The Effect of Inactivation of Aldehyde Dehydrogenase on Pheromone Production by the Gut Bacteria of an Invasive Bark Beetle, *Dendroctonus Valens* (LeConte)

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Research Article

Keywords: Transcriptome, Gut facultative anaerobes, Verbenone, Mutants

Posted Date: February 10th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1309915/v1

Abstract

Semiochemical-based strategies are important for bark beetle management worldwide. One of the most destructive invasive bark beetles, the causal agent of mass pine (Pinus sp.) mortality in China, is the red turpentine beetle (RTB) *Dendroctonus valens* (LeConte), originating from North America. For this species, verbenone pheromone regulates the beetles' attack density in a dosage dependent manner. In addition, we have previously shown that RTB's gut bacteria is involved in the pheromone production under both anaerobic and micro-oxygen environments. However, although some investigations of anaerobic gut bacteria of bark beetles have been made, functional verification of their role at molecular level is still lacking. To clarify the function of gut bacteria in verbenone production, we investigated the activity of key genes of the primary gut bacteria involved in verbenone production in *D. valens* under anaerobic environment. These key genes (aldehyde dehydrogenase) identified by transcriptome analysis were then knocked out by homologous recombination to obtain mutant bacteria strains. Our results show that these mutants had significantly decreased ability to convert the monoterpene precursor to verbenone compared to the wild type bacteria. Our findings provide further evidence of the mechanism of pheromone production of *D. valens* and a new perspective for functional studies of gut bacteria in general.

Introduction

Insect gut microbiome performs myriad ecological functions (Dillon and Dillon 2004; Engel and Moran 2013) such as absorption and utilization of nutrients (Kaufman and Klug 1991; Breznak 2002), improving resistance against invasion of pathogens and natural enemies (Shao et al. 2017; Koch and Schmid-Hempel 2011), boosting host immune response (Mikonranta et al. 2014), degrading harmful xenobiotics (Tago et al. 2015; Mason et al. 2016) and mediating plant-insect interactions by detoxificaticating host defense chemicals (Mason et al. 2014; Wang et al.2020). Microbiota is also involveld in other key functions in insects' life, such as pheromone production. For example, gut microbiota is largely responsible for the production of aggregation pheromones of locusts, sex pheromones of cockroaches (Dillon et al. 2002), and influence the mating preference of *Drosophilla meladella* (Sharon et al. 2010).

Bark beetles are economically important insect pests of conifer and broadleaf trees, and pheromone-based mass trapping and push-pull techniques are essential for their successful management (Wermelinger 2004; Witzgall et al. 2010). A large proportion of bark beelte pheromones are composed of monoterpenoid oxides (Wood 1982; Blomquist et al. 2010). For example, α-pinene is not only an important host defensive monoterpene, but also a precursor for the bark beetle pheromone verbenone (Blomquist et al. 2010; Gitau et al. 2013). Many micro-organisms have been implicated to be involved in the bioconversion of precursors to terpenes and in the synthesis of terpenoid pheromones. In *Ips paraconfusus*, for instance, gut bacteria oxidize α-pinene to pheromone verbenol (Brand et al. 1975). Pheromone synthesis by microorganisms is conducted by degrading plant compounds, and some investigation of the mechanisms behind this process have also been made. For example, Merlin et al. (2005) suggested that aldehyde oxidase could degrade aldehyde odorant compounds, such as pheromones or plants volatiles. Comparison of *in vivo* and *in vitro* physiological data from *Drosophila*

melanogaster adults confirmed that aldehyde dehydrogenase (ALDH) was responsible for the detoxification effect of acetaldehyde in v*ivo* (Leal and Barbancho 1992).

Red turpentine beetle *Dendroctonus valens* LeConte (Coleoptera: Scolytidae), was introduced to China from North America in the 1980s. Despite *D.valens* is an innocuous secondary pest in its native range, has become a primary tree-killer of Chinese red pine *Pinus tabulaeformis* (Carr) in the new invasive range, causing substantial economic losses and recieving its status as China's most destructive forest pest. Semiochemical-based management strategies, including pheromone traps, has been successfully implemented in *D. valens*' control (Sun et al. 2013; Liu et al. 2019). Several bioactive volatiles, such as *cis*-verbenol, *trans*-verbenol, myrtenol, myrtenal, and verbenone have been idenitified from the gut of this beetle (Zhang et al. 2006; Sun et al. 2013). Verbenone, in general, is an aggregation pheromone of *D. valens* but also acts as an anti-aggregation pheromone in high concentrations (Zhang et al. 2006). The concentration of verbenone in *D. valens* mainly depends on the conversion rate of *cis*-verbenol and *trans*-verbenol to verbenone (Xu et al. 2015).

Previous studies investigating pheromone conversion of *D.valens* have shown that oxygen concentration inside the gut affecst the rate of pheromone synthesis (Cao et al. 2018). However, Cao et al. (2018) showed that nine out of ten species of gut facultative anaerobic bacteria were able to convert *cis*-verbenol into verbeone under both anaerobic and micro-oxygen environments, facultative anaerobic gut bacteria *Enterobacter xiangfangensis* having the strongest ability to synthesize this pheromone.

Althought pheromones of *D. valens* have been extensively studied, molecular level confirmation of the function of its gut bacteria in pheromone production is still lacking. To address this knowledge gap, we identified the key bacterial genes involved in *D. valens*' verbenone production under anaerobic conditions. More specificially, we analyzed the ability of the most effective facultative anaerobic gut bacteria *E. xiangfangensis* of *D. valens* to covert *cis*-verbenol to verbenone. We then identified the key genes responsible for this task via transcriptome analysis, knocked them out by homologous recombination to obtain mutant strains and determined the ability of these mutants to convert the monoterpene precursor into the pheromone product. Our findings will shed light on the molecular basis how gut bacteria participate in the synthesis of pheromones, and provide a new scientific basis for the functional study of gut bacteria and the mechanism of pheromone synthesis of *D. valens*.

Materials And Methods

Bacterial strains, plasmids, and growth conditions

Strains used in this study are listed in Table 1. *E. xiangfangensis* were cultured in Luria-bertani medium (LB medium, per liter, 10 g of trypton, 5 g of yeast extract) at 30 ℃. *E. coli* strains were cultured in LB medium at 37 ℃.

Table 1
Bacterial strains and plasmids used in this work

Strain plasmid	Genotype, properties	Source or reference
Strain *E.Xiangfangensis* *E.coli*	Wild type	(Cao *et al.*, 2018)
S17-1 (λpir)	TpR SmR recA, thi-1, pro, hsdR-M+RP4:2-Tc:Mu:Km Tn7 λpir	School of Pharmaceutical Science and Technology Tianjin University
Plasmid		
pUC19	Amp^R	School of Pharmaceutical Science and Technology Tianjin University
pRE112	Suicide vector, Cm^R, SacB,oriT,oriV	School of Pharmaceutical Science and Technology Tianjin University

RNA-Seq library construction and sequencing

We selected the *E. xiangfangensis* strain as the key strain. After the activation, *E. xiangfangensis* were cultured in 5 mL LB medium at 30 ℃, 180 rpm for 24 hours until the light absorption value reached 0.5 at the wavelength of 600 nm. Then, 40 ng/L *cis*-verbenol was added to the final concentration as the treatment group, and DMSO was added to control.

The bacteria were cultured at 30 ℃, 180 rpm for another 16 hours and then were collected in a 1.5 mL tube by centrifugation at 12000 rpm for 3 min. Three replicates were conducted for each group. RNA extraction was performed using the RNeasy Mini Kit (QIAGEN, USA). RNA-Seq library construction and sequencing was completed by Beijing Novo Zhiyuan Sci-Tech Company Limited.

Transcriptome data analysis and synthetic pheromone related gene identification

Reads were mapped to the reference genome. The reference genome was completed by Beijing Novo Zhiyuan Sci-Tech Company Limited. Building of the reference genome index and alignment of clean reads to the reference genome were done using Bowtie2-2.2.3.

Because the pheromones of *D. valens* are synthesized by the oxidation process (Brand et al. 1975; Blomquist et al. 2010), the genes that belonged to oxidation-related gene families were selected for further analysis. Finally, pheromone-related genes were identified according to results from gene annotation. Based on these results, we chose ALDH as a target gene (see Results) for generating mutant strains without a functioning copy of this candidate gene potentially responsible for pheromone conversion.

Construction of the gene-deficient mutants

ALDH gene-deficient mutant strains of *E. xiangfangensis* were constructed using the homologous, double-cross-over method with the suicide vector pRE112 as previously described (Edwards et al. 1998; Yu et al. 2010; Deng et al. 2018).

Briefly, the upstream and downstream genomic sequences of the ALDH coding sequence were separately amplified using primers listed in Table 2. After ligating using restriction enzyme and digesting pRE112 using ClonExpress II One Step Cloning Kit (Vazyme, China) (Liu et al. 2020), the recombinant plasmid was transferred to the *E.coli* S17-1 (λpir) component cells. The positive clones were confirmed by PCR using primers in Table 2 and sequenced.

Table 2
Primers used in this work

Primer name	Primer sequence
Ex-ALDH1-up-F	CGACGGCCAGTGCCAAGCTTCGGTCATACCGAGCATCT
Ex-ALDH1-up-R	CGGCAAGAAAGAGGTGCGTTCTTCTCTCCAGATGTTTCGT
Ex-ALDH1-down-F	ACGAAACATCTGGAGAGAAGAACGCACCTCTTTCTTGCCG
Ex-ALDH1-down-R	ATGACCATGATTACGAATTCAGTAGTTACCGTCGCCCA
Ex-ALDH1-T1	GATCAGCGTCTTGCGGTA
Ex-ALDH1-T2	TTCCCATCGCAGACCTCA
Ex-ALDH1-T3	TTCTGCTCGAGATCCACCA

The positive *E.coli* S17-1 (λpir) colony was cultured in 5 mL LB medium at 37 ℃, 180 rpm for 16 hours, meanwhile the wild type *E. xiangfangensis* were cultured in 5 mL LB medium at 30 ℃, 180 rpm. Then, we mixed 1mL of each of the above two strains with 3 mL LB medium and cultured at 30℃, 180 rpm for 24 hours, after which the bacteria were collected and spread in the LB plate (50mg/L chloramphenicol, 50mg/L Ampicillin). After culturing overnight, the putative single-cross-mutant clones were confirmed by PCR, and cultured in LB medium for second-round homologous cross-over. The ALDH mutants were obtained by spreading the media in the LB agar plate containing 10% sucrose and confirmed by PCR with two pairs of primers.

Conversion experiments

Wild type *E. xiangfangensis* and its ALDH gene-deficient mutants were cultured in LB medium and incubated for 24 h. A dilution of 1:100 of each isolate was made when cultures were adjusted to an optical density (OD_{600}).0.5. concentration (40 ng/μl and 200 ng/μl) of *cis*-verbenol was then added into 4 ml bacterial suspensions and shaken for a further 36 h. Both the wild (control) and mutant *E.*

xiangfangensis bacteria suspensions contained an equivalent amount of *cis*-verbenol. All solutions were extracted with hexane and then stored for later chemical analysis to determine verbenone concentration. The conversion experiments followed previously described methods (Cao et al. 2018), with slight modification of incubation and shaking times.

Statistical analysis

R software (version 3.0.3) was used to for pearson's correlation analysis, and significant correlations were declared at $r > 0.8$ or < -0.8. Conversion experiment results were analyzed using Dunnett's T^3 test, and significances were determined at $P<0.05$.

Results

Transcriptome data analysis

Statistics of sequencing data of *E. xiangfangensis* shown in Table 3. These sequence data have been submitted to the GenBank databases under accession number PRJNA798447. The genome of the *E. xiangfangensis* was used as a reference genome (reference genome completed by Beijing Novo Zhiyuan Sci-Tech Company Limited) shown in Table 4.

Table 3
Statistics of sequencing data of *Enterobacter xiangfangensis*

Sample name	Raw reads	Clean reads	clean bases	Error rate	Q20	Q30	GC content
ExCK1	8847502	8756726	1.32G	0.02	98.55	95.27	54.5
ExCK2	7906310	7843966	1.18G	0.02	98.58	95.32	54.17
ExCK3	7901156	7803404	1.18G	0.02	98.65	95.56	54.68
ExV1	7755232	7685084	1.16G	0.02	98.75	95.71	53.86
ExV2	7709364	7629624	1.15G	0.02	98.64	95.55	54.61
ExV3	7635610	7575258	1.14G	0.02	98.63	95.43	54.23
ExCK= The control group (DMSO was added); ExV= The treatment group (*cis*-verbenol was added)							

Table 4
Transcriptome sequencing and comparison with reference genomes

Sample name	ExCK1	ExCK2	ExCK3	ExV1	ExV2	ExV3
Total reads	8756726	7843966	7803404	7685084	7629624	7575258
Total mapped	8735517 (99.76%)	7827046 (99.78%)	7784461 (99.76%)	7670468 (99.81%)	7611704 (99.77%)	7559157 (99.79%)
Multiple mapped	53548 (0.61%)	42025 (0.54%)	48758 (0.62%)	37661 (0.49%)	44344 (0.58%)	39930 (0.53%)
Uniquely mapped	8681969 (99.15%)	7785021 (99.25%)	7735703 (99.13%)	7632807 (99.32%)	7567360 (99.18%)	7519227 (99.26%)
ExCK= The control group (DMSO was added);ExV= The treatment group (*cis*-verbenol was added)						

Overall distribution of expressed genes showed that 262 genes were differentially expressed in the control group (DMSO was added) compared to treatment (*cis*-verbenol was added), whereby 199 DEGs were upregulated and 63 DEGs were downregulated (Fig.1). The expression of control and treatment also displayed a clear separation based on PCA analysis, and the samples in the group were clustered together (Fig.2). These results show that *D. valens* gut strains have a complex oxidative defense mechanism against monoterpenes.

Pheromone related gene identification

Gene expression patterns and phylogenetic analysis from transcriptomic data of gut bacteria revealed that aldehyde dehydrogenase (ALDH, Gene ID: GM002204) genes were predominantly expressed in oxidoreductase activity (Fig. 3), and clustered with genes involved in pheromone synthesis and detoxification in other species. Furthermore, the transcription abundance of ALDH and aldehyde-alcohol dehydrogenase (ADH) was significantly upregulated when verbenol was added, and the expression level of ALDH was significantly higher than that of ADH. We therefore considered ALDH as the main candidate gene for *D. valens* pheromone synthesis.

ALDH knock-out

Following these results from candidate gene identification, we subsequently generated ALDH mutants of *E. xiangfangensis* by homologous recombination (Edwards et al. 1998; Yu et al. 2010; Deng et al. 2018). ALDH mutant *E. xiangfangensis* showed a shorter PCR product than wild type when using primer T1/T2 or T1/T3 (Fig. 4).

Conversion experiments

Comparison of *cis*-verbenol conversion results between the mutant and the wild-type strains of *E. xiangfangensis* at 40 ng/μl and 200 ng/μl concentrations showed that the wild-type strains (control group) had the ability to convert *cis*-verbenol, whereas this ability was significantly decreased in the ALDH-deficient mutant strain (Fig. 5).

Discussion

In our previous work, our group has demonstrated that the gut bacteria of *D. valens* have the ability to convert monoterpenes to pheromone products. For example, under aerobic environment, intestinal bacteria isolated from *D.valens* converted *cis*-verbenol to verbenone (Xu et al. 2015), and nine out of ten intestinal facultative anaerobic bacteria were able perform this function under different oxygen environments, but the conversion rate increased with increasing oxygen concentration (Cao et al. 2018). In order to investigate the mechanisms of phernomone conversion ability at molecular level, we selected a bacterial strain with the strongest conversion ability (*E. xiangfangensis*, a facultative anarobe) to analyze its transcriptome with or without *cis*-verbenol, screened the target genes responsible for the pheromone conversion and investigated the converision ability of the bacteria in the absence of function of these genes.

To our knowledge, our current study is the first one to provide molecular evidence to verify the function of intestinal bacterial in the conversion of monoterpenes to pheromone products under anaerobic conditions. When identifying candidate genes responsible for *cis*- verbenol conversion, our transcriptome analysis showed that the highest expression level was detected in ALDH gene, which is a functional gene commonly found in intestinal bacteria of *D. valens* as well as other insects (Maeno et al. 2016; Martino et al. 2017). This gene is verified to be involved in detoxification of plant secondary chemicals, and can therefore help the insect to adapt to the chemical defense substances released by the host tree (Zhang et al. 2020). Following a knock-down of the target gene using homologous recombination strains, we observed a strong reduction in pheromone synthesis ability, strongly suggesting the primary role ALDH in pheromone synthesis of *E. xiangfangensis*. Several pine monoterpenes, such as α-Pinene and *cis*-verbenol are toxic to *D. valens*, but are rendered to non-toxic when converted into aggregation pheromone verbenone by its intestinal bacteria (Xu et al. 2015). Therefore, conversion of plant monoterpernes to pheromones is likely a dual process resulting in both detoxification and pheromone synthesis. ALDH is a key gene in the metabolic pathway (involved also in Limonene degradation) of monoterpene α-Pinene through redox reaction. Since there are no relevant metabolic pathway for the conversion of *cis*-verbenol to verbenone in *D. valens's* own metabolic machinery, our results indicate that ALDH may be the key gene of intestinal facultative anaerobic bacteria of *D. valens*. involved in the pheromone transformation and potentially detoxification.

This result is in contrast with other studies where gut bacteria does not enhance insect's pheromone synthesis (Hunt et al. 1989; Bell et al. 2003). These inconsistencies might result from different experimental settings, such as use of diverse bacterial communities and nutritional media, which in turn influence both microbial communities and host physiology. Another potential reason for differences

among current and previous studies investigating pheromone converision of insects is that the cultivation and validation of intestinal facultative anaerobes in this study was carried out in an anaerobic environment, whereas some earlier studies have used atmospheric oxygen concentration (Keeling et al. 2016; Nadeau et al. 2017). Therefore, our simulated intestinal microenvironment may be more accurately reflect the oxygen conditions in insect integtines.

Previous studies on the function of intestinal bacteria mainly focused on their ecological roles (Cao et al. 2018), whereas our current study provides a new methodology for molecular verification of the functions of insects' intestinal microbes. Future work should consider external factors that influence intestinal facultative anaerobic bacteria, such as insect gut hydrogen ion concentration (pH). To better understand the complex symbiotic relationships among bark beetles and their micro-organisms, future studies should also address the functions of these bacteria to *D. valens* development, detoxification, and chemical communication.

Declarations

Acknowledgements

This work was supported by the National Natural Science Foundation of China (32088102 and 32061123002), Natinal Key Reserach and Development Program of China (2021YFC2600100) and the Program of the Natural Science Foundation of Hebei Province of China (C2019204244).

Author Contributions

Sun conceived and designed research; Cao conducted experiments; Cao and Li analyzed data; Cao and Koski wrote this manuscript. All authors read, corrected and approved the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Figures

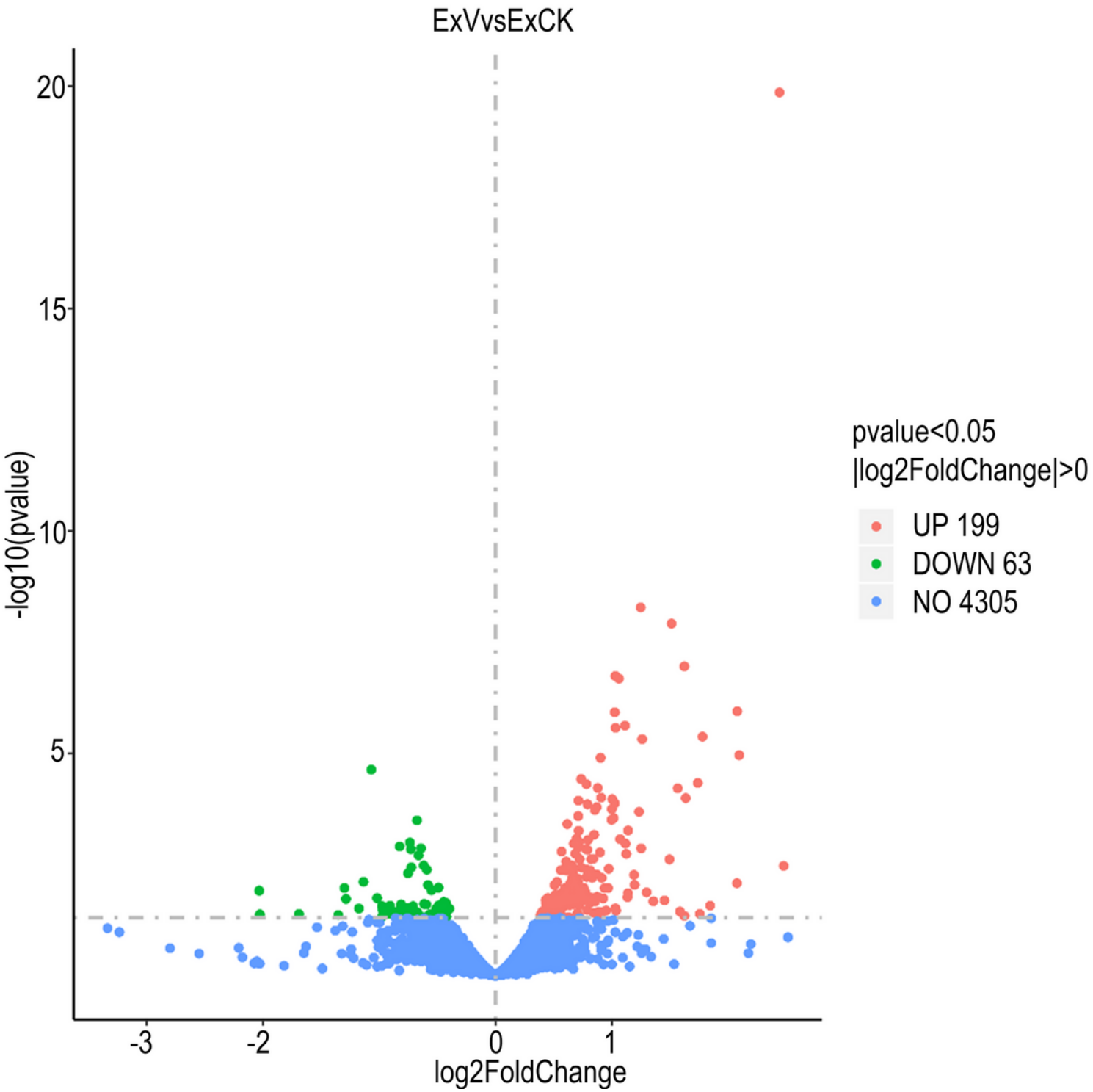

Figure 1

Overall distribution of differently expressed genes of *Enterobacter xiangfangensis*, the primary bacteria invoveld in verbanone conversion of *D. valens* in the presense and absence of the pheromone precursor *cis*-verbenol. Significantly differentially expressed genes marked with red (elevated) and green (degreased) dots. Non-significant differences are indicated by blue dots. ExCK vs ExV= The difference genes of control group (DMSO was added) and treatment group (*cis*-verbenol was added) were compared.

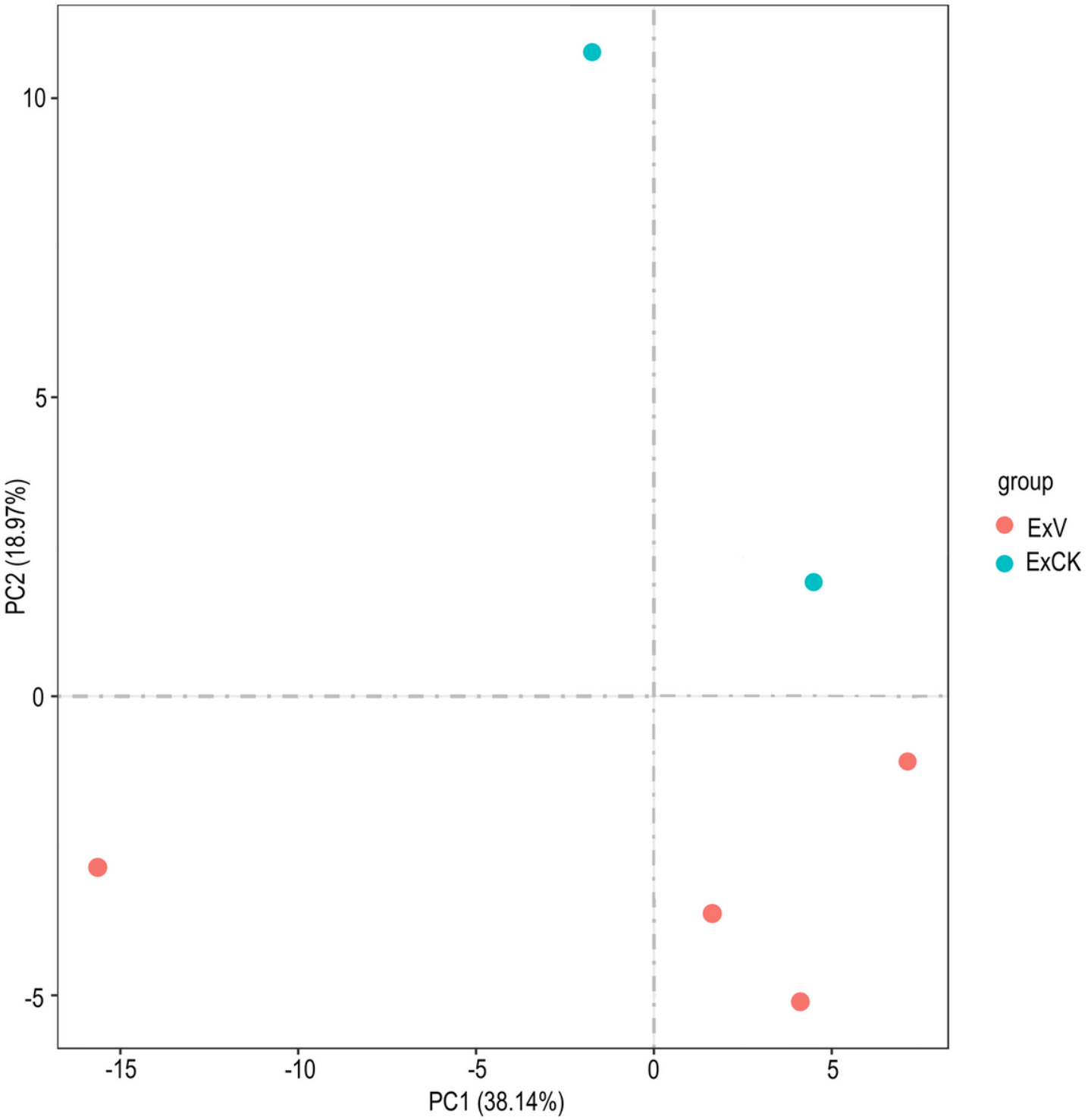

Figure 2

PCA plot of expressed genes of *Enterobacter xiangfangensis,* the primary bacteria invoveld in verbanone conversion of *D. valens*, in the presense and absence of the pheromone precursor *cis*-verbenol. PCA adopts the calculation method of linear algebra. ExCK= The control group (DMSO was added)，ExV= The treatment group (*cis*-verbenol was added).

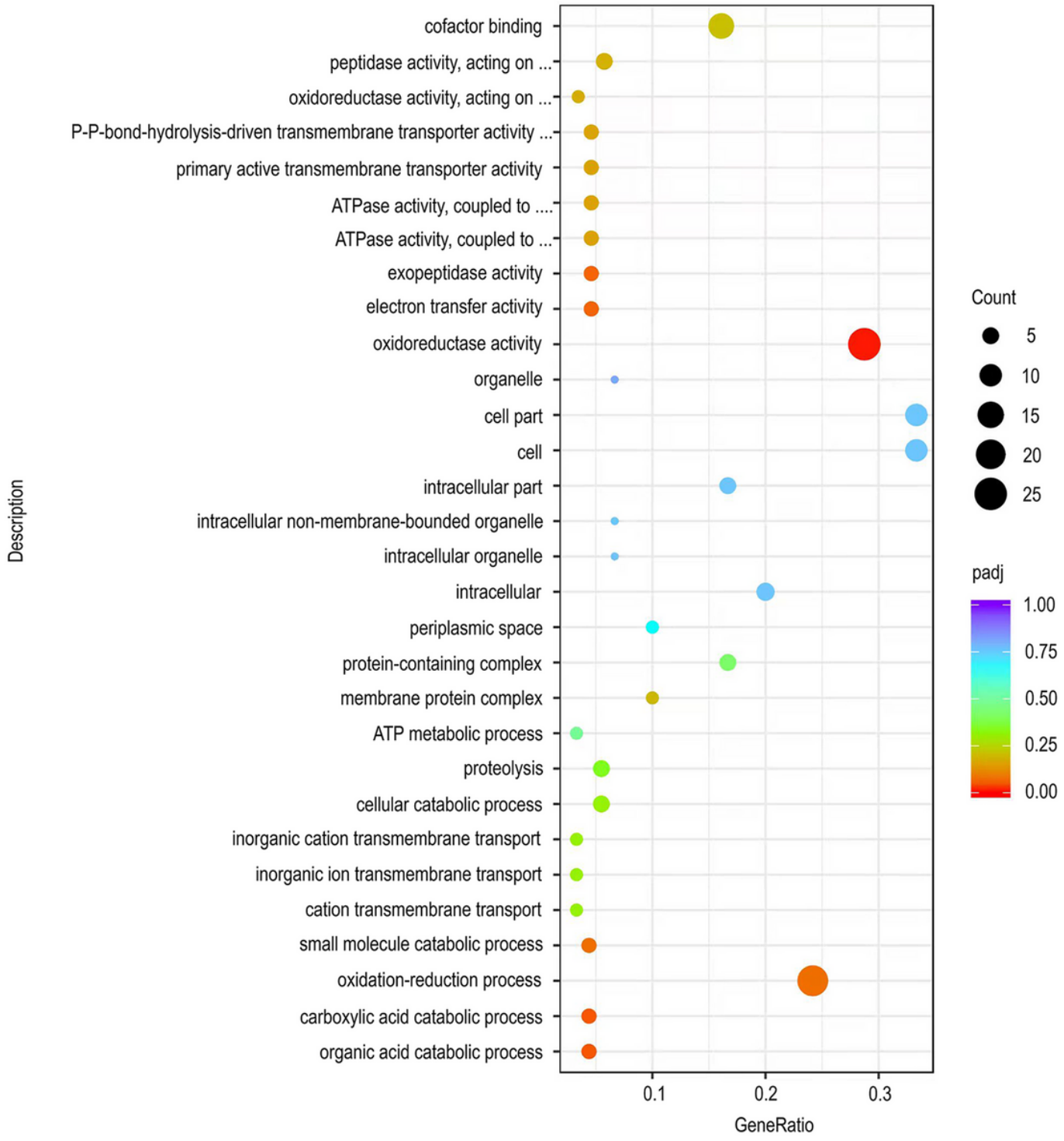

Figure 3

Cluster and phylogenetic analysis of gene expression patterns of *Enterobacter xiangfangensis*. Red indicates high gene expression, blue indicates low gene expression. Hence, colour change from red to blue indicate log10 (FPKM+1) change from large to small. The log10 (FPKM+1) values were normalized and clustered.

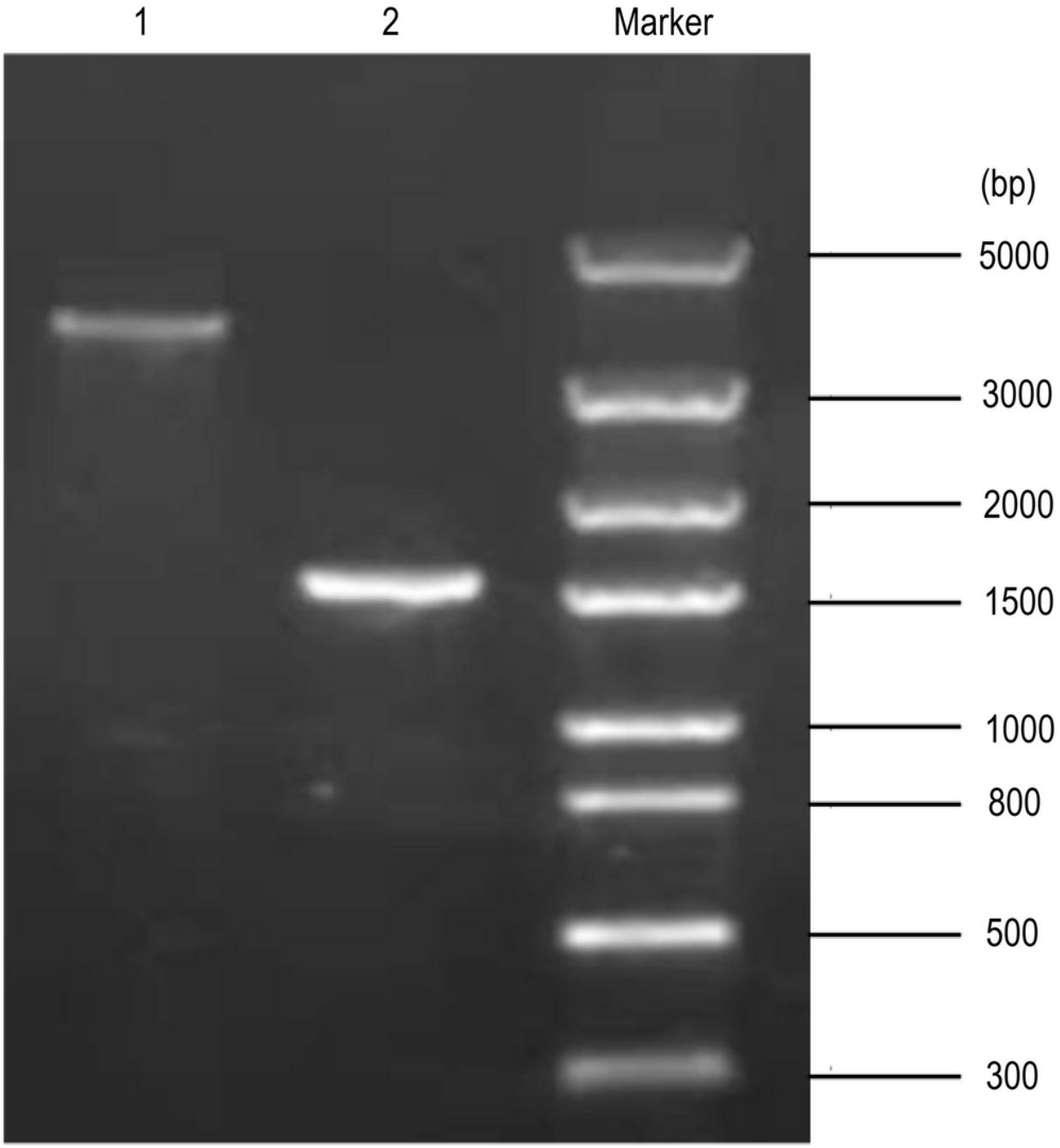

Figure 4

Construction of the ALDH gene-deficient *Enterobacter xiangfangensis* mutants. PCR identification of mutants with T1/T2 or T1/T3; Marker. DL5000 DNA Ladder; 1, wild-type**;** 2, mutant.

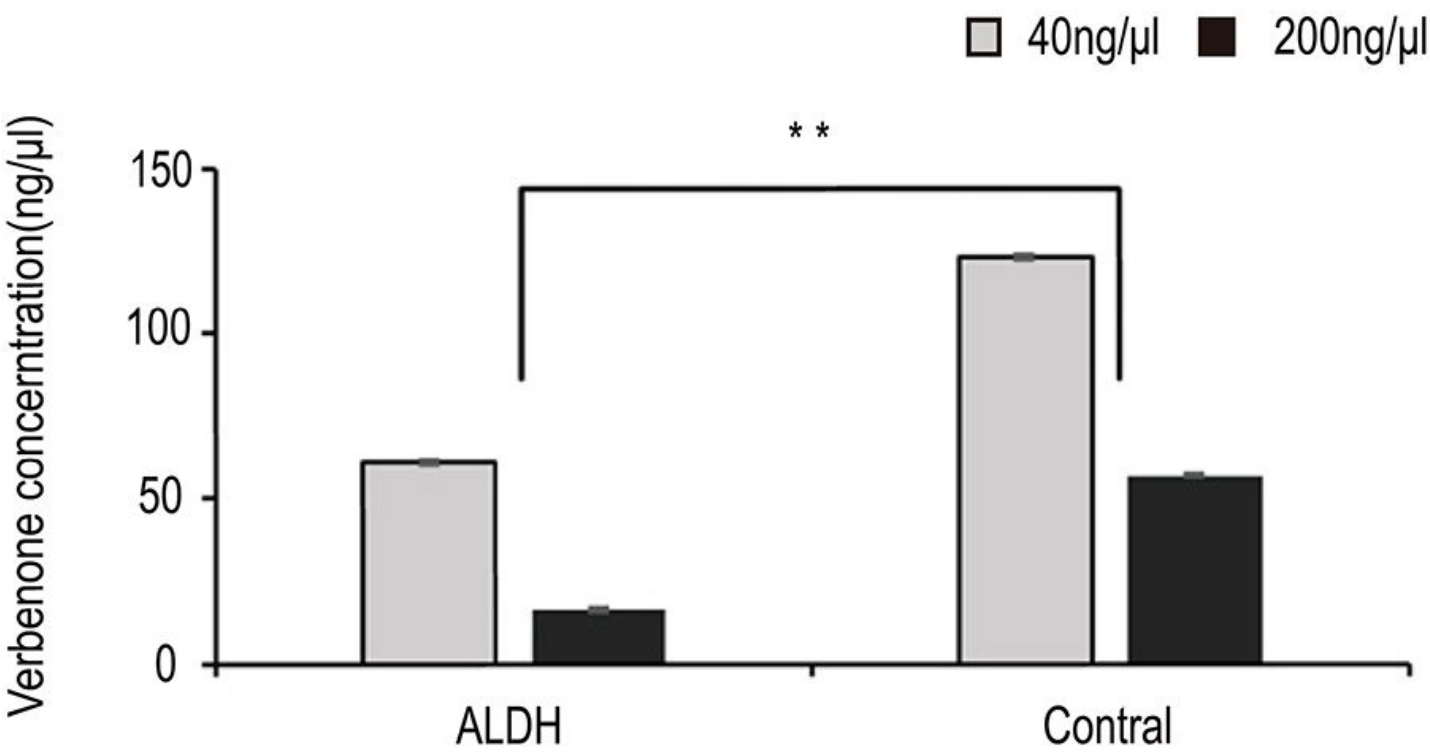

Figure 5

Verbenone production by ALDH gene mutant *E. xiangfangensis* under anaerobic environment. The amounts of verbenone produced by facultative anaerobic ALDH mutant (ALDH) and wild-type strains (control) at 40 ng/μl (light grey bars) and 200 ng/μl (black bars) *cis*-verbenol concentrations. Statistical analysis was performed using Dunnett's T^3 test. ** are significantly different at P=0.05.