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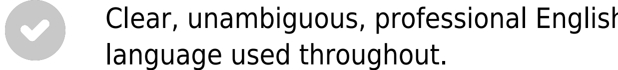

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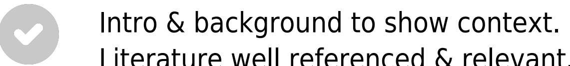

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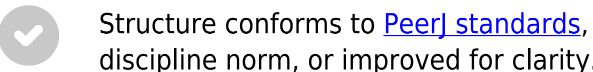

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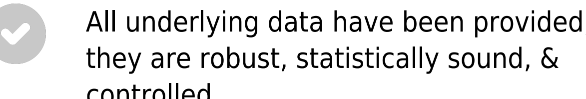

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Cbl upregulates *cysH* for hydrogen sulfide production in SmpB deletion to survive under the nutrient deficiency stress of *Aeromonas veronii*

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Endogenous hydrogen sulfide (H_2S) is generated in many metabolism pathways, and has been recognized as a second messager against antibiotics and reactive oxygen species (ROS). In *Aeromonas veronii*, Small Protein B (SmpB) plays an important role in resisting stress. The absence of *smpB* could trigger sulfate assimilation pathway to adapt the nutrient deficiency, of which was mediated by up-regulation of *cbl* and *cys* genes and followed with enhancing H_2S production. To figure out the mutual regulations of *cbl* and *cys* genes, a series of experiments were performed. Compared with the wild type, *cysH* was down-regulated significantly in *cbl* deletion by qRT-PCR. The fluorescence analysis further manifested that Cbl had a positive regulatory effect on the promoter of *cysJIH*. Bacterial one-hybrid analysis and electrophoretic mobility shift assay (EMSA) verified that Cbl bound with the promoter of *cysJIH*. Collectively, the tolerance to adversity could be maintained by the production of H_2S when SmpB was malfunctioned, of which the activity of *cysJIH* promoter was positively regulated by upstream Cbl protein. The outcomes also suggested the enormous potentials of *Aeromonas veronii* in environmental adaptability.

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37 ## Abstract
38 Endogenous hydrogen sulfide (H_2S) is generated in many metabolism pathways, and has been
39 recognized as a second messager against antibiotics and reactive oxygen species (ROS). In
40 *Aeromonas veronii*, Small Protein B (SmpB) plays an important role in resisting stress. The
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43 production. To figure out the mutual regulations of *cbl* and *cys* genes, a series of experiments
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47 mobility shift assay (EMSA) verified that Cbl bound with the promoter of *cysJIH*. Collectively,
48 the tolerance to adversity could be maintained by the production of H_2S when SmpB was
49 malfunctioned, of which the activity of *cysJIH* promoter was positively regulated by upstream
50 Cbl protein. The outcomes also suggested the enormous potentials of *Aeromonas veronii* in
51 environmental adaptability.
52 ## Introduction
53 *Aeromonas veronii* is widely present in fresh water, sewage, soil and even sea water (Hickman-
54 Brenner et al., 1987), which endows with strong resistance to multiple antibiotics (Liu et al.,
55 2018). Small Protein B (SmpB) acts as a small RNA binding protein in the trans-translation
56 system to help transfer messenger RNA (tmRNA) to rescue the retained ribosomes in bacteria
57 (Wali Karzai, Susskind & Sauer, 1999). Also, SmpB performs many significant functions in
58 biological regulation. For example, the expression of ribonuclease R (RNase R), an exonuclease
59 molecule that recognizes and degrades RNA, depends on SmpB in *Streptococcus pneumoniae*
60 (Moreira et al., 2012). SmpB protein promotes the binding and degradation of RNase R by
61 HslUV and Lon in *Escherichia coli* (Liang & Deutscher, 2012). Moreover, SmpB has similar
62 effects with the known RNA chaperone proteins such as CrsA and Hfq. The loss of SmpB affects
63 4% transcription changes of genes in *salmonella*, of which involves in biological processes,
64 including invasion, bacterial movement, central metabolism, lipopolysaccharide (LPS)
65 biosynthesis, two-component regulatory system and fatty acid metabolism (Sittka et al., 2007;
66 Ansong et al., 2009). In all, SmpB is essential for intra-macrophage proliferation and the strong
67 adaptability to stress (Ansong et al., 2009).
68 Hydrogen Sulfide (H_2S) is an unpleasant smell with toxicity (Lindenmann et al., 2010).
69 However, the low concentration of H_2S participates in bacterial defense against reactive oxygen
70 species (ROS) and antibiotics-induced oxidative damage (Lindenmann et al., 2010). One of the
71 basic H_2S synthesis includes sulfate assimilation pathway, which is catalyzed by *cysNDC* and
72 *cysJIH* (Shatalin et al., 2011; Kimura, 2014; Wu et al., 2015). In *Salmonella typhimurium*, the
73 expression of *cysJIH* is regulated by CysB which has 41% amino acid sequence homology with
74 Cbl (Iwanicka-Nowicka et al., 2007; Álvarez et al., 2015). Both CysB and Cbl are LysR-type
75 transcriptional activator. In sulfur metabolism, Cbl acts as a sensor of the intracellular sulphate
76 level, and activates *tau* and *ssu* promoter in *vivo* and in *vitro* (Van Der Ploeg et al., 1999; Van

77 Der Ploeg, Eichhorn & Leisinger, 2001; Bykowski et al., 2002). In addition, Cbl activates sulfate
78 starvation-induced genes under sulfate starvation(Van Der Ploeg et al., 1999). Taking together,
79 there may be a potential connection between Cbl and *cys* genes in sulfate assimilation pathway.
80 In *Aeromonas veronii*, both SmpB and H_2S play important roles in adverse stress. The absence of
81 SmpB induced the generation of H_2S helping to survive. The transcriptomic analysis revealed
82 that both*cbl* and *cys* genes were up-regulated in SmpB deletion strain. To clarify the regulatory
83 relationship between *cbl* and *cys* genes in sulfate assimilation pathway, Real-time PCR
84 experiment and fluorescence analysis were performed, showing that Cbl positively regulated
85 *cysH* gene. Furthermore, bacterial one-hybrid system and EMSA verified that Cbl regulated *cysH*
86 by binding to the promoter of *cysJIH*. In brief, Cbl bound and activated *cysJIH* promoter directly
87 to increase H_2S production, remedying the survival ability after *smpB* deficiency. Our study
88 elucidated the strong vitality and adaptability of *Aeromonas veronii*.
89

90 Materials & Methods

91 **1.Bacterial strains, plasmids and culture conditions**

92 The bacterial strains and plasmids used in this study were shown in supplemental Table S1. The
93 *smpB* deletion strain of *Aeromonas veronii* C4 was constructed previously(Liu et al., 2015). The
94 derivative *Aeromonas veronii* C4 strains were grown in LB/M9 medium supplemented with 50
95 mg/mL ampicillin at 30℃, and *E. coli* strains were grown in LB medium supplemented with 50
96 mg/mL kanamycin and 25 mg/mL chloramphenicol at 37℃. And all plasmids were sequenced
97 for verification.

98 **2. H_2S Detection**

99 The $Pb(Ac)_2$ detection (Shatalin et al., 2011) method and WSP5 fluorescent H_2S probe (Peng et
100 al., 2014) were performed for monitoring H_2S production. Bacteria were grown in M9 at 30℃
101 for 48h with $Pb(Ac)_2$ paper strips, and 5mg/L Na_2SO_3 was added as a source of sulfur. $Pb(Ac)_2$-
102 soaked paper strips showed a PbS brown stain as a result of the reaction with H_2S. The color
103 length of 1mm represented 12 μg/L of H_2S production. After 10 μM WSP5 was added to liquid
104 bacterial culture, the samples were incubated at 37℃ for 30 min and then washed in PBS buffer
105 to remove excess probe. Synergy H1 (BioTek) was used to take fluorescent readings at excitation
106 500 nm and emission 533 nm.

107 **3. RNA Extraction and qRT-PCR**

108 The qPCR reaction was conducted with ABI Prism® 7300 (ABI, New York, NY, USA) for
109 fluorescent detection utilizing SYBRR® Green real time PCR Master Mix (Toyonbo, Shanghai,
110 China). The cDNA was synthesized by RNA reverse transcription reaction and was used as the
111 template for real-time PCR. The primers used to monitor expression of the objective genes were
112 summarized in supplemental Table S2. Each reaction was performed at least in triplicate. And
113 the data was analyzed by the comparative CT method (Schmittgen & Livak, 2008).

114 **4. Fluorescence analysis**

115 The promoter of *cysJIH* was fused with eGFP and inserted into pUC19 plasmid. The *cbl* gene
116 was cloned into pTRG plasmid simultaneously. Both the above plasmids were co-transformed

117 into *E. coli* Reporter strain. In the meanwhile, the recombinant pUC19 plasmid and the empty
118 pTRG plasmid were co-transformed as the negative control. After bacteria were grown in LB at
119 37°C, the total amount of 1 OD_{600} cells were harvested at interval time. The samples were
120 washed with PBS twice, and placed on Synergy H1 (Biotek) for the fluorescent readings at
121 excitation 425 nm and emission 525 nm.
122 **5. Bacterial one-hybrid analysis**
123 To identify whether the transcription factor Cbl interacted with the promoter of *cysJIH*, Cbl was
124 inserted into pTRG, and the promoter of *cysJIH* was ligated with pBXcmT, following with both
125 the recombinant plasmids were cotransformed into *E. coli* Reporter strain. The transformants
126 were placed on a selective NM medium plate containing 5 mM 3-amino-1, 2, 4-triazole (3-AT)
127 and 12.5 mg/mL streptomycin for incubation at 37°C for 48 h.
128 **6. Protein expression and purification**
129 The *cbl* gene was inserted into pET28a plasmid and transformed into *E. coli* BL21 strain. The
130 expression and purification were performed according to previous procedure (Bykowski et al.,
131 2002).
132 **7.Electrophoretic mobility shift assay (EMSA)**
133 Double stranded DNA probes were radiolabeled with Fluorophore 6-carboxy-fluorescein (FAM)
134 and purified by FastPure Gel DNA Extraction Mini Kit (Vazyme). For the EMSA, DNA probe
135 was incubated with Cbl protein samples in reaction buffer (10 mM Tris–HCl, 1 mM $MgCl_2$, 1
136 mM DTT, 40 mM KCl, 0.1 mg/mL BSA, 5% (w/v) glycerol) at 37°C for 30min. After the
137 samples were separated using a native 6% native acrylamide gel (Zhang et al., 2020), the gel was
138 then exposed to a phosphorscreen and visualized on Typhoon FLA 9500.
139 **8. Transcriptome analysis**
140 To perform the whole-transcriptome analysis, the wild type and *smpB* deletion of *Aeromonas*
141 *veronii* veronii C4 were grown in M9 at 30°C for 20h, and 2 OD_{600} of cells were collected.
142 Illumina HiSeq-X ten based on the service of RNA-Seq Quantification library at BGI-Shenzhen
143 (China) was used to obtain the transcriptome sequencings. And the RNA-seq raw data was
144 assembled and analyzed by comparing with the translational region of the annotated DNA
145 sequence in reference genome (GCA_001593245.1) using HISAT (Kim, Langmead & Salzberg,
146 2015). The DESeq. 2 package in R was used for the estimation of fold changes and other
147 analysis (Love, Huber & Anders, 2014).
148 **9. Statistical analyses**
149 Statistical significance was determined by t test (two-tailed distribution with two-sample, equal
150 variance) when directly comparing two conditions or a one-way analysis of variance (ANOVA)
151 followed by pairwise comparisons.
152
153

154 Results

155 **1. Transcriptomic analysis**

156 Based on the transcriptomic analysis, the deletion of SmpB mainly caused the changes in 20
157 biological pathways, including two-component system, sulfur metabolism, plant pathogenic
158 bacteria interaction, and phenylalanine metabolism. Sulfur metabolism was the most influential
159 on metabolic pathways (Fig.1A).
160 In *Aeromonas veronii* C4, H_2S synthesis pathway included the sulfate assimilation pathway, the
161 organic pathway, and the 3-MST pathway. But compared with others, *Aeromonas veronii* C4
162 lacked cystathionine β-synthase (CBS) in the transsulfuration pathway and cysteine
163 aminotranferase (CAT) in the 3MST pathway. The deletion of SmpB mainly up-regulated the
164 transcription levels of *cysN*, *cysD*, *cysC*, *cysH*, *cysJ*,*cysI* and *cbl* (Fig.1B). And these genes were
165 mainly involved in sulfate assimilation pathway (Fig.1C). Therefore, we speculated that SmpB
166 deficiency was able to increase H_2S synthesis.
167 **2.SmpB deficiency promotes H_2S biosynthesis and increases the tolerance to oxidative**
168 **stress under nutritionally deficient conditions**
169 To figure out how sulfur metabolism was affected by SmpB deficiency, H_2S production was
170 measured in rich and deficient nutrition conditions by $Pb(Ac)_2$ detection test. There is no
171 difference between wild type (WT) and *smpB* deletion in a rich medium (LB medium) (Fig.2A).
172 Under the condition of nutritional deficiency (M9 medium), the *smpB* deletion produced less
173 amount of H_2S in the early stage of growth, but it enhanced to synthesize H_2S in the stationary
174 stage (Fig.2B). The final H_2S yield of *smpB* deletion was significantly higher than that of WT.
175 This suggested that the absence of SmpB enhanced the synthesis of H_2S during auxotrophic
176 conditions, especially predominant during the stationary phase of bacterial growth. And previous
177 study has proved that *smpB* deletion survives better than WT when treated with appropriate
178 concentration of H_2O_2 (Wang et al., 2019). In view of the function of H_2S in oxidative resistance,
179 we speculated that H_2S was responsible for the strong viability of *smpB* deletion.
180 **3. Cbl affects the generation of H_2S**
181 Using both the classic $Pb(Ac)_2$ detection test and a fluorescent-based probe WSP5(Zhang et al.,
182 2020), we confirmed that, the production of H_2S in *cbl* deletion strain was significantly lower
183 than that of WT in M9 medium (Fig.3A; Fig.3B). And the difference was offset when Cbl
184 protein was complemented (Fig.3A; Fig.3B). All the results were consistent with the
185 transcriptome data, implying that Cbl had a positive regulatory effect on the synthesis of H_2S
186 under nutritional deficiency.
187 **4. Cbl promotes the transcription of *cysH***
188 The amino acid sequence of *cbl* gene was highly homologous to the *cysB* family, and CysB was
189 proved to binding with the promoter of sulfur reductase (CysJIH) as a transcription factor for
190 regulation. Therefore, it was speculated that *cbl* regulated the transcription of genes such as
191 *cysH*, *cysJ* and *cysI*. The relative expression of *cysH* decreased significantly compared WT with
192 cbl deletion by RT-qPCR, while those of *cysI*, *cysJ* revealed no differences (Fig.3C).
193 Furthermore, the fusion of the promoter *cysJIH* (P*cysJIH*) and eGFP was constructed as the
194 indicator plasmid for the fluorescent measurement. When co-expressed with Cbl, the

195 fluorescence value was extremely significantly higher than that of the strain containing only
196 P*cysJIH* (Fig.3D). Collectively, Cbl had a positive regulation on P*cysJIH*.
197 **5. Cbl regulates downstream *cysH* by binding to the P*cysJIH***
198 To confirm whether Cbl bound to P*cysJIH*, the P*cysJIH* promoter sequence and Cbl coding
199 sequence were cloned into pBXcmT and pTGR plasmids respectively, and then co-transformed
200 into *E. coli* XL 1-Blue MRF' reporter strain for bacterial one-hybrid experiment. Only the co-
201 expressed strain and the positive control grew on the minimum medium supplemented with 6mM
202 3-AT and streptomycin (Fig.4A), suggesting that the strong interaction between P*cysJIH* and
203 Cbl.
204 Next P*cysJIH* was labelled with Fluorophore 6-carboxy-fluorescein (6-FAM) for electrophoretic
205 mobility shift assay (EMSA). The Cbl protein reduced the mobility of the 6-FAM-P*cysJIH* DNA
206 probe corresponding to the increased Cbl concentration with the enhanced Cbl–DNA complex
207 (Fig.4B). So Cbl protein was able to bind with P*cysJIH* following with the regulation of H_2S
208 production.
209 **6. Determination of the binding region of P*cysJIH* with Cbl protein**
210 To confirm the binding region of P*cysJIH* with Cbl protein, we truncated the full length of
211 P*cysJIH* to 150bp and 50bp upstream of transcriptional initiation which were named as
212 P*cysJIH*^{150} and P*cysJIH*^{50}. P*cysJIH*^{150} was able to form a complex with Cbl protein, while
213 P*cysJIH*^{50} lost the binding ability (Fig.4C). The result suggested that the regions between 50bp
214 and 150bp upstream of transcriptional initiation in P*cysJIH* were responsible for the binding of
215 Cbl.
216 ## Discussion
217 SmpB protein is involved in the regulation of multiple biological processes such as protein
218 invasion, bacterial movement, central metabolism, lipopolysaccharide biosynthesis, two-
219 component system, fatty acid metabolism, high temperature tolerance, cell cycle, and stress
220 response (Shin & Price, 2007; Ansong et al., 2009; Barends et al., 2010). And the destruction of
221 SmpB reduces the tolerance and adaptability of bacteria (Ansong et al., 2009).
222 H_2S is able to resist oxidative stress by reacting with reactive oxygen species (ROS), H_2O_2, etc.
223 or stimulate catalase and superoxide dismutase to scavenging free radicals (Kimura, 2014;
224 Mironov et al., 2017). Besides, the oxidative stress effect of H_2S is also related to the defense of
225 bacteria against antibiotics, because many antibiotics also trigger the production of ROS when
226 they targeting inhibit their targets (Kohanski et al., 2007). So, the effect of H_2S in scavenging
227 ROS can make it more resistant to antibiotics.
228 In our study, the *smpB* deletion of *Aeromonas veronii* C4 was significantly higher in H_2S
229 production than wild type under M9 culture condition (Fig.2B), implying that SmpB deficiency
230 enhanced the H_2S generation. Indeed, *smpB* deletion up-regulated multiple genes in sulfate
231 assimilation pathway, including *cysN,cysD*, *cysC*, *cysH*, *cysJ,cysI* and *cbl* (Fig.1B; Fig.1C).
232 In *Salmonella Typhimurium*, the promoter of *cysJIH* (P*cysJIH)* is regulated by CysB (Álvarez et
233 al., 2015), which is homologous with Cbl (Kertesz, 2000). Therefore, we presumed that Cbl was

234 responsible for the regulation of *cysH*, *cysJ* and *cysI* in *Aeromonas veronii* C4. Really, Cbl bound
235 to P*cysJIH* and positively regulated the transcription of *cysH* (Fig.3D; Fig.4A; Fig.4B; Fig.4C).
236 Previously *smpB* deletion exhibits more tolerance to aminoglycosides antibiotic and oxidative
237 stress under M9 culture (Fig.2C) (Liu et al., 2018; Wang et al., 2019). Logical, we proposed that
238 Cbl-regulated H_2S generation compensated for the resistance and survival of SmpB damage
239 under nutrient deficiencies, contributing to the adaptation and evolution of *Aeromonas veronii*
240 against extreme environment.

241 ## Conclusions

242 This study provided the first demonstration for the regulatory between Cbl and *cysJIH*, and
243 innovatively proposed the mechanism of Cbl-mediated H_2S synthesis to compensate for
244 tolerance defects caused by SmpB deficiency. The results expanded the function of Cbl in
245 pathogenic bacteria, and systematically explained the the dynamic role of H_2S in protecting
246 bacteria from oxidative stress. These findings provide potential drug targets for aquatic diseases,
247 offers theoretical basis for better understanding of bacterial pathogens resistance to
248 environmental stress and supplies new ideas for clinical prevention and control of bacterial
249 pathogens.

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253

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347

Figure 1

Transcriptomic analysis between wild type (WT) and *smpB* knockout.

(A) The KEGG pathways for the different metabolites between WT and the *smpB* deletion (Δ*smpB*). (B) The relative expression of the correlated H_2S synthesis genes in WT and Δ*smpB* cells. Values represented means ± SD (n = 3). ***p < 0.001 was determined by one-way ANOVA and Tukey post-test. (C) The deletion of *smpB* enhanced the expression of genes in sulfate assimilation pathway.

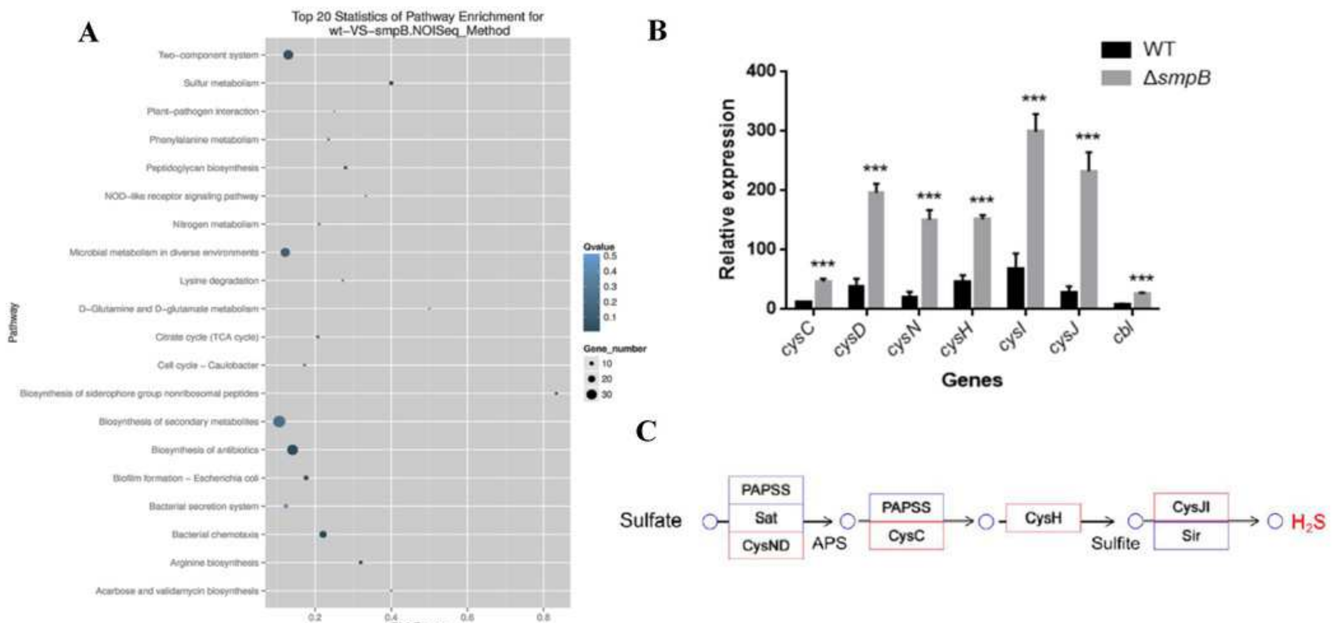

Figure 2

SmpB deficiency promotes H_2S biosynthesis and increases the tolerance to oxidative stress under nutritionally deficient conditions

(A) H_2S production was measured by $Pb(Ac)_2$-soaked paper strips in LB medium supplemented with 5mg/L Na_2SO_3. No significant differences existed between WT and Δ*smpB* strains. (B) H_2S production was measured by $Pb(Ac)_2$-soaked paper strips in M9 medium supplemented with 5mg/L Na_2SO_3. Values represented as means ± SD (n = 3). **$p < 0.005$ was determined by one-way ANOVA and Tukey post-test.

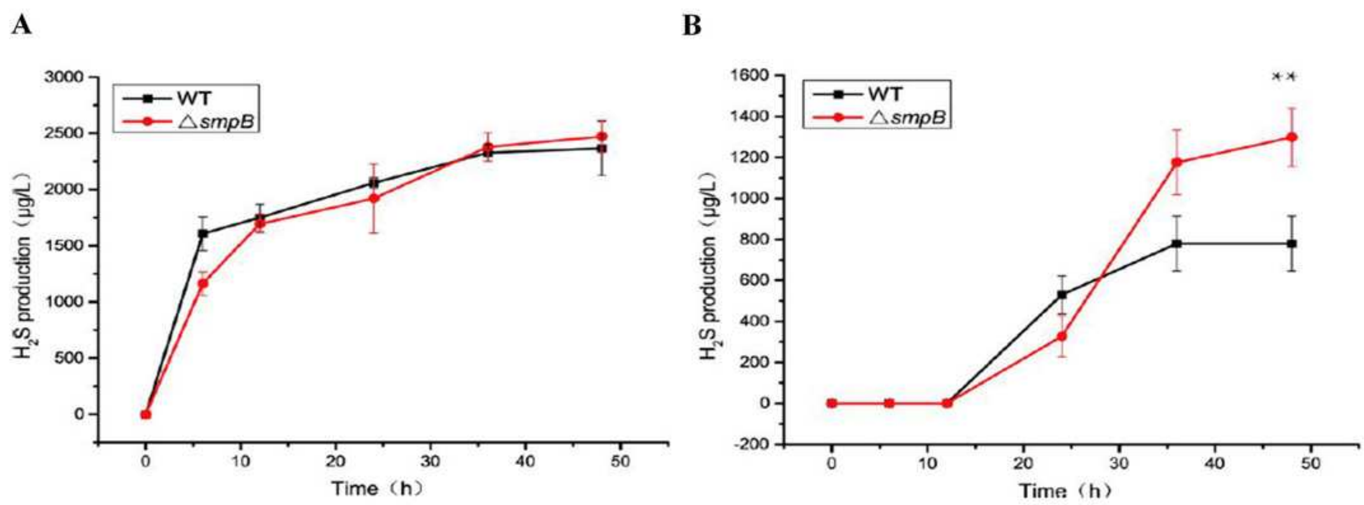

Figure 3

Cbl affected H_2S production by promoting the transcription of *cysH*

(A) H_2S production was measured by $Pb(Ac)_2$-soaked paper strips in M9 medium supplemented with 5mg/L Na_2SO_3. The tested strains included WT, Δ*cbl* and the complemented strain (Δ*cbl*-P*cbl*). Values represented as means ± SD (n = 3). $^{**}p < 0.005$ was determined by one-way ANOVA and Tukey post-test. (B) Fluorescence intensities were detected by Synergy H1 (BioTek) after the tested strains were treated with fluorescent H_2S probe in M9 medium. Values represented as means ± SD (n = 3). $^{*}p < 0.01$ was determined by one-way ANOVA and Tukey post-test. (C) The relative expressions of H_2S synthesis genes were detected by qRT-PCR. Values represented as means ± SD (n = 3). $^{*}p < 0.01$ was determined by one-way ANOVA and Tukey post-test. (D) Fluorescence intensities were detected by Synergy H1 (BioTek). The tested strains expressed P*cysIJH* only (pTRG+pUC19-P*cysIJH* -eGFP), and co-expressed both Cbl and P*cysIJH* (pTRG-Cbl+pUC19- P*cysIJH* -eGFP), respectively. Values represented means ± SD (n = 3). $^{*}p < 0.01$ was determined by one-way ANOVA and Tukey post-test.

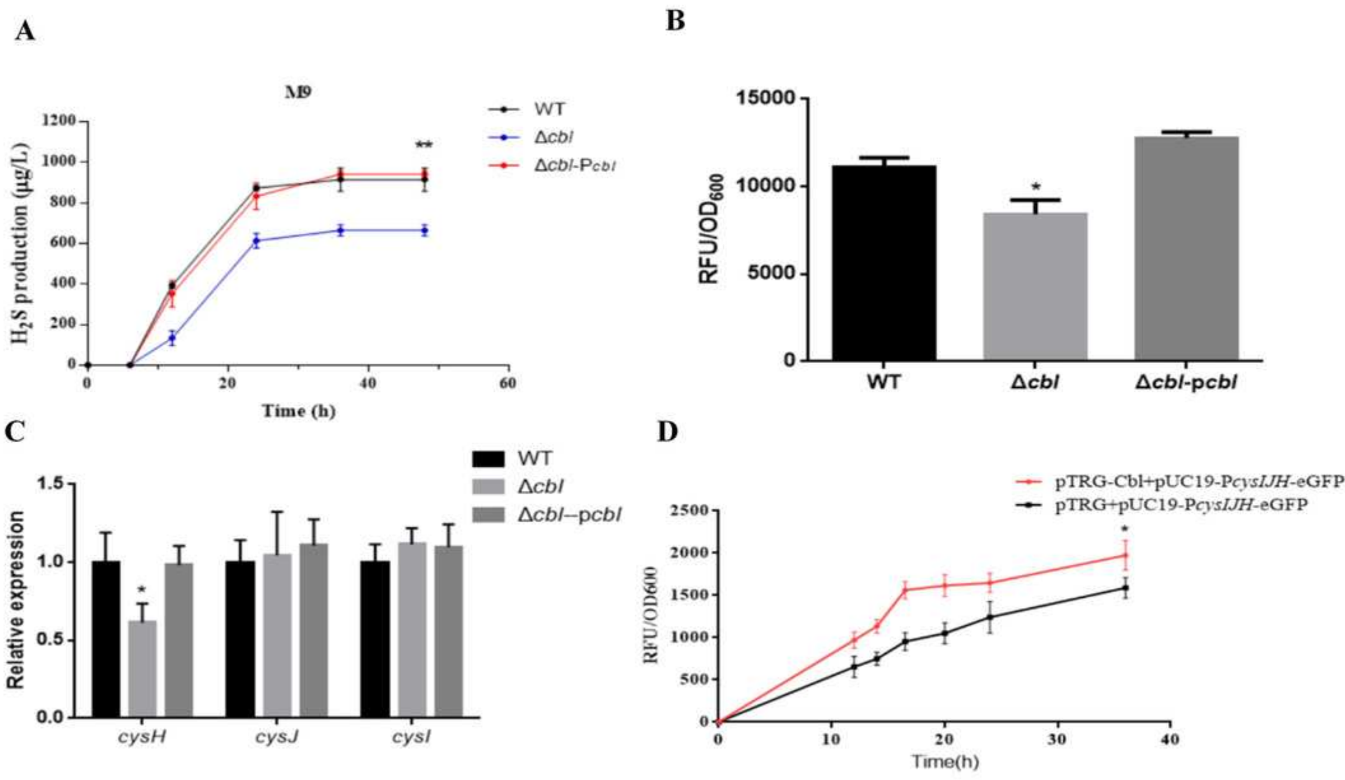
A
M9
WT
Δcbl
Δcbl-Pcbl
H2S production (μg/L)
Time (h)
**
B
RFU/OD600
WT
Δcbl
Δcbl-pcbl
*
C
WT
Δcbl
Δcbl--pcbl
Relative expression
cysH
cysJ
cysI
*
D
pTRG-Cbl+pUC19-PcysIJH-eGFP
pTRG+pUC19-PcysIJH-eGFP
RFU/OD600
Time(h)
*

Figure 4

Cbl regulated downstream *cysH* by binding to the P*cysJIH.*

(A) Results of bacterial one-hybrid. (B) Electrophoretic mobility shift assay (EMSA) for Cbl binding with P*cysJIH* . The 25 nM FAM-labeled P*cysJIH* was incubated with the increased amounts of Cbl protein. Cbl protein was titrated to the concentration of 0μM, 10μM, 20μM, 30μM, 40μM, 50μM and 60μM. (C) Electrophoretic mobility shift assay (EMSA) for Cbl binding with the varied size of P*cysJIH.* P*cysJIH* contained 226 bp upstream of transcriptional initiation site, P*cysJIH*^{150}contained 150bp upstream of transcriptional initiation site, and P*cysJIH*^{50} contained 50bp upstream of transcriptional initiation site. The 25 nM FAM-labeled probe DNA was incubated with 60μM Cbl protein. The experiments were repeated in triplicate.