on purified pGEM-SH*::gfp* vectors from white colonies employing the pUC/M13 Sequencing Forward and Reverse Primers.

The pJQ200mp18 suicide vector was used in this work to carry out gene replacement and mobilization experiments (Quandt and Hynes, 1993). Mini-prep pJQ200mp18 suicide vector and

pGEM-SH*::gfp* vector DNA were digested with PstI restriction endonuclease and dephosphorylated using Antarctic Phosphatase for further ligation to yield the recombinant vector pJQ200mp18-SH*::gfp*, which was used to transform *E. coli* JM109. Following blue/white screening (LB/gentamicin /IPTG/X-Gal selective plates), the recombinant vector purified from *E. coli* JM109 was used to transform *E. coli* S17-1 competent cells via a heat shock at 42°C for 1 min. The transformed competent cells were plated onto LB/gentamicin/trimethoprim/IPTG/X-Gal plates, as *E. coli* S17-1 harbouring pJQ200mp18 is resistant to gentamicin (Quandt and Hynes, 1993) and trimethoprim (Simon et al., 1983). The mobilisable suicide vector, pJQ200mp18-SH*::gfp*, was transferred from *E. coli* S17-1 to *C. necator* H16 by spot mating. Single colonies from the donor strain *E. coli* S17-1 and recipient strain *C. necator* were used to inoculate 5 mL of LB broth containing gentamicin/trimethoprim and FGN media, respectively. The donor and recipient cultures were incubated overnight at 37°C and 30°C, respectively, on a rotary shaker at 200 rpm. Subculturing of both overnight cultures was performed by a 10 fold dilution in corresponding growth media. Donor and recipient cells were then grown to log phase and pelleted by centrifugation at 3,000 x g for 5 min. Donor *E. coli* S17-1 culture was washed in 5 mL of LB broth to remove antibiotics and re-centrifuged. Both cell pellets were resuspended in 50 µL of LB broth and subsequently mixed at a ratio of 1:1. The conjugation mix was carefully spread onto a sterile 0.22 µM filter (Millipore) on top of a pre-warmed LB agar plate. The matings were incubated at 30°C for 2 days. The cells were washed from the mating filter into 1 mL of LB medium with vortexing. Transconjugants were selected by plating serial dilutions on low-salt LB plates containing 5% sucrose. After 3-5 days of incubation at 30°C, transconjugants appeared and colony PCR was used to screen transconjugants, with primers *F-gfp* and *R-recombination*. Transconjugants were inoculated into 5 mL of FGN media and incubated

overnight at 30°C. Genomic DNA from select transconjugants was subjected to final PCR using the primers *F-gfp* and *R-recombination*, and primers *F-upstream* and *R-gfp-truncated* to amplify DNA fragments of approximate 800 bps and 1.14 kbps, respectively, in order to confirm final successful recombination.

Transcriptional analysis

Total RNA extraction and subsequent cDNA synthesis were performed using the TRIzol Plus RNA Purification Kit (Life Technologies, USA) and the SuperScript III First-Strand Synthesis System (Life Technologies, USA), respectively, as described in Jugder et al (Jugder et al., 2015). Expression levels of the *hoxF* gene encoding HoxF protein (NAD-reducing hydrogenase diaphorase moiety large subunit) of the SH in different growth phases of wild-type *C. necator* were analysed using qRT-PCR with primers *hoxF_fwd* and *hoxF_rev*. In the conjugated strains, expression of *gfp* gene was examined with *gfp_fwd* and *gfp_rev* primers. The *gyrB* gene was used as an internal reference gene due to its constitutive expression. qRT-PCR was performed on a Rotor-Gene RG-3000A cycler (Qiagen, Australia) using the SensiFAST SYBR No-ROX Kit (Bioline, Australia) as described elsewhere (Jugder et al., 2015).

Fluorescence microscopy examination of the transconjugants to detect the presence of GFP

The cultures that were inoculated from single colonies from the conjugated strains were grown overnight in 5 mL GN (Glycerol as sole carbon source, hydrogenase de-repressing condition) and FN media (Fructose as sole carbon source, hydrogenase repressing condition). The overnight cultures were placed on glass slides with cover slips and examined for brightfield imaging under light microscope settings with 10x and 50x objectives for locating the cells. Subsequently, the cells were examined for fluorescence by using the “WB” filter tube, which is a combination of a

BP450-480 excitation filter, a DM500 dichroic mirror and a BA515 barrier filter (filter cube WB). This filter provides excitation light between 450 nm and 480 nm (BP450-480), transmits a high percentage of light at wavelengths above 515 nm but passes little light below 515 nm (BA515) and 50% of the maximum transmission is 500 nm (DM500). This combination elicited a green fluorescence of the transconjugants expressing GFP. The images of the GFP-expressing cells under fluorescence settings were obtained using DP Manager v3.3.1.222 software (Olympus).

Flow cytometry analysis of GFP

The cultures that were inoculated from single colonies from the conjugated strains were grown overnight in 5 mL GN and FN media. After two successive 400-fold dilutions, 5×10^4 cells from each pool were analyzed using a Becton-Dickinson FACS Caliber flow cytometer, and fluorescence (488-nm excitation, 520-nm emission) was scaled by scattering to compensate for differences in cell morphology and size. One hundred thousand events (cells) were counted for each sample. Experiments were performed in triplicate unless otherwise stated.

Purification of GFP isolated from transformed *C. necator*

The cell pellets were harvested by centrifugation at 5,500 *g* for 15 min at 5 °C, and stored at -80°C. Cells were disrupted by sonication and the cell-free extract was centrifuged (100,000*g*, 30 min at 5 °C). The remaining supernatant was loaded onto a 10-ml volume metal affinity resin (Talon resin, Clontech) equilibrated in buffer containing 150 mM NaCl, 100 mM HEPES-NaOH, pH 7.5. Unbound proteins were washed off using the same buffer containing 10 mM imidazole. The bound protein was then eluted with a buffered solution composed of 200 mM imidazole, 150 mM NaCl, 100 mM HEPES-NaOH, pH 7.5. The solution containing the precipitated protein was

centrifuged, and the supernatant was discarded. The precipitate was progressively dissolved in 20 mM HEPES-NaOH, pH 7.5. The protein solution was dialyzed overnight against a 500-fold (vol/vol) excess of the same solution.

Absorption and fluorescence excitation and emission spectra

Samples of purified GFP were diluted to approximately 4.5 µM in buffered solution (containing 10 mM glycine, 10 mM sodium citrate, 10 mM sodium phosphate, and 5 mM Tris-HCl). A fluorometer (Fluorostar Optima) was used to obtain the emission spectrum of the commercial GFP and the GFP extracted and purified from the transconjugant *C. necator* H16. Measurements were obtained using excitation and emission wavelengths, bandpass, and integration times of 392 nm, 510 nm, 3 nm, and 0.5 s, respectively.

Fluorescence quantitation in wildtype and transformed *C. necator*

The fluorescence intensity of GFP in fixed cells was measured with a Fluoromax-2 spectrofluorometer using the Datamax for Windows software interface (Instruments S.A. Inc., Edison, N.J.). A protein assay on lysates of the cell samples was carried out prior to normalise cell loading for gfp fluorescence determination, using the Pierce BCA Protein Assay Kit (Thermo Scientific, Illinois USA)**.** The relative fluorescence unit (RFU) is defined as the culture fluorescence relative to culture concentration (OD_{600nm}).

RESULTS AND DISCUSSION

In this study, the transcriptional reporter method was employed to construct the P_{SH} promoter-*gfp* fusion in the megaplasmid pHG1 of *C. necator* H16 to analyse promoter activity. The molecular cloning method was designed to generate, by PCR, the entire sequence of the 5' upstream elements which were subsequently fused to the *gfp* gene that was combined with 3' downstream

238 elements of the SH operon by establishing a rapid and robust cloning approach which is
239 summarised in Fig. 1. The *gfp* gene from a commercially available pGLO vector was fused to the
240 P_{SH} promoter of the SH operon in place of the first ORF (*hoxF*) followed by 3' downstream
241 elements following the final ORF (*hypF2*) of the same operon. The results confirmed that the
242 fusion elements recombined with the pHG1 megaplasmid of wild-type *C. necator* by a means of
243 gene replacement at the site of the SH operon elements. The resulting reporter construct was
244 capable of being induced under the hydrogenase de-repressing condition (GN medium) in the
245 transconjugant derivative cells which led to detectable fluorescence signals from the GFP
246 expressed.

247 Initially, the 784 bp amplicon representing a GFP product combined with the region
248 downstream of *hypF2* (using the primers *F-gfp* and *R-gfp* as well as pGLO vector) and the 353
249 bp amplicon from the region upstream of *hoxF* (using the primers *F-upstream* and *R-upstream*,
250 and *C. necator* H16 chromosomal DNA as template) were obtained (Fig. S2A). The ligation
251 reaction of these two fragments theoretically can result in three possible ligated products joined
252 via the 5'-phosphorylated ends (Fig. S2B) as follows: i) between two N-terminal products, ii)
253 between an N-terminal product and a C-terminal product and iii) between two C-terminal
254 products. The second product is the desired ligation product with a calculated size of 1137 bp
255 which was excised from a gel for further PCR amplification by using the primers *F-upstream*
256 and *R-gfp-truncated* (Fig. S2B). Following further subcloning of this fragment into the pGEM-T
257 Easy vector for subsequent transformation of *E. coli* JM109, blue/white colour screening was
258 undertaken and the presence of an amplicon band of the expected theoretical size (1137 bp) for
259 the cloned fragment in white colonies was confirmed by colony PCR (Fig. S3A). The isolated
260 pGEM-SH*::gfp* vector and the pJQ200mp18 suicide were restricted with the PstI endonuclease

and confirmed on an agarose gel (Fig. S3B) prior to the ligation to yield the vector pJQ200mp18-SH*::gfp*. Successful sub-cloning of the resulting recombinant suicide vector, following *E. coli* JM109 transformation and blue/white screening, was confirmed with an insert fragment (SH operon elements fused to *gfp*) with an estimated size of 1.1 kb separated from the remaining vector backbone (approximately 5.55 kb), as shown in Fig. S3C. The purified pJQ200mp18-SH*::gfp* vector subsequently used to transform freshly prepared *E. coli* S17-1 competent cells was confirmed in white colonies using the primers *F-upstream* and *R-gfp-truncated* enabling rapid screening for successful transformation (Fig. S3D). Following transformation with the recombinant pJQ200mp18-SH::*gfp* suicide vector, the donor strain *E. coli* S17-1 was mated with wild-type *C. necator* H16. The colonies on low-salt LB/sucrose plates screened by using primers *F-gfp* and *R-recombination* generated a band of the estimated size of 800 bp (Fig. S3E) on a gel indicating successful gene replacement on the megaplasmid pHG1. These transconjugants were designated as *C. necator* H16*::gfp* cells. PCR was performed to further confirm the gene replacement by using two primer pairs: *F-upstream* and *R-gfp-truncated*, as well as *F-gfp* and *R-recombination* on genomic DNA prepared from the transconjugant colonies. Following PCR with the former and latter pairs, the amplicon bands with the expected estimated size of 1.14 kb and 800 bp, respectively, were successfully visualized on an agarose gel (Fig. S3F).

Following the confirmation of the successful final recombination event, the performance of the transconjugant (*C. necator* H16*::gfp*), in producing GFP under control of the P_{SH} promoter, was determined using fluorescence microscopy. Glycerol stocks were subsequently prepared from cultures derived from single colonies that demonstrated green fluorescence when grown in GN media.. Quantitative RT-PCR, flow cytometry and quantitative fluorescence analysis was subsequently undertaken using cultures from these glycerol stocks. Images and flow

cytometry data of the cells expressing GFP under the hydrogenase-repressing condition (fructose; FN media) and the hydrogenase-de-repressing condition (glycerol; GN media) were obtained (Fig. 2). GFP expression was observed visually and by a significant shift in the population, verifying that the P_{SH} promoter from the transconjugated *C. necator* successfully induced GFP production under the selected hydrogenase de-repressing growth condition.

The emission characteristics of the recombinant GFP isolated from the transconjugants confirmed its authenticity, with emission maxima observed at excitation wavelengths of 392 and 475 nm (Fig. S4) coinciding exactly with that of the native GFP. In the fluorescence plate assay, a significant increase in GFP expression was demonstrated under P_{SH} de-repressing conditions (growth in GN media) for the transformed population (Fig. 3).

A time course study in FGN media (Fig. 4) showed increasing protein expression (soluble hydrogenase in the WT strain and GFP fluorescence in the transformed strain) and increased fold change in respective mRNA levels, as cells switched from growth on fructose (t=10h) to growth on glycerol (t=16h, 24h and 36h). The gene *hoxF* was approximately 1.4, 2.1 and 3.5-fold up-regulated in the cells harvested at 16h, 24h and 36h where the expression of SH was assumed to be induced, in comparison to the cells at 10h (Fig. 4A). The SH expression was also demonstrated as specific SH activity increased in accordance with the increase in abundance of *hoxF* mRNA. In parallel, the *gfp* gene expression was investigated in the conjugated cells at the transcriptional level (Fig. 4B). We observed the up-regulation of the *gfp* gene with an approximate 8.9-fold increase at 36h. Observations made in the time course of the expression pattern of the genes *hoxF* and *gfp* confirmed that P_{SH} promoter, in our constructed strain, is responsive to the de-repression upon carbon source change in a similar manner. Together, these

findings confirm the utility of the transformed *C. necator* H16*::gfp* for future P_{SH} activity screening.

To our knowledge, this is the first report of a successful fluorescent reporter system to study P_{SH} promoter activity in *C. necator* H16. Understanding the environmental factors in the regulation of SH expression is of increasing interest and the availability of versatile monitoring methods is crucial. The system developed in this study should allow for the conduct of factorial experiments and high-throughput assays in a microplate format that employs the recombinant *C. necator* H16*::gfp* cells to explore alternative growth conditions and rapidly estimate SH promoter activity. Furthermore, there is potential to use this construct in transposon mutagenesis experiments to identify new SH regulators by monitoring a simple fluorescence read-out. This tool has the potential to further assist in investigating the sigma factor, σ^{54}, which recognises the P_{SH} promoter (Schwartz et al., 1998). Possible carbon sources could theoretically be identified as ideal candidates to induce strongly the σ^{54}-dependent P_{SH} promoter. Also, evaluation of site-directed mutagenesis of the P_{SH} promoter or the replacement of the P_{SH} promoter with a more strongly inducible promoter could be facilitated by this reporter system. The generation of these reporter strains is based on recombination events; further characterization of a range of the recombinant transconjugants may also reveal as yet unidentified variants that possess useful traits that may assist in the identification of inducing conditions.

CONCLUSION

In the present study, a system to investigate soluble hydrogenase P_{SH} promoter activity in *C. necator* H16 was constructed and its functionality was confirmed, developing a P_{SH}-GFP fusion protein reporter. A series of molecular cloning steps were employed to replace the ORF of the SH with a *gfp* gene in the megaplasmid pHG1, and the expression of GFP in response to the de-

329 repression of the SH genes was demonstrated under fluorescence and transcriptional analyses.
330 This construct will enable future studies to design simple screening methods for SH promoter
331 activity in *C. necator* H16 cells, further investigations on growth-related optimisation with
332 alternative cultivation conditions and functionality of SH promoter mutants in *C.necator*.

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Table and Figure Legends

Table 1 Strains, plasmids and oligonucleotides used in this study.

Figure 1 Overview of the molecular cloning method employed in this study

(A) Flow diagram of the steps involved in the generation of the target sequence to be fused. Step 1: The PCR amplification of the region upstream of *hoxF* up to the translational stop codon of the previous ORF (phosphorylated at the 5' end of the non-coding strand) by using primers #1 and #2 and template *C. necator* H16 DNA and a *gfp* plus the portion of the region post *hypF2* amplicon (phosphorylated at the 5' end of the coding strand) by using primers #3 and #4 and template pGLO. Step 2: Ligation of PCR products. Step 3: Secondary amplification of the ligated product to generate the target DNA. **(B) Flow diagram of the steps involved in the construction of a P_{SH} promoter-*gfp* fusion system.** Step 1: Cloning of the target sequence to pGEM-T Easy vector to generate *pGEM-SH::gfp*vector. Step 2: Restriction enzyme digestion of *pGEM-SH::gfp* vector and pJQ200mp18 vectors at the PstI endonuclease site (shown by the orange arrows). Step 3: Ligation of the digested target sequence to the digested pJQ200mp18 vector to generate *pJQ200mp18-SH::gfp* vector. Step 4. Conjugation of the recombinant vector *pJQ200mp18-SH::gfp* from *E. coli* S17-1 to *C. necator* H16 to construct the integrated final P_{SH} promoter-*gfp* fusion system.

Figure 2 Detection of GFP-expressing *C. necator* H16::*gfp* cells

The fluorescence images (left column) of the cells with corresponding flow cytometry fluorescence histograms (right column). The GFP signal was not detected from wild-type *C. necator H16* cells (A) and the transformed *C. necator* H16*::gfp* cells under the hydrogenase-repressing condition (growth on fructose) (B), whereas the GFP signal was detectable in transformed *C. necator* H16*::gfp* cells under the hydrogenase-de-repressing condition (growth on glycerol)(C).

Figure 3 Fluorescence of wildtype (WT) and recombinant *C. necator* (transformed) in fructose (FN) media and glycerol (GN) media

Specific fluorescence response (RFU) of *C. necator* H16::*gfp* (transformed) and non-transformed (WT- wild-type) cells excited at 392 nm under repressing conditions (fructose) and de-repressing conditions (glycerol). Histogram bars represent the mean ± S.E gfp relative fluorescence units

obtained from triplicates for each treatment group. Significance $^{*}p<0.01$ compared to wild-type *C. necator* H16 cells in glycerol and fructose, and the transformed *C. necator* H16*::gfp* cells under the hydrogenase-repressing condition (fructose).

Figure 4 Transcriptional analyses of SH operons

Differential expression of **(A)** *hoxF* gene (P value 0.0039) and NAD^{+} reducing soluble hydrogenase (SH) activity (P value 0.0012) from wild-type *C. necator* H16 cells and **(B)** *gfp* gene (P value 0.0493) and GFP (P value 0.0303) in *C. necator* H16::*gfp* cells, respectively. These graphs are based on three technical replicates and represent their mean values with standard deviation indicated by the error bars . Constructed and analysed by GraphPad Prism, v 6.07.

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Overview of the molecular cloning method employed in this study

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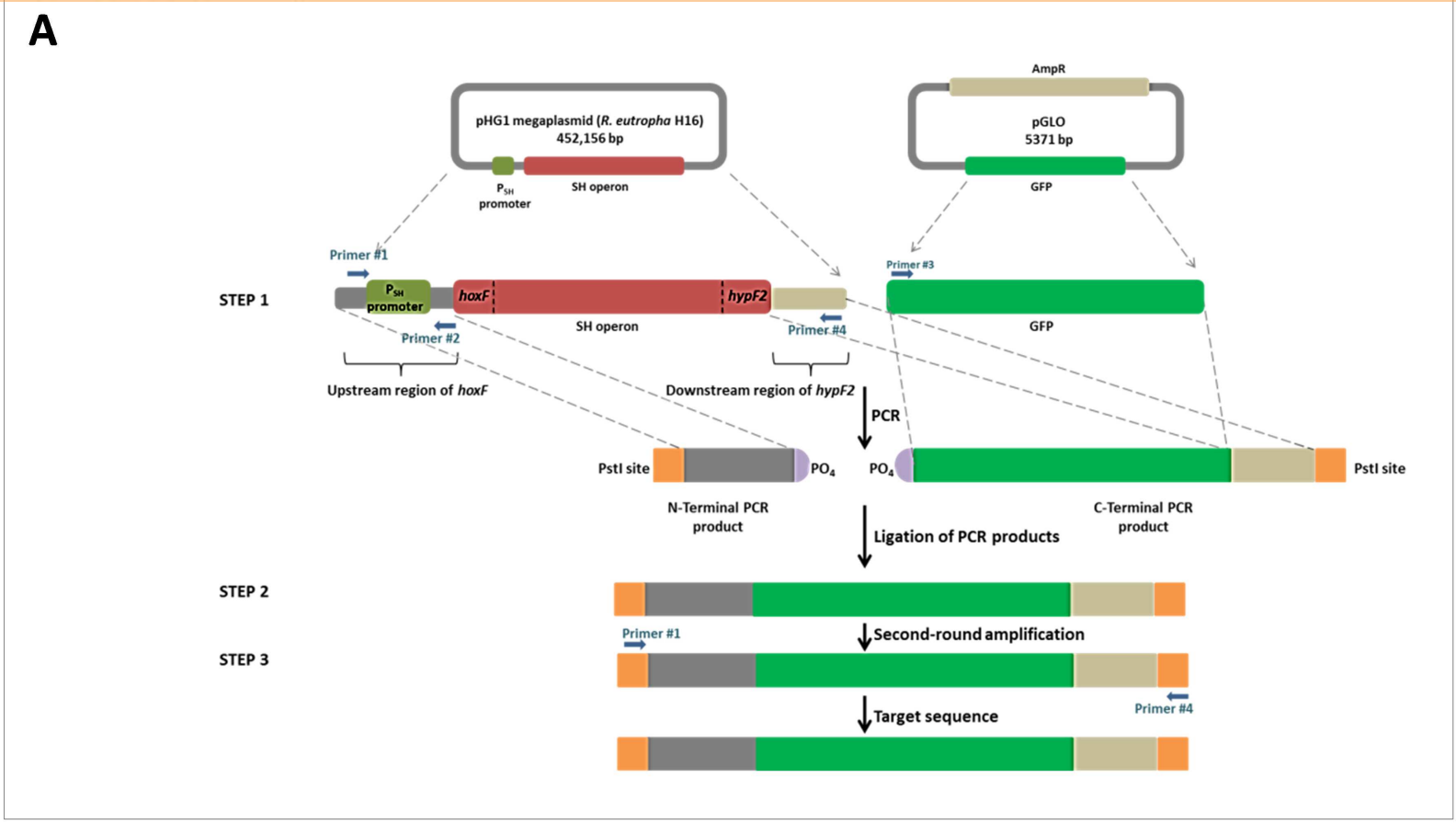

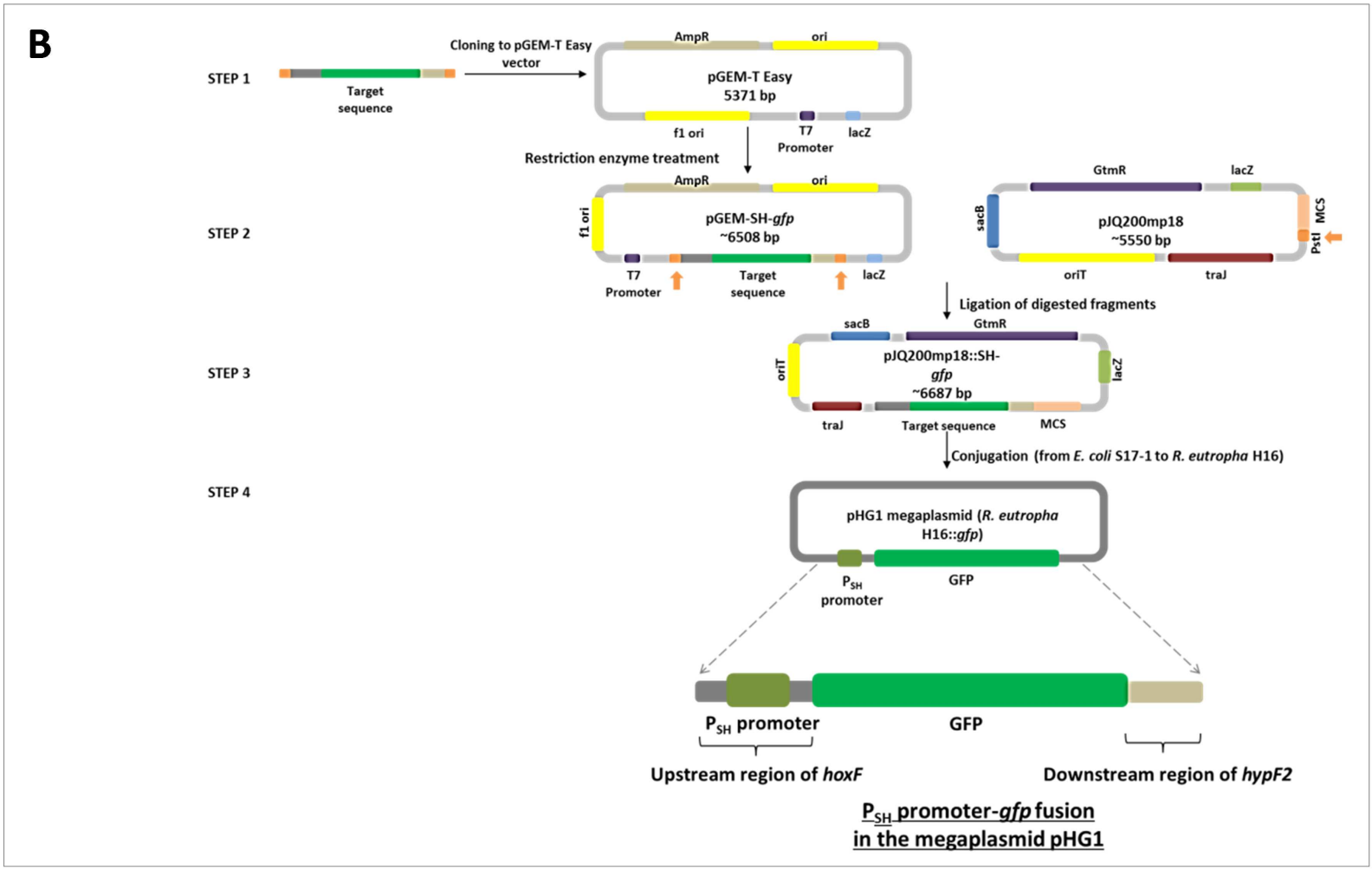

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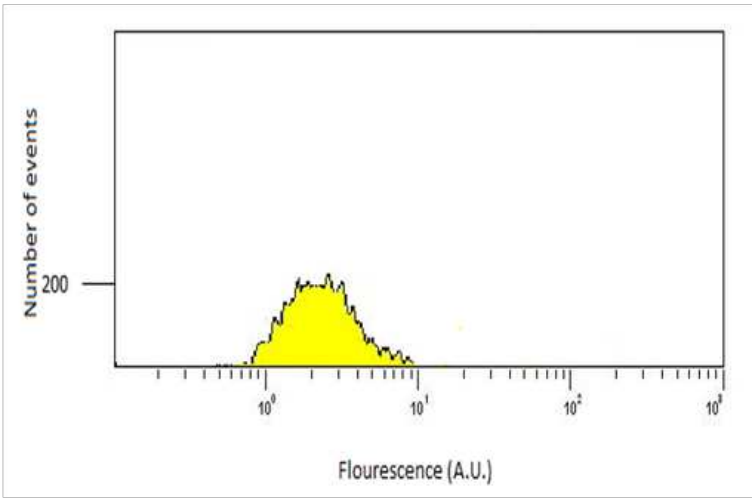

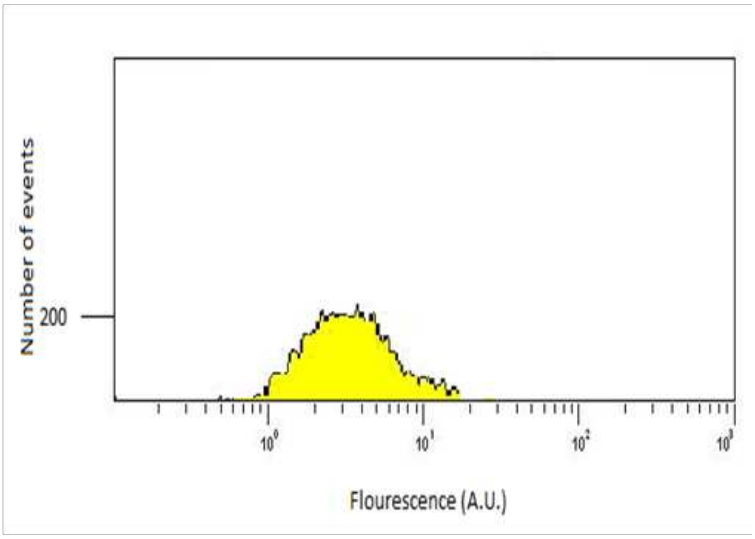

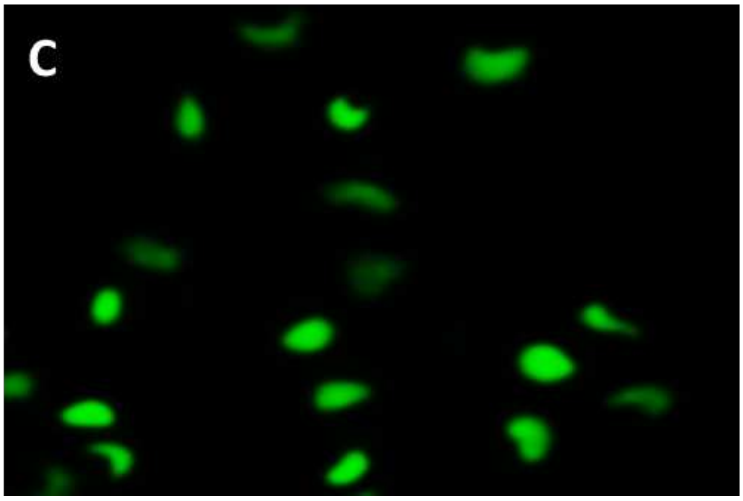

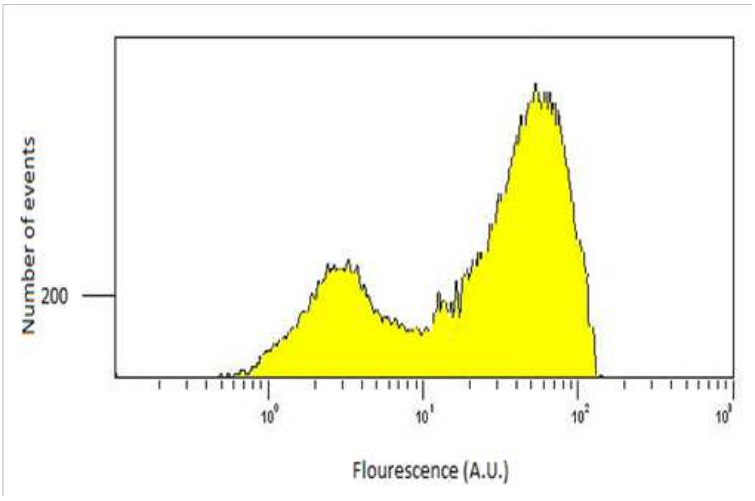

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Fluorescence of wildtype (WT) and recombinant *C. necator* (transformed) in fructose (FN) media and glycerol (GN) media

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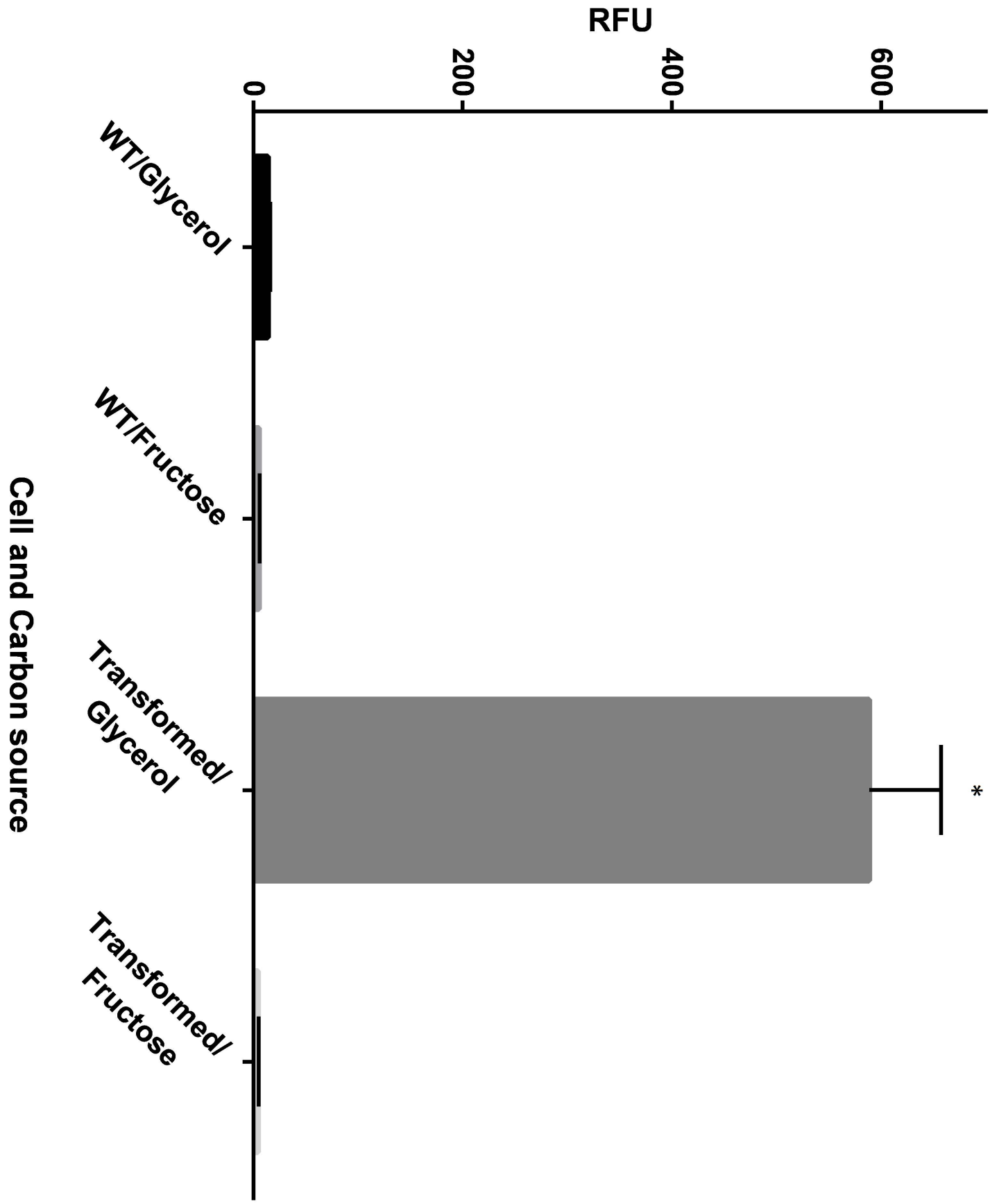

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Transcriptional analyses of SH operons

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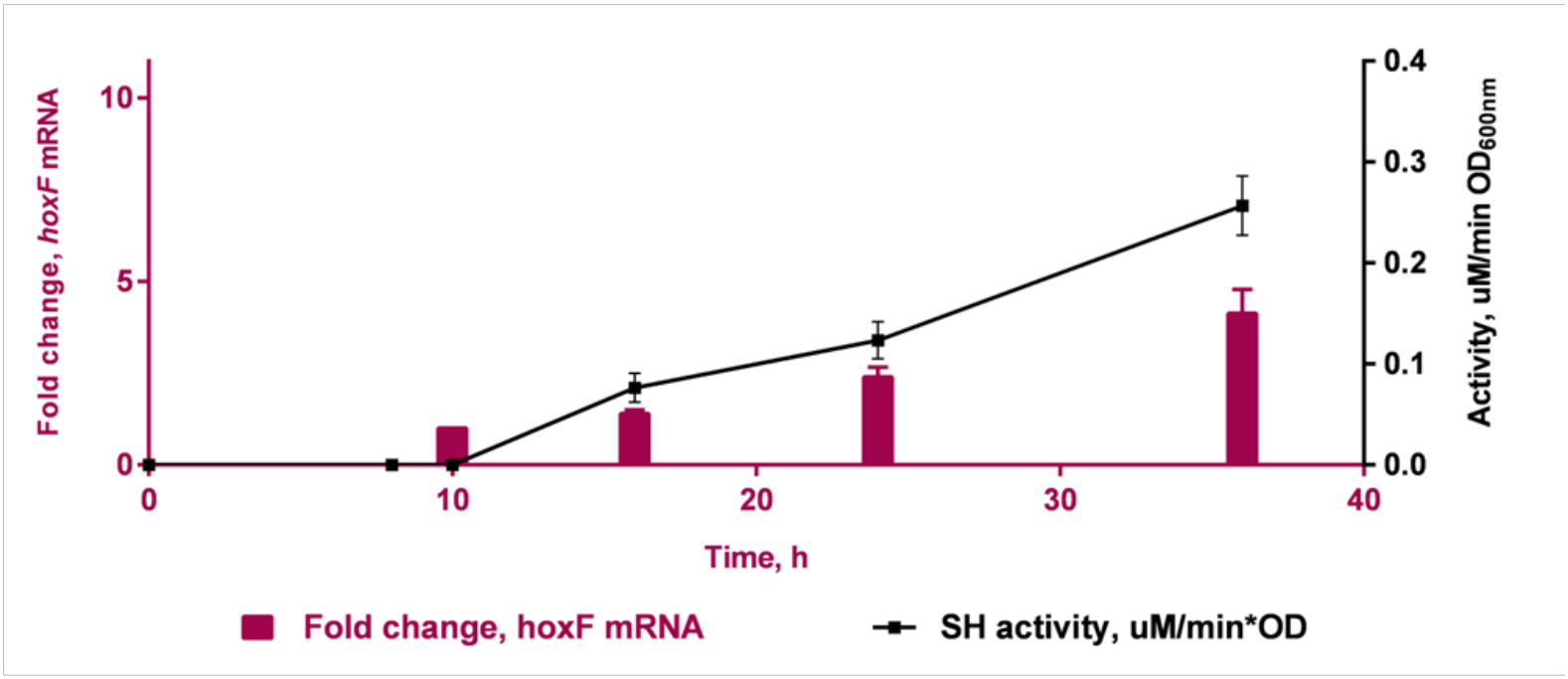
A



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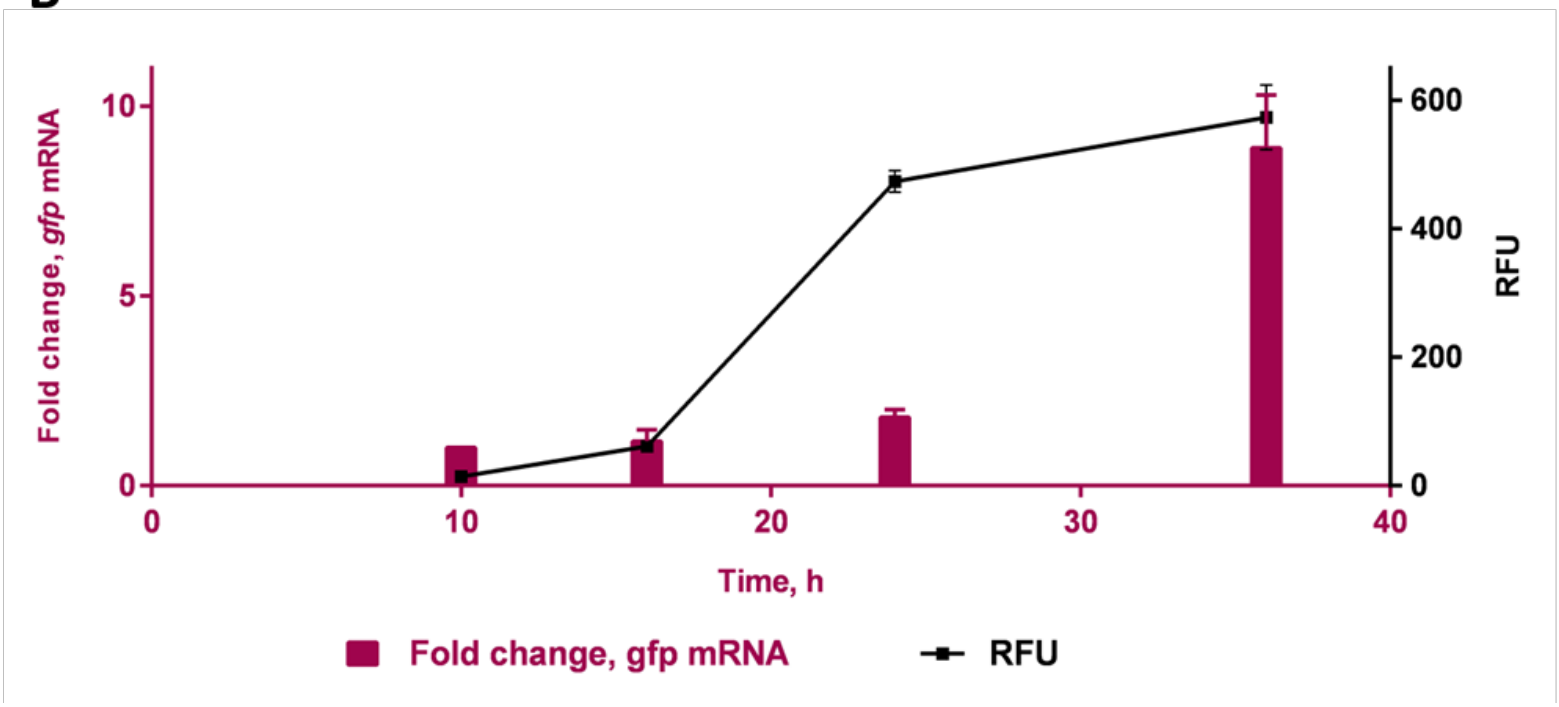

Table 1(on next page)

Strains, plasmids and oligonucleotides used in this study

1 **Table 1** Strains, plasmids and oligonucleotides used in this study.

Strain or plasmid	**Description**	**Reference or source**
C. necator strains		
H16	Wild-type (wt), DSM 428 (*Cupriavidus necator*)	DSMZ
H16:: *gfp*	Recombinant strain containing *gfp* fusion vector, derivative of H16	This study
E. coli strains		
S17-1	Strain (ATCC 47055) for conjugative transfer of vectors to *C. necator,* recA pro hsdR, RP4-Tc::Mu-Km::Tn7 integrated into the chromosome, tmpR, spcR, strR	ATCC, (Simon et al., 1983)
JM109	High Efficiency Competent Cells (>10^8cfu/µg) for transformation	Promega
Vectors		
pGEM®-T Easy	PCR TA cloning vector, ampR	Promega
pGEM-SH*::gfp*	Derivative of pGEM®-T Easy containing P_{SH}*::gfp* fusion elements	This study
pGLO	Vector carrying the *gfp* gene	Bio-Rad
pJQ200mp18	Suicide vector in *E. coli* DH10B, ATCC 77485. gtmR – EcoRI/MCS/HindIII/PstI – P15A – traJ – oriT – sacB	ATCC, (Quandt and Hynes, 1993)
pJQ200mp18-SH::*gfp*	Derivative of pJQ200mp18 containing P_{SH}*::gfp* fusion elements	This study
Oligonucleotides	**Sequence**	**Restriction site**
Cloning		
F-upstream	CTGCAGCTGCCTCCGGTCACCCGG	PstI

R-upstream	GTTGTCTCCTCCTTACTAATGTTCGCC 5' phosphorylated	
F-gfp	ATGGCTAGCAAAGGAGAAGAACT 5' phosphorylated	-
R-gfp	CTGCAGTTGTCAAATTTTTTCGCGATG CGTGCAGGTATGGCCAGGCACATGTTC TACCCTCTCGTCATTTGTAGAGCTCAT CCATGC	PstI
R-gfp-truncated	CTGCAGTTGTCAAATTTTTTCGCGATG	PstI
Confirmation		
pUC/M13 Sequencing Forward Primer (Promega)	GTTTTCCCAGTCACGAC	-
pUC/M13 Sequencing Reverse Primer (Promega)	CAGGAAACAGCTATGAC	-
R-recombination	CAGGTCGATGAGGGCCATGTCG	-
RT-qPCR		
hoxF_fwd	CTGTTCGACACCCCCTGTAT	
hoxF_rev	ATAGGCGATGTCCTGACTGG	
gfp_fwd	AGTGGAGAGGGTGAAGGTGA	
gfp_rev	ACGGGAAAAGCATTGAACAC	
gyrB_fwd	GCCTGCACCACCTTGTCTTC	
gyrB_rev	TGTGGATGGTGACCTGGATCT	

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