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ORIGINAL RESEARCH PAPER

Improvement of desulfurizing activity of haloalkaliphilic *Thialkalivibrio versutus* SOB306 with the expression of *Vitreoscilla* hemoglobin gene

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Abstract
Objective To construct efficient transformation and expression system and further improve desulfurizing activity of cells through expression of *Vitreoscilla* hemoglobin (VHb) in haloalkaliphilic *Thialkalivibrio versutus* SOB306.
Results We transferred plasmids pKT230 and pBBR-sm^r into *T. versutus* SOB306 via a conjugation method. We identified four promoters from among several predicted promoters by scoring for streptomycin resistance, and finally selected *tac* and p3 based on the efficiency of expression of red fluorescent protein (RFP). Expression of RFP when regulated by *tac* was more than three times that of p3 in SOB306. Further, we expressed VHb under the control of *tac* promoter in SOB306. Expression of VHb was verified using CO-difference spectra. The results showed that VHb expression can boost sulfur metabolism, as evidenced by an increase of about 11.7 ± 1.8% in the average rate of thiosulfate removal in the presence of VHb.
Conclusion A conjugation transfer and an expression system for *Thialkalivibrio*, has been developed for the first time and used for expression of VHb to improve desulfurizing activity.

Keywords Biodesulfurization · Conjugative transfer · Desulfurization · Hemoglobin · Gene expression · *Vitreoscilla* hemoglobin

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Introduction

A chemolithotrophic microorganism species that can utilize reduced sulfur compounds as a substrate for growth, was isolated from halophilic and alkaliphilic environments and classified as belonging to the genus *Thialkalivibrio* by Sorokin et al. (2001). A member of this genus, *Thialkalivibrio versutus* is a Gram-negative, halophilic, alkaliphilic, sulfur-oxidizing and chemolithotrophic bacterium. This bacterium derives its energy by oxidizing reduced or partially reduced sulfur compounds and also fixes CO_2 from the

atmosphere. *Thialkalivibrio* is of interest for exploring the treatment of industrial waste water from sulfide (Klok et al. 2012; van den Bosch et al. 2008). Moreover, *T. versutus* is preferred as a model haloalkaliphilic organism owing to its ability to grow well in defined medium with reduced sulfur compounds as substrates (Banciu et al. 2004).

The physiology of *T. versutus* has been well studied. So far, however, no methods have been developed to introduce foreign genes into this extremely haloalkaliphilic bacterium. Conjugation is an important method of horizontal gene transfer in bacteria. The genetic material is transferred from one bacterium to another via direct cell-to-cell contact. Liu et al. (2007) developed a conjugative transfer system between *E. coli* and the moderately thermophilic, extremely acidophilic bacterium, *Acidithiobacillus caldus* MTH-04. Tan et al. (2014) transformed plasmids into target bacteria via conjugative transfer to engineer *Halomonas* TD01. Conjugation was originally a robust method of introducing foreign genes into recipient cells before the development of transformation methods by heat shock or electroporation.

In this study, we have developed a set of conjugation transfer systems for *Thialkalivibrio* and subsequently also devised an effective expression system based on the broad-host-range plasmid pBBRMCS1. The derived plasmids can be transferred into host cells by conjugation. High-efficiency promoters were selected based on the observed expression levels of red fluorescent protein (RFP) from a reporter construct. Subsequently, a plasmid carrying the *Vitreoscilla* hemoglobin (VHb) gene (*vgb*) under the control of a highly efficient *tac* promoter was transformed into SOB306 strain and the effect of the expression of this gene was further evaluated. To our knowledge, this is the first time that a plasmid transfer method and expression system have been developed for *Thialkalivibrio* strains, which will lay the foundation for further studies at the genetic level.

Materials and methods

Strains and medium

The bacterial strains and plasmids used in this study are listed in Supplementary Table 1. *Thialkalivibrio versutus* SOB306 is a mutant strain of *T. versutus* D301. The recipes of TD and MTD media are described in Supplementary Table 2. *E. coli* strains were cultured in lysogeny broth at 37 °C and 220 rpm. *T. versutus* SOB306 strains grew in a defined medium with thiosulfate (TD) at 30 °C and 180 rpm. The solid medium was made by adding 1.5% agar in liquid media.

Conjugation transfer

Conjugation between *T. versutus* SOB306 and *E. coli* was performed on MTD solid medium by filter mating. The donor and recipient cells were harvested by centrifugation at 7000×*g* for 10 min when the OD_{600} reached 0.6–0.7. Both donor and recipient cells were washed twice or thrice with MTD liquid medium without yeast extract, resuspended in the same solution, and mixed at a ratio of 1:1 (v/v). One hundred microliters of this mixture were then transferred to filter membranes (0.45 μm pore size, 50 mm diameter) which were placed on MTD solid medium plates. The conjugation plates were cultured at 37 °C for 6, 12 18 and 24 h. The cultures were washed from the membrane with 5 ml washing liquid mentioned above and plated on streptomycin selective and nonselective TD solid media. The donor and recipient cells were also plated on respective selective and non-selective media as controls. The selection plates containing the exconjugants and the *T. versutus* SOB306 parent were incubated at 30 °C for 5 days while plates containing the donor *E. coli* were incubated at 37 °C for 24 h. Finally, colonies recovered in the experimental groups were counted and the transfer frequency was calculated using the following formula:

$$TF_r = \frac{N_S}{N_T} \times 100\%, \quad (1)$$

where TF_r represents the transfer frequency, N_S represents the number of colonies on the selective medium, and N_T represents the number of colonies on the nonselective medium.

Primers used in this study are listed in Supplementary Table 2.

Observation of RFP fluorescence and analysis of fluorescence intensity

Fluorescence of cells was observed at 550 nm (excitation). The fluorescence intensity of different cells

was analyzed by Spectramax M5, Molecular Devices. The relative fluorescence intensity was calculated by actual fluorescence intensity of bacterial liquids dividing by the OD_{600} value.

The characterization of VHb and determination of sulfur compounds

The expression of gene was detected by CO difference spectra at 419 nm (Liao et al. 2014). Concentration of sulfate and thiosulfate were determined by an ICS-900 ion chromatograph system (Xu et al. 2015).

Results and discussion

Conjugative transfer between *E. coli* and *T. versutus* SOB306

To assess the effect of conjugation time, the donor and recipient cells were mixed and plated on MTD solid medium at 6, 12, 18 and 24 h, respectively. The exconjugants were then washed out and plated on solid TD medium containing streptomycin. After 5 days, all plates had colonies, with the greatest number of colonies on the plate corresponding to 6 h conjugation (Supplementary Fig. 1). To confirm the transfer of the two plasmids into *T. versutus* SOB306, colony PCR was performed by primers HX-smF/R and M13F/R. Transfer frequency of pBBR-sm^r was less than that of pKT230 within the same contact time (Supplementary Table 3). Compared to *E. coli* sm10, *E. coli* s17 had a greater ability to act as donor to transfer broad-range-host plasmids into recipient cells. The results were caused due to the diversity of physiological characteristics between the two donor cells. Finally, the stability of plasmids pBBR-sm^r and pKT230 in *T. versutus* SOB306 was tested. After five passages, about 59% SOB306 cells still carried pKT230, but only 43% cells retained pBBR-sm^r (Supplementary Table 3). These results indicated that pKT230 was more stable than pBBR-sm^r in *T. versutus* SOB306.

We constructed an expression system for *T. versutus* SOB306 based on broad-host-range plasmid pBBR1MCS owing to its small size and abundant multiple cloning sites. A few high-scoring promoters were selected based on the prediction results of online software mentioned in Supplementary Table 4 (Reese 2001). The efficiency of these promoters was then tested by reporter constructs. Positive colonies were selected for resistance to chloramphenicol and then screened for resistance to streptomycin. We noted that the cells did not show resistance to streptomycin after modifying the construct. Eight promoters were inserted into pBBR-sm and then transformed in SOB306 strain by conjugative transfer. Of the eight clones obtained, those carrying four promoters (pBBR-sm-p3/6/32/tac) regained resistance to streptomycin, while the other four were still sensitive. We verified that these promoters could promote transcription of the downstream *strAB* gene. RNA polymerase from *E. coli* may have poor affinity with some promoters of *T. versutus* SOB306, which is a likely explanation as to why pBBR-sm-p1/2/4/5 clones were not resistant to streptomycin in *E. coli*. To test the strength of these promoters, we employed another plasmid, pBBR-rfp. Like the previous constructs in pBBR-sm, all promoters were cloned into *Kpn*I/*Hin*dIII sites of pBBR-rfp and transformed into DH5α competent cells.

We could visually identify expression of *rfp* only in cells carrying pBBR-rfp-p3 and pBBR-rfp-ptac, which developed cherry red color (Supplementary Fig. 2). Cells carrying three clones (pBBR-rfp-p1/p5/p32) were bright cherry red only when concentrated by centrifugation. Additionally, SOB306 carrying pBBR-rfp-p3 and pBBR-rfp-ptac clones were cherry red with an intensity similar to those in *E. coli*. Fluorescence intensity of strains carrying *rfp* gene under the control of p3 and *tac* promoters was checked to evaluate the effectiveness of these promoters. As shown in Table 1, p3 and p*tac* can effectively promote the expression of *rfp* gene. In *E. coli*, the fluorescence intensities of s17(pBBR-rfp-p3) and s17(pBBR-rfp-ptac) were 234 and 635 times that of wild type s17, respectively. The values in corresponding *T. versutus* SOB306 were 281 and 982 times, respectively. Therefore, we concluded that *tac* was more effective than p3 in both bacteria.

Expression of Vgb in *T. versutus* SOB306

Wakabayashi et al. (1986) reported that a Gram negative bacterium, *Vitreoscilla*, expressed a hemoglobin (VHb) encoded by *Vitreoscilla* globin gene (*vgb*). VHb can bind O_2 and deliver it to the cell respiratory chain, thus enhancing respiration under hypoxic conditions and facilitating cell growth and

Table 1 Fluorescence intensity of cell with different promoters

Strains	Fluorescence intensity	Strains	Fluorescence intensity
E. coli S17	42 ± 3.78	*T. versutus* SOB306	9.8 ± 0.86
E. coli S17 carrying pBBR-rfp-p3	9840 ± 141	*T. versutus* SOB306 carrying pBBR-rfp-p3	2762 ± 89
E. coli S17 carrying pBBR-rfp-ptac	26,705 ± 557	*T. versutus* SOB306 carrying pBBR-rfp-ptac	9626 ± 146

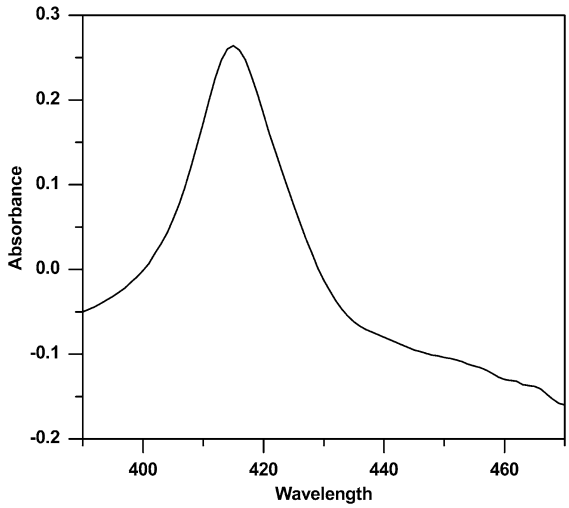

Fig. 1 CO-difference spectra of *T. versutus* SOB306-vgb$^+$ for check vgb gene expression

accumulation of metabolic products (Geckil et al. 2001).

Plasmid pBBR-vgb-ptac was transferred into *T. versutus* SOB306 by conjugation. CO difference spectrum assay was used to determine expression of VHb in *T. versutus* SOB306. CO binds to the expressed bioactive VHb and generates a CO–VHb chromophore complex. This complex can be detected at ~419 nm. The non-recombinant SOB306 strain was used as a negative control in the assay. The results showed that the CO-binding complex appeared at ~419 nm (Fig. 1). This indicated that *vgb* was expressed in *T. versutus* SOB306 and had potent CO-binding activity.

Growth of *T. versutus* SOB306 expressing VHb and its desulfurization activity

Wild type and recombinant SOB306 strains were cultured in TD medium in a shake-flask under aerobic conditions. Growth curves were derived based on viable cell number and OD_{600} values (Fig. 2). As shown in Fig. 2a, OD values of both strains were almost the same during the initial growth phase. However, the curve of recombinant SOB306 showed a steep rise from 25 h. Finally, OD of the recombinant strain reached the highest OD value of 16 ± 0.75 at 40 h and 7.6 ± 0.86 at 48 h (the end of culture). By contrast, the highest value of OD of wild type was only 2.41 ± 0.11. The viable cell density of the recombinant strain was close to that of the wild strain during the entire growth phase (Fig. 2b). SOB306 carrying and expressing v*gb* did not have an obvious growth advantage over the wild type strain. Furthermore, the expression of *vgb* gene appeared to cause a slight inhibition of cell growth, which is similar to that observed in *E. coli* (Geckil et al. 2001) and *Ganoderma lucidum* (Li et al. 2016). The highest value of OD of a vgb$^+$ strain is approx. 6.7 times of that of a vgb$^-$ strain. The growth conditions of the two strains for different culture times are shown in Supplementary Fig. 3. Liquid transmission of light containing of *T. versutus*-vgb$^+$ was weaker than that of *T. versutus*-vgb$^-$. This may be attributed to hemoglobin mediated changing metabolism of cells by enhanced transport of oxygen into the cellular respiratory chain (Yang et al. 2015).

The concentrations of thiosulfate and sulfate were measured to determine the change of sulfur metabolism of these two kinds of strains. As shows in Fig. 3a, the vgb$^+$ strain can metabolize thiosulfate faster than the vgb$^-$ strain. *Thialkalivibrio* species mainly derive energy from the oxidization of low-valence sulfur compounds. The rate of thiosulfate oxidation is closely related to cell growth. Moreover, thiosulfate was removed completely by the vgb$^+$ strain late in growth, but 4% of thiosulfate was not utilized by the vgb$^-$ strain in the same phase of growth. The average rate of removal of thiosulfate was increased from 43 ± 1.6 to 55 ± 1.9% owing to VHb expression during the entire growth period. Therefore, the vgb$^+$ strain had higher

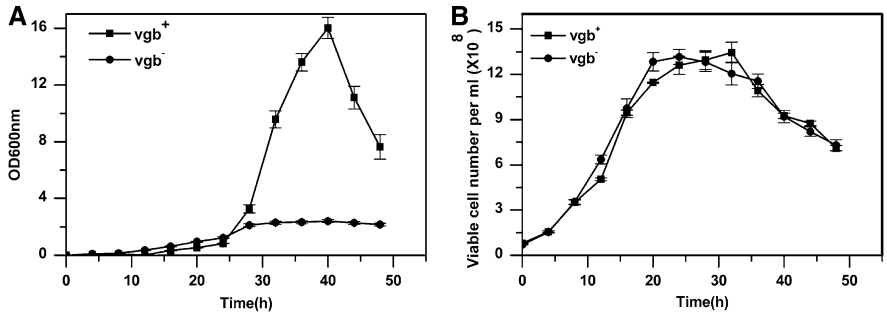

Fig. 2 Comparison of growth of OD_{600} of cultures of *T. versutus* SOB306-vgb$^+$ and wild type (vgb$^-$) under aerobic conditions, **a** OD_{600} values and **b** cell numbers

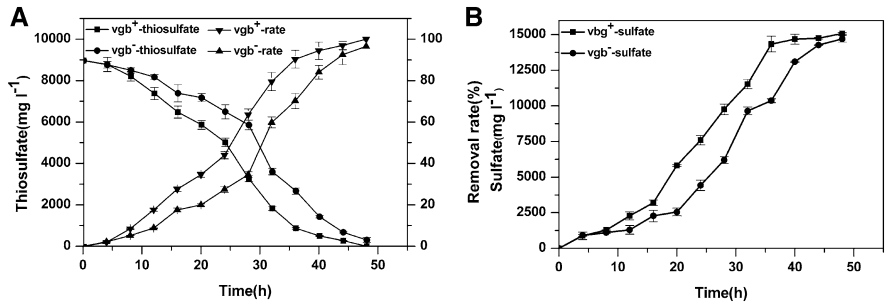

Fig. 3 Comparison of desulfurizing activity of *T. versutus* SOB306-vgb$^+$ and wild type (vgb$^-$) under aerobic conditions, **a** the consumption and removal rate of thiosulfate and **b** production of sulfate

desulfurization activity when compared to that of the vgb$^-$ strain owing to its better performance in utilization of thiosulfate. As in case of thiosulfate oxidation, the formation of sulfate in the vgb$^+$ strain is also faster than that in the vgb$^-$ strain (Fig. 3b). Xiong et al. reported that the expression of *vgb* gene in *Rhodococcus erythropolis* LSSE8-1 markedly improved its desulfurizing activity (Xiong et al. 2007).

The supply of O_2 is very important for cell growth and product formation in aerobic microorganisms. Although the cell density of the SOB306 strain was not very high owing to its chemolithotrophic properties, a large number of sulfur globules produced by the strain could potentially hinder O_2 transfer. Therefore, VHb could help SOB306 utilize the dissolved O_2 more efficiently.

Conclusion

A highly efficient genetic transformation system based on conjugative transfer method in the genus *Thialkalivibrio* was developed. A broad-range-host plasmid pBBRMCS1 was used to select strong promoters for SOB306 strain. Two promoters, *tac* (generally used in *E. coli*) and p3, regulated transcription of peroxiredoxin gene in SOB306 strain and promoted expression of the *rfp* gene in SOB306. The strength of the *tac* promoter was about three times of p3. Plasmid pBBR-tac-vgb expressing VHb was then constructed and transformed into SOB306 through conjugation transfer and this boosted oxidation of thiosulfate as well as cell metabolism. However, cell growth was slightly decreased, which is different from results reported earlier. To our knowledge, this is the first time that a genetic transformation strategy and expression system have been built in the genus *Thialkalivibrio*. It lays a foundation to boost research into metabolic engineering and helps to develop potentially high activity strains for biodesulfurization.

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Supporting information Supplementary materials—construction of plasmids, information of bacteria and plasmids, detailed information of primers, promoters and expression of RFP.

Supplementary Table 1—Strains and plasmids used in this study.

Supplementary Table 2—The primers used in this paper.

Supplementary Table 3—Statistic of transfer frequency and plasmid stability.

Supplementary Table 4—Information of predicted promoters from SOB306 strain.

Supplementary Fig. 1—Check of conjugative transfer between *E. coli* and *T. versutus* SOB306.

Supplementary Fig. 2—Expression of *rfp* gene promoted by different promoters in SOB306 strain.

Supplementary Fig. 3—The growth conditions of the two kinds of strains at different culture times.

References

Banciu H, Sorokin DY, Kleerebezem R, Muyzer G, Galinski EA, Kuenen JG (2004) Growth kinetics of haloalkaliphilic, sulfur-oxidizing bacterium *Thialkalivibrio versutus* strain ALJ 15 in continuous culture. Extremophiles 8:185–192

Geckil H, Stark BC, Webster DA (2001) Cell growth and oxygen uptake of *Escherichia coli* and *Pseudomonas aeruginosa* are differently effected by the genetically engineered *Vitreoscilla* hemoglobin gene. J Biotechnol 85:57–66

Klok JB, van den Bosch PL, Buisman CJ, Stams AJ, Keesman KJ, Janssen AJ (2012) Pathways of sulfide oxidation by haloalkaliphilic bacteria in limited-oxygen gas lift bioreactors. Environ Sci Technol 46:7581–7586

Li HJ, Zhang DH, Yue TH, Jiang LX, Yu X, Zhao P, Li T, Xu JW (2016) Improved polysaccharide production in a submerged culture of *Ganoderma lucidum* by the heterologous expression of *Vitreoscilla* hemoglobin gene. J Biotechnol 217:132–137

Liao B, Wang Y, Su J, Liu F, He J (2014) Expression of *Vitreoscilla* hemoglobin in *Bacillus thuringiensis* BMB171 can promote manganese(II) oxidation under oxygen-restricted conditions. Ann Microbiol 64:1865–1868

Liu X, Lin J, Zhang Z, Bian J, Zhao Q, Liu Y, Lin J, Yan W (2007) Construction of conjugative gene transfer system between *E. coli* and moderately thermophilic, extremely acidophilic *Acidithiobacillus caldus* MTH-04. J Microbiol Biotechnol 17:162–167

Reese MG (2001) Application of a time-delay neural network to promoter annotation in the *Drosophila* melanogaster genome. Comput Chem 26:51–56

Sorokin DY, Lysenko AM, Mityushina LL, Tourova TP, Jones BE, Rainey FA, Robertson LA, Kuenen GJ (2001) *Thioalkalimicrobium aerophilum* gen. nov., sp. nov. and *Thioalkalimicrobium sibericum* sp. nov., and *Thialkalivibrio versutus* gen. nov., sp. nov., *Thialkalivibrio nitratis* sp. nov., novel and *Thialkalivibrio denitrificans* sp. nov., novel obligately alkaliphilic and obligately chemolithoautotrophic sulfur-oxidizing bacteria from soda lakes. Int J Syst Evol Microbiol 51:565–580

Tan D, Wu Q, Chen JC, Chen GQ (2014) Engineering *Halomonas* TD01 for the low-cost production of polyhydroxyalkanoates. Metab Eng 26:34–47

van den Bosch PL, Sorokin DY, Buisman CJ, Janssen AJ (2008) The effect of pH on thiosulfate formation in a biotechnological process for the removal of hydrogen sulfide from gas streams. Environ Sci Technol 42:2637–2642

Wakabayashi S, Matsubara H, Webster D (1986) Primary sequence of a dimeric bacterial haemoglobin from *Vitreoscilla*. Nature 322(322):481–483

Xiong X, Xing J, Li X, Bai X, Li W, Li Y, Liu H (2007) Enhancement of biodesulfurization in two-liquid systems by heterogeneous expression of *Vitreoscilla* hemoglobin. Appl Environ Microbiol 73:2394–2397

Xu X, Cai Y, Song Z, Qiu X, Zhou J, Liu Y, Mu T, Wu D, Guan Y, Xing J (2015) Desulfurization of immobilized sulfur-oxidizing bacteria, *Thialkalivibrio versutus*, by magnetic nanoparticles under haloalkaliphilic conditions. Biotechnol Lett 37:1631–1635

Yang JK, Xiong W, Xu L, Li J, Zhao XJ (2015) Constitutive expression of *Campylobacter jejuni* truncated hemoglobin CtrHb improves the growth of *Escherichia coli* cell under aerobic and anaerobic conditions. Enzym Microb Technol 75:64–70