J. Ocean Univ. China (Oceanic and Coastal Sea Research)
DOI 10.1007/s11802-015-2344-3
ISSN 1672-5182, 2015 14 (1): 84-88
http://www.ouc.edu.cn/xbywb/
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Floating *Escherichia coli* by Expressing Cyanobacterial Gas Vesicle Genes

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(Received April 3, 2013; revised May 22, 2013; accepted September 25, 2014)

Abstract Gas vesicles are hollow, air-filled polyprotein structures that provide the buoyancy to cells. They are found in a variety of prokaryotes. In this study, we isolated a partial gas vesicle protein gene cluster containing *gvpA* and *gvpC20Ψ* from *Planktothrix rubescens*, and inserted it into an expression vector and expressed it in *E. coli*. The gas vesicle was developed in bacterial cells, which made bacterial cells to float on medium surface. We also amplified *gvpA* and *gvpC20Ψ* separately and synthesized an artificial operon by fusing these two genes with the standardized gene expression controlling elements of *E. coli*. The artificial operon was expressed in *E. coli*, forming gas vesicles and floating bacteria cells. Our findings verified that the whole set of genes and the overall structure of gas vesicle gene cluster are not necessary for developing gas vesicles in bacteria cells. Two genes, *gvpA* and *gvpC20Ψ*, of the gas vesicle gene cluster are sufficient for synthesizing an artificial operon that can develop gas vesicles in bacteria cells. Our findings provided a wide range of applications including easing the harvest of cultured microalgae and bacteria, as well as enriching and remediating aquatic pollutants by constructing gas vesicles in their cells.

Key words gas vesicle; gene cluster; gas vesicle gene; buoyancy

1 Introduction

Gas vesicles are the components of gas vacuoles, which develop in cells of >150 unicellular aquatic prokaryotes including bloom forming cyanobacteria, *Bacillus megatherium*, Archaea and others (Pfeifer, 2012; Walsby, 1994). Gas vesicles have multiple functions, including preventing oxygen limitation and promoting light harvesting. Gas vesicles enable cyanobacteria and others to colonize the air-liquid interface and resist to the low-oxygen conditions. These functions of gas vesicles are regulated by the quorum sensing system (Oren, 2013; Ramsay and Salmond, 2012). In some species, the proteins forming gas vesicle embed in the cellular membrane, serving as a delivery vehicle there (Childs and Webley, 2012). Gas vesicles are hollow polyprotein structures, performing as the swimming bladders and providing the buoyancy to cells. Gas vesicles in some species take cylindrical structures with hollow conical end-caps. The most obvious difference between the vesicles of different species is their widths (Belenky *et al*., 2004). The genes encoding gas vesicle proteins are organized into a cluster in cyanobacterial genomes. The gas vesicle genes of *Planktothrix* sp. from Lake Zürich encode two proteins, a small hydrophobic GvpA, about 7.5 kDa in weight, which forms the ribs of the cylindrical gas vesicles (Offne *et al*., 1998), and a larger hydrophilic GvpC which attaches to the outer surface of the integrating gas vesicles (Strunk *et al*., 2011; Sivertsen *et al*., 2010; Englert and Pfeifer, 1993; Hayes *et al*., 1992, 1988) (Fig.1A). The *gvpA* genes in the cluster are two base pairs different each other; while *gvpC* genes vary in length and are characterized by either an internal *Ω* or an internal *Ψ* region (Beard *et al*., 2000, 1999). Different *gvpC* genes encode proteins which form gas vesicles with different diameters, making cells adapt to different pressures. For example, GvpC16 in combination with GvpC20 forms a narrow gas vesicle of

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50 nm in diameter, which float cells at a critical pressure of 1.1 MPa; while GvpC20, either alone or in combination with GvpC28, forms a gas vesicles of 60 nm in diameter, which float cells at 0.9 MPa (Beard *et al*., 2002). *P. rubescens* 9402 contains *gvpC16Ω*, *gvpA* and *gvpC20Ψ*, and produces gas vesicles functioning at higher pressures than gas vesicles produced by others (Beard *et al*., 1999, Fig.1B).

A fragment containing *gvpA* and *gvpC* has been tentatively expressed in *E. coli* previously (Hayes and Powell, 1995); however, expressing the partial gas vesicle gene cluster and synthesizing artificial functional operons with the genes of gas vesicles have not been reported to our knowledge. The gas vesicle proteins may assemble automatically into gas vesicles (Ezzeldin *et al*., 2012); however, the assembling progress is not clearly described. In this study, we isolated a partial gas vesicle gene cluster from *P. rubescens* 9402, which contained *gvpA* and *gvpC20Ψ* and a putative promoter ahead of *gvpA*, and expressed the partial cluster in *E. coli*. We also amplified *gvpA* and *gvpC20Ψ* separately and synthesized an artificial operon by fusing these genes with standardized gene expression controlling elements of *E. coli*. It was found that both the partial gas vesicle gene cluster and the synthesized operon determined gas vesicle development in *E. coli*.

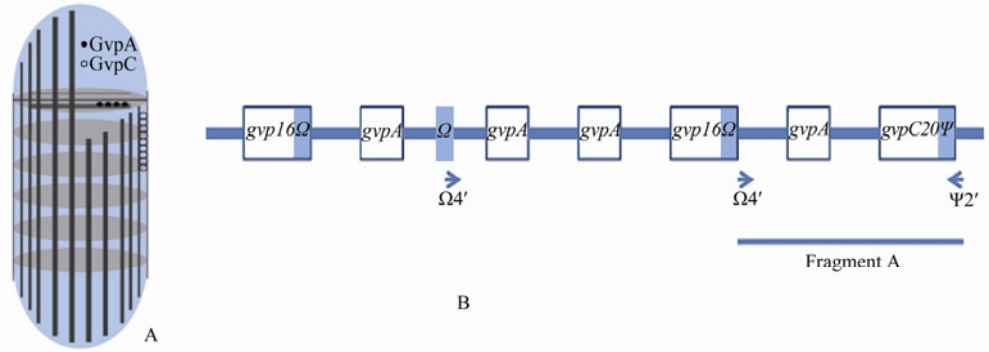

Fig.1 The sketch map of a gas vesicle (A) and the gas vehicle gene cluster (B) of *P. rubescens* 9402. The gas vesicle is a hollow structure. Horizontal ribs consisting of GvpA stratify inside; while vertical piles consisting of GvpC coat outside. Air is entrapped into the gas vesicle providing buoyancy to the vesicles themselves and bacteria containing the vesicles indirectly. Boxes in B indicate the open reading frames; while lines indicate the intergenic spacers (not proportional). Bars represent either *Ω* region alone or at the 3' end of *gvpC16Ω* or *Ψ* region at the 3' end of *gvpC20Ψ*. Primer Ω4' and Ψ2' amplify fragment A which is expected to be 1207 bp in length. To *gvpA* from left and to *gvpC20Ψ* from right, two regions, 131 bp and 179 bp in length, respectively, flank fragment A. Primer Ω4' matches two positions; however only the second near *gvpA* works in PCR amplification.

2 Materials and Methods

2.1 Culture of *P. rubescens*

P. rubescens 9402 (CCAP1460/9) was purchased from CCAP (NERC Culture Collection of Algae and Protozoa) and cultured in BG11 (Bright and Walsby, 2000) at 16℃ and under 3000 lux following a rhythm of 12 h light and 12 h dark.

2.2 Amplification of Gas Vesicle Gene(s)

As shown in Fig.1B, primers Ω4' (5'-CCT TAA GTG ATA TAA AAG ATC TCC AAG CCA TTC CCC ATT CTA T-3') and Ψ2' (5'-AAC TGC AGA TAA TAA TAC TAC TAG TCT GCC GAG TTA GGG ATT AGC-3') (Beard *et al*., 2000, 1999) were used to amplify the genomic DNA of *P. rubescens* 9402. The amplification was carried out by denaturing at 94℃ for 4 min followed by 30 cycles of denaturing at 94℃ for 30 s, annealing at 65℃ for 30 s and extending at 72℃ for 1 min and an extra extension at 72℃ for 5 min. The PCR product was phenol-chloroform extracted and precipitated with ethanol and stored at −20℃.

We also amplified *gvpA* and *gvpC20Ψ* separately with primer pair A1 (5'-GAA TTC GCG GCC GCT TCT AGA GTC ACA CAG GAA AGT ACT ATA TGG CCG TTG A-3') and A2 (5'-CTG CAG CGG CCG CTA CTA GTA TTA GAC CGA AGG AAC AGC CGC CTG-3') and primer pair C-20-1 (5'-GAA TTC GCG GCC GCT TCT AGA GTC ACA CAG GAA AGT ACT AGA TGG CTT TAA AAG ACG A-3') and C-20-2 (5'-CTG CAG CGG CCG CTA CTA GTA TTA ACA GGA ATA TAA ACG CGA TGG-3'). The amplification of *gvpA* was carried out by denaturing at 94℃ for 4 min followed by 30 cycles of denaturing at 94℃ for 30 s, annealing at 70℃ for 30 s and extending at 72℃ for 20 s and an extra extension at 72℃ for 5 min. The amplification of *gvpC20Ψ* was carried out by denaturing at 94℃ for 4 min followed by 30 cycles of denaturing at 94℃ for 30 s, annealing at 68℃ for 30 s and extending at 72℃ for 30 s and an extra extension at 72℃ for 5 min.

2.3 Construction of Recombinant Plasmids

A recent study showed that some strong promoters like P_A function in the expression of *gvpACNO* cluster which encodes the major gas vesicle structural proteins GvpA and GvpC in *Haloarchaea* (Marschaus and Pfeifer, 2012). We believed that our partial gene cluster contained also

its own promoter(s). Therefore, we did not place our partial cluster under the control of an *E. coli* promoter but rather fused this partial cluster with a terminator only. The fragment A was double digested with *Eco* RI and *Spe* I, ligated with a double terminator, BBa_B0015, an iGEM part (http://partsregistry.org/) consisting of two concatenate transcriptional terminators, BBa_B0010 and BBa_B0012, double digested with *Xba* I and *Pst* I in advance. In order to avoid intensive expression which may cause the death of host cells (Hayes, 1995), the ligation product was inserted into a low-copy number expression vector pSB4K5 (http://partsregistry.org/) double digested with *Eco* RI and *Pst* I early, yielding a recombined plasmid pSB4K5-*gvpA*-*gvpC20Ψ* (Fig.2A).

We synthesized an artificial operon by using a three antibiotic assembly (Shetty *et al*., 2011), which contained *gvpA* and *gvpC*, a promoter J23106 (an iGEM part, http://partsregistry.org/), an introduced RBS and a transcriptional terminator B0015 in order to verify the feasibility of forming gas vehicle by expressing gas vehicle genes under the control of standardized elements of *E. coli*. The construct was named as p5B1K3-gvpA-*gvpC20Ψ* (Fig.2B).

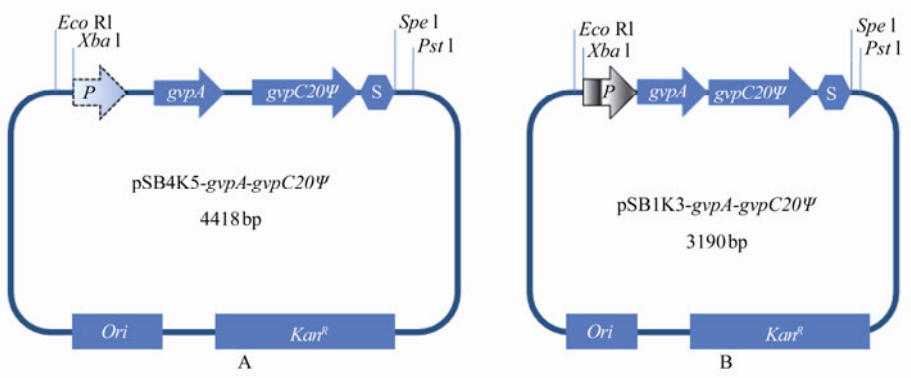

Fig.2 Two recombination plasmids, pSB4K5-*gvpA*-*gvpC20Ψ*(A) and pSB1K3-*gvpA*-*gvpC20Ψ*(B), constructed in this study. Fragment A, a partial gas vesicle gene cluster amplified from the genome of *P. rubescens* with primer Ω4' and Ψ2' contains *gvpA*, *gvpC20Ψ* and a putative promoter in front of *gvpA*, and *Eco* RI and *Xba* I sites in left and *Spe* I and *Pst* I sites in right. The transcriptional terminator BBa_B0015, a double terminator in a plasmid with Amp^R and Kan^R marker, contains *Eco* RI and *Xba* I sites in left and *Spe* I sites in right. The low copy plasmid pSK4K5-I52002 contains *Eco* RI and *Xba* I sites in the left and *Spe* I and *Pst* I sites in the right of an insert I52002. After double digestion (Fragment A with *Eco* RI and *Spe* I, BBa_B0015 with *Xba* I and *Pst* I and pSK4K5-I52002 with *Eco* RI and *Pst* I) and purification, fragment A, BBa_B0015 and pSB4K5 are merged together, yielding recombinant pSB4K5-*gvpA*-*gvpC20Ψ*. With the same strategy, *gvpA* was ligated with promoter BBa_J23106 in plasmid J61002, and *gvpC20Ψ* was ligated with terminator BBa_B0015 first, and then two genes and two control elements were inserted into high copy vector pSB1K3, yielding recombinant pSB1K3-*gvpA*-*gvpC20Ψ*.

2.4 Bacterial Transformation

Bacterial transformation was carried out following the standard protocols (Berger and Kimmel, 1987).

2.5 Observation of *E. coli* Floating

The constructs were transferred into either *E. coli* JM109 (pSB4K5-*gvpA*-*gvpC20Ψ*) or *E. coli* Top10 (pSB1K3-*gvpA*-*gvpC20Ψ*). In order to quantify the cell numbers at different layers of medium, the transformant was inoculated into a sampling apparatus we devised ourselves and cultured without rotation to the logarithmic growth phase (about 24 h). The cells were sampled by punching needles of a few small syringes into the large syringe at different heights and drawing a desirable amount of culture into small syringes each in an up-to-bottom order. The sampled cells were diluted to 1 mL with the bacteria cell number counted with blood counting plate. The engineered bacterial cells were treated with lysozyme and SDS, stained with uranyl acetate and observed under an electron microscope (JEM-1200EX-TEM, JEOL) for checking the existence of gas vesicles.

3 Results

3.1 Isolation of Partial Gas Vesicle Gene Cluster, *gvpA* and *gvpC20Ψ*

Fragment A (1207 bp in length) was amplified with primers Ω4'and Ψ2'. To the left and right ends of fragment A, the reorganization sites of *Eco* RI, *Xba* I, *Spe* I and *Pst* I, were introduced, respectively, as were underlined in primer Ω4' and Ψ2', which facilitated the construction of recombination plasmid. The *gvpA* and *gvpC20Ψ* were amplified with primer pairs A1 and A2, C-20-1 and C-20-2, which were 0.3 kb and 0.6 kb in length, respectively.

3.2 Gas Vesicle Development in Bacterial Cells

The recombinant plasmid pSB4K5-*gvpA*-*gvpC20Ψ* was constructed with fragment A, which was introduced into *E. coli* JM109. When the transformant was cultured for 24 h, the cells were found to float on the top of medium (Fig.3A). The transformant was also cultured in a sampling device with cells at different heights. It was found

that most bacteria cells containing the recombinant plasmid aggregated at the top of medium, which was different from the control containing pSB4K5 (Fig.3B). The recombinant plasmid pSB1K3-*gvpA-gvpC20Ψ* was transferred into *E. coli* Top10. It was found that bacteria cells floated on the top of medium (Fig.3C). Cell counting showed that most cells aggregated at the top of medium; while the control cells containing pSB1K3 appeared at different heights with the most at the bottom (Fig.3D). The development of gas vesicles in *E. coli* containing pSB4K5-*gvpA-gvpC20Ψ* was also verified under an electron microscope (Fig.4).

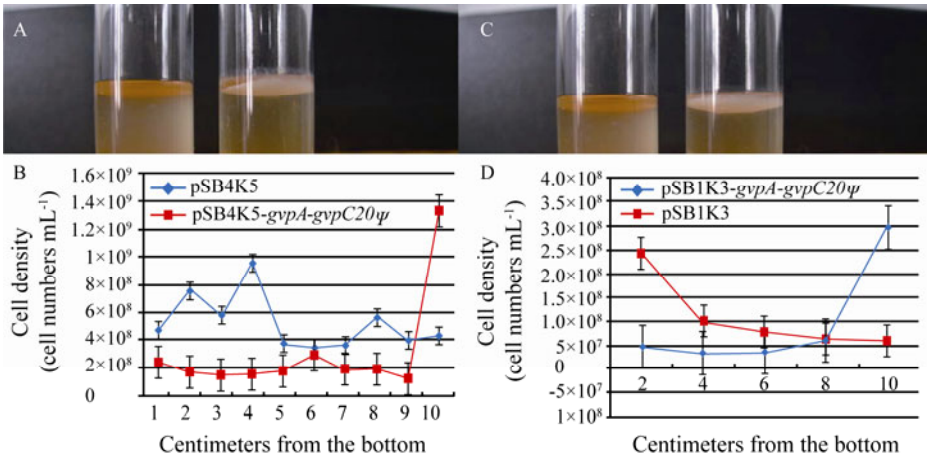

Fig.3 Observation and counting of floating *E. coli* JM109 cells containing pSB4K5-*gvpA-gvpC20Ψ* and *E. coli* Top10 cells containing pSB1K3-*gvpA-gvpC20Ψ*. *E. coli* JM109 cells containing pSB4K5-*gvpA-gvpC20Ψ* float on the top of medium; while the control cells do not (A, observation; B, cell number counting). *E. coli* Top10 containing pSB1K3-*gvpA-gvpC20Ψ* float on the top of medium; while the control cells do not (C, observation; D, cell number counting).

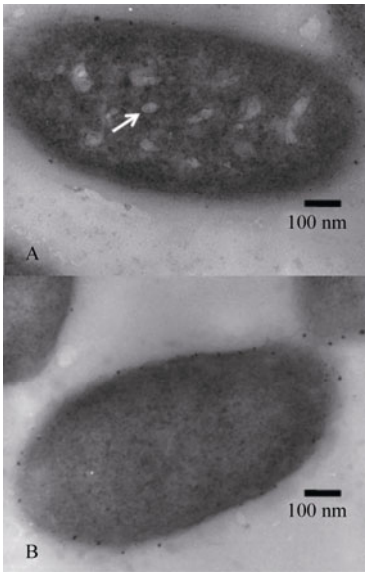

Fig.4 Gas vesicles developed in *E. coli* JM109 containing pSB4K5-*gvpA-gvpC20Ψ* (A). The white arrow points to a gas vesicle; Magnification, 60000; No gas vesicles were found in control cells containing pSB4K5 (B).

4 Discussion

We isolated a partial gas vesicle gene cluster containing *gvpA* and *gvpC20Ψ* from *P. rubescens* and synthesized an artificial operon using *gvpA* and *gvpC20Ψ* and standardized gene expression controlling elements of *E. coli*. The expression of both partial gas vesicle gene cluster and artificial operon developed the gas vesicles in *E. coli*. In a recent research in haloarchaea, a few over expressed gas vesicle genes (*e.g.*, *gvpG*, *H* and *M*) were found to associate with the formation of gas vesicles (Tavlaridou *et al*., 2013). Our findings verified that the whole gene set and the overall structure of the gas vesicle gene cluster were not necessary for developing gas vesicles in bacteria cells. Moreover, *gvpA* and *gvpC20Ψ*, two genes of the gas vesicle gene cluster were sufficient for synthesizing an artificial operon controlling the development of gas vesicles in bacteria.

Cyanobacterial genes may be controlled by expression regulating elements different from those functioning in bacteria (Chungjatupornchai *et al*., 1999). Both the partial gas vesicle gene cluster and *gvpA* and *gvpC20Ψ* genes we isolated may contain regulating elements in their flanking regions; however, these regions did not interfere with the expression of both the partial gene cluster and the artificial operon in *E. coli*. It is necessary to verify the possible influence of these regions and delete them completely in our future studies. It is well known that over expression of a protein may cause the death of host cells (Hayes, 1995). In this study, we expressed both the partial gas vesicle gene cluster in *E. coli* by inserting it into a low copy plasmid, pSB4K5, and an artificial operon in a high copy plasmid, pSB1K3. Expression of gas vesicle proteins was not found to cause the death of host cells.

The gas vesicle can be applied in measuring cellular turgor pressure and determining phylogeny (Miklaszewska *et al*., 2012; Holland and Walsby, 2009). Our research provides a wide range of applications. For example, the partial gas vesicle gene cluster or the artificial operon could be transferred into cultured algae, easing their harvesting. It is also expected that algal or bacterial species modified genetically with the gas vesicle genes may grow in water and float on surface, which can help to enrich

nutrients or remediate pollutants in aquatic environments.

5 Conclusions

A partial gas vesicle protein gene cluster containing *gvpA* and *gvpC20Ψ* was isolated from *P. rubescens*, and an artificial operon was synthesized containing *gvpA* and *gvpC20Ψ* and standardized gene expression controlling elements of *E. coli*. The expression of both partial gas vesicle gene cluster and artificial operon was sufficient to develop gas vesicles in *E. coli*. The whole gene set and the overall structure of gas vesicle gene cluster were not necessary for the development of gas vesicles in bacterial cells.

Acknowledgements

This work was partially supported by Undergraduate Institution of Marine Biological Science, Ocean University of China (OUC). Prof. Xiaohua Zhang, College of Marine Life Sciences, OUC, provided *Planktothrix rubescens* 9402. Prof. Xiaohua Zhang and Prof. Guanpin Yang, College of Marine Life Sciences, OUC, provided the cost of sequencing. Prof. Guanpin Yang helped prepare the manuscript. This work has won International Genetically Engineered Machine Competition (iGEM) Group of OUC a Gold Award in 2012. Authors appreciate all those who have helped iGEM Group of OUC directly or indirectly.

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(Edited by Qiu Yantao)