

# Frequency-dependent response of *Chromobacterium violaceum* to sonic stimulation and altered gene expression associated with enhanced violacein production at 300 Hz

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**In this study, *Chromobacterium violaceum* was subjected to sonic (100–2000 Hz) stimulation. Sound waves of 300 Hz frequency promoted bulk production of the quorum-regulated pigment, violacein. Whole transcriptome analysis indicated that a total of 342 genes (i.e. 4.63% of the whole genome) were significantly upregulated in the sonic stimulated culture. Enhanced violacein production in the sound-stimulated culture seems to have stemmed from enhanced expression of the genes involved in pentose phosphate pathway, resulting in an increased availability of erythrose-4-phosphate to be used in the synthesis of tryptophan – the precursor of violacein synthesis. This study is a good demonstration of the ability of sound waves to alter bacterial metabolism.**

**Keywords:** Altered gene expression, *Chromobacterium violaceum*, sonic stimulation, violacein.

SOUND is an omnipresent environmental factor. Higher organisms, particularly animals, have well-developed auditory organs (ears) and hence are capable of sensing and responding to any sonic stimulation within their reception range<sup>1</sup>. It is interesting to study whether lower forms of life, e.g. microorganisms, which are not widely regarded to possess an equivalent system for reception and response to sound, are affected by it. Ultrasound has long been known to have damaging effects on microbial cells, and it is also used for achieving bacterial cell lysis in laboratory experiments<sup>2</sup>. What needs to be investigated is the interaction of sound of the audible/sonic range (20–20,000 Hz) with prokaryotic cells. Though the interaction of microbial cells with the audible range of sound has not been studied much, there are quite a few reports descri-

bing microbial growth and metabolism being affected by sound stimulation<sup>3–7</sup>. Whether microbes respond to sonic stimulation in a frequency-dependent fashion, is an interesting problem to examine. It is also possible that different microorganisms may respond differently to sound of the same frequency. Microbes have been reported to be capable of producing sound and responding to it after sensing the same<sup>8</sup>. Sound has also been proposed as a possible signal for communication among microbes<sup>9,10</sup>.

Only limited knowledge is available regarding the influence of sonic waves on single cells and cellular metabolism. How can the microorganisms possibly perceive sound? There may be a possible role for their mechanosensory channels here. Audible sounds passing through the microbial growth medium in the form of oscillating pressure waves can stimulate mechanosensory cells<sup>3,11</sup>.

While sufficient evidences for sonic-signal based intercellular communication among bacterial populations are yet to be reported, chemical-signal based intercellular communication known as quorum sensing (QS) is a well-accepted and well-understood phenomenon in the microbial world. QS is used to describe the phenomenon whereby microbial cells communicate among themselves for regulation of their behaviour as a population. This process is believed to be largely mediated through chemical signals like acyl homoserine lactone (AHL), or small peptide signals. Many of the traits, including pigment production among bacteria have been shown to be regulated by QS<sup>12</sup>. *Chromobacterium violaceum* is one of the most widely used model bacterium for the study of QS in Gram-negative bacteria. Production of the violet pigment violacein in this bacterium is regulated by its QS machinery<sup>13</sup>. Violacein is a bioactive molecule with potential commercial and therapeutic importance. Various research groups are seeking to improve the fermentative yields of violacein through genetic/metabolic engineering and synthetic biology<sup>14,15</sup>.

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In this study, we report results regarding the influence of audible sound (100–2000 Hz) on growth and QS-regulated pigment production of *C. violaceum*, wherein we have characterized the differential gene expression in sonic-stimulated *C. violaceum* culture overproducing violacein.

## Materials and methods

### Bacterial culture

*C. violaceum* (MTCC 2656) was procured from the microbial type culture collection (MTCC), Institute of Microbial Technology Chandigarh. The organism was grown in nutrient broth (HiMedia, Mumbai) supplemented with 1% v/v glycerol (CDH<sup>®</sup>, New Delhi), and incubated at 35°C.

### Sound generation

Sound beep(s) of the required frequency was generated using NCH<sup>®</sup> tone generator. The sound file played during the experiment was prepared using Wave Pad Sound Editor Masters Edition v.5.5, in such a way that there is a time gap of one second between two consecutive beep sounds.

### Sound stimulation

Inoculum of the test bacterium was prepared from its activated culture, in sterile normal saline. Optical density (OD) of the inoculum was adjusted to 0.08–0.10 at 625 nm (Agilent Technologies Cary 60 UV-Vis, Bengaluru) to make it equivalent to the McFarland 0.5 turbidity standard<sup>16</sup>. The test tubes (Borosil, 18 × 150 mm; 27 ml) containing 6 ml of growth medium (including 5% v/v inoculum) were placed in a glass chamber (Actira, L : 250 × W : 150 × H : 250 mm). A speaker (Lenovo) was put in this glass chamber at the distance of 15 cm from the inoculated test tubes. Sound delivery from the

speaker was provided throughout the period of incubation (48 h). This glass chamber was packed with a glass lid and one layer of loose-fill shock absorber polystyrene, in such a way that the polystyrene layer gets placed below the glass lid. Silicone grease was applied on the periphery of the glass chamber coming in contact with the polystyrene material. This type of packaging was done to minimize any possible leakage of sound from inside the chamber, and also to avoid any interference from external sound. Similar chamber was used to house the ‘control’ (i.e. not subjected to sound stimulation) group test tubes. One speaker was also placed in the glass chamber used for the control tubes at a distance of 15 cm, where no electricity was supplied and no sound was generated<sup>17</sup>. Table 1 lists the intensity of sound, measured with a sound-level meter (ACD Machine Control Ltd, Mumbai) at a distance of 15 cm from the speaker at each of the test frequencies. Sound level in the control chamber was found to be below detection level (<40 dB) of the sound-level meter. Figure 1 shows a schematic of the whole experimental set-up.

Intermittent mixing of the contents of the test tubes to minimize heterogeneity was achieved by vortexing the tubes at an interval of every 3 h using a cyclomixer. Whenever the tubes were taken out of the cyclomixer, the positions of the tubes of a single chamber were interchanged, and their direction with respect to the speaker was changed by 180° rotation. This was done to ensure almost equal sound exposure to all the tubes.

### Growth and pigment estimation

At the end of incubation, after quantifying the cell density at 764 nm (this wavelength was chosen to avoid interference from the pigment)<sup>18</sup>, the culture tubes were subjected to extraction of the pigment violacein<sup>19</sup>. Briefly, 1 ml of the culture broth was centrifuged (REMI CPR-24 Plus, Ahmedabad) at 12,000 rpm (13,520 g) for 15 min at 25°C and the resulting supernatant was discarded. The remaining cell pellet was resuspended in 1 ml of dimethyl sulphoxide (Merck, Mumbai), and incubated at room temperature for 30 min, followed by centrifugation at 12,000 rpm for 15 min. The violacein extracted in the supernatant was estimated by measuring OD at 585 nm. Violacein unit (VU) was calculated as  $OD_{585}/OD_{764}$ , to nullify the effect of change in cell density on pigment production.

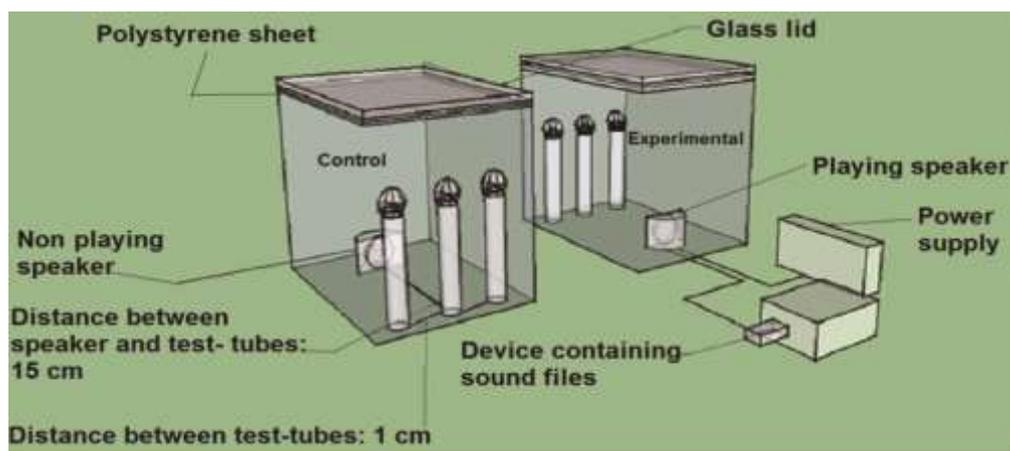
### Gene expression analysis

Before RNA isolation, taxonomic identity and purity of culture were confirmed by 16S rRNA sequencing. Cell pellets obtained from centrifugation of 1 ml of culture broth at the end of incubation of 48 h were used for RNA isolation.

**Table 1.** Level of sound intensity at different frequencies

Frequency of the test sound (Hz)	Intensity as measured by the sound-level meter (dB) <sup>#</sup>
100	80
200	85
300	90
400	86
500	84
600	75
700	82
1000	86
2000	99

<sup>#</sup>Speakers were set at their full volume and the USB player (Expeed Magic Box) was set at a constant level of 36 (volume indicating unit) for all frequencies.



**Figure 1.** Schematic of the experimental set-up.

### *Total RNA isolation, and its qualitative and quantitative analysis*

Total RNA was isolated from the samples using Trizol (Invitrogen) method for RNA extraction. The quality of total RNA was assessed on 1% denatured agarose gel and quantified using Qubit Fluorometer. The samples were treated with MICROB Express kit to deplete the bacterial ribosomal RNA and enrich the mRNA population to be used for whole transcriptome analysis (WTA) library preparation. The enriched mRNA was fragmented and the libraries were prepared using Illumina TruSeq RNA Sample Preparation V2 kit. The means of the library fragment size distributions were 725 bp and 734 bp for ‘control’ and ‘experimental’ samples respectively. The libraries were sequenced using  $2 \times 150$  PE chemistry generating  $\sim 1.5$ – $2.0$  GB of data per sample.

### *Illumina $2 \times 150$ PE library preparation, cluster generation and sequencing*

The samples were initially treated with MICROB Express kit (Ambion/Life Tech, Ahmedabad) to deplete the ribosomal RNA and enrich the bacterial mRNA population. Enriched bacterial mRNA was then subjected to reverse transcription into first-strand cDNA using SuperScript II reverse transcriptase (Invitrogen) and random hexamer primer according to Illumina TruSeq RNA Sample Preparation V2 kit protocol. The single-stranded cDNA was then converted to ds cDNA and the ds cDNA samples were subjected to Covaris shearing followed by end-repair of overhangs resulting from shearing. The end-repaired fragments were A-tailed, adapter-ligated and then enriched by limited number of PCR cycles. Library quantification was achieved using DNA high-sensitivity assay kit (Thermo Fisher Scientific, Ahmedabad). The next-generation sequencing for control and experimental samples was performed on the Illumina platform and

approximately  $\sim 5$ – $6$  GB of data has been generated per sample. The high-quality paired-end reads of both the samples were assembled together using SOAPdenovo-Trans (version 1.03). The final assembly contained 779 transcripts with an N50 scaffold length of 22,087 bp; the largest transcript assembled measured 119,734 bp. A total of 7403 coding regions were identified using TransDecoder (version 2.0.1) program. The amplified libraries were analysed in Bioanalyzer 2100 (Agilent Technologies) using high sensitivity (HS) DNA chip, in accordance with the manufacturer’s instructions.

Once the Qubit concentration for the libraries and average peak size were obtained from the Bioanalyser profile, the library was loaded into Illumina platform for cluster generation and sequencing. Paired-end sequencing allowed the template fragments to be sequenced in forward as well as reverse directions. The library molecules bind to complementary adapter oligos on paired-end flow cell. The adapters were designed to allow selective cleavage of the forward strands after resynthesis of the reverse strand during sequencing. The copied reverse strand was utilized to sequence from the opposite end of the fragment.

All the raw sequence data have been deposited at Sequence Read Archive; the relevant accession number is SAMN06606485.

### *Functional annotation of predicted protein coding genes*

Each of the predicted protein-coding genes was annotated evaluating the homology by BLASTX search against NR (non-redundant) database. To locate the biological pathways that are active in both (control and experimental) samples, the predicted open reading frames (ORFs) were mapped to reference pathways in Kyoto Encyclopedia of Genes and Genomes (KEGG) using KEGG automatic annotation server (KAAS). The DESeq package was used to identify significantly differentially expressed genes between control and experimental samples.

### Statistical analysis

All experiments were performed in triplicate, and measured values reported as mean  $\pm$  standard deviation (SD). Statistical significance of the data was assessed by applying *t*-test using Microsoft Excel®. Data with *P*-value  $\leq 0.05$  were considered to be statistically significant.

## Results and discussion

### Effect of different sonic frequencies on *C. violaceum*

Figure 2 summarizes the response of *C. violaceum* to sound stimulation at different frequencies in terms of growth and pigment production (see [Supplementary Appendix A](#)). Out of nine test frequencies, eight caused significant change in bacterial growth. In all these eight cases, the effect on growth was stimulatory. Significant change in VU was observed at seven of the test frequencies. This can be considered as an indication of true QS-modulatory effect of sound treatment, largely independent of the alteration in growth. In five cases (i.e. 300, 400, 600, 700 and 2000 Hz), the magnitude of alteration in QS-regulated violacein production owing to sound stimulation was much higher than that in cell density. This is to say that the QS-regulated trait (i.e. pigment production) was affected more than the bacterial growth. Among the five QS-modulatory sound frequencies, maximum (51.28%) positive effect on QS-regulated pigment production with least (4.54%) effect on growth was observed at 300 Hz. Thus, we subjected the cells exposed to 300 Hz sound to transcriptome profiling, along with the 'control' cells which received no sound stimulation. The QS-regulated trait, i.e. violacein production was maximally affected at 300 and 2000 Hz. However, the effect of 2000 Hz sound on VU being negative, the sample treated with 300 Hz sound was selected for WTA.

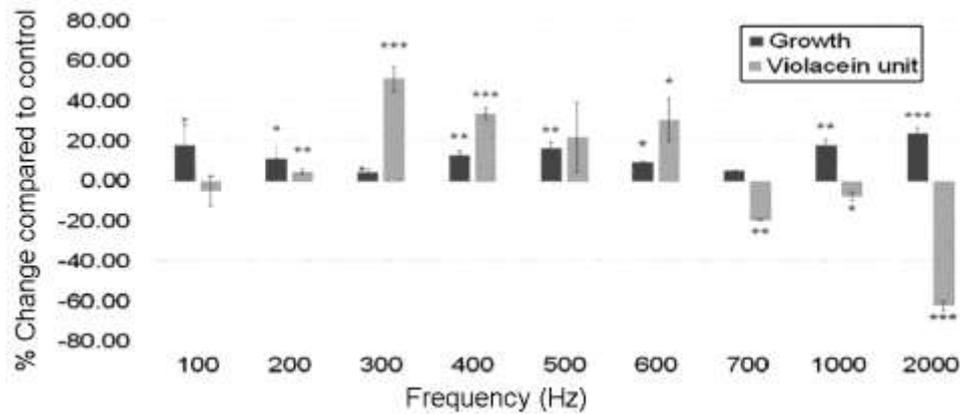
### Differential gene expression in *C. violaceum* exposed to 300 Hz sound

Whole transcriptional profiling of *C. violaceum* culture resulted in the identification of totally 7403 coding regions. A total of 425 genes were significantly ( $P < 0.05$ ) differentially expressed, of which 342 were found to be upregulated and 83 were found to be downregulated. However, applying the dual criteria for screening, i.e.  $P < 0.05$ , and a cut-off value of 1.5 for fold change, none of the downregulated genes (see [Supplementary Appendix B](#)) was found to be significant. All the upregulated genes passed both the significance criteria. [Supplementary Appendix C](#) provides a list of all the differentially upregulated genes including the 143 hypothetical proteins whose functions are not known. Figure 3 presents function-wise categorization of the significantly upregulated genes.

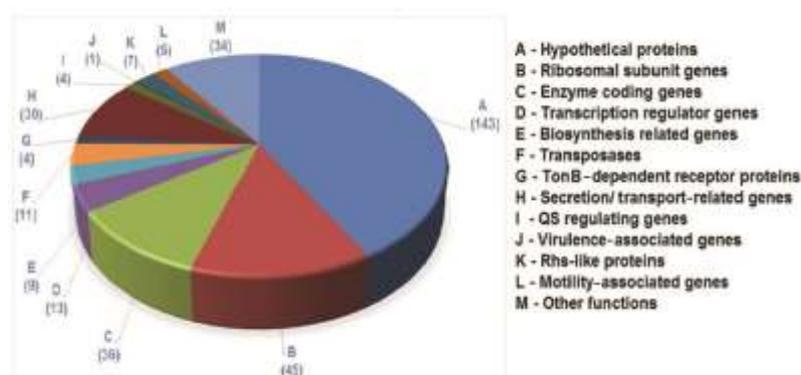
In this study, selection of the test frequency was based on observations during our previous studies<sup>5,7</sup>, where we found that sound (corresponding roughly to a frequency range 50–750 Hz) applied in the form of music could notably alter the growth and production of certain metabolites by the test microorganisms. Frequencies of 1000 and 2000 Hz were selected based on results of other studies. For example, Gu *et al.*<sup>6</sup> reported that 2000 and 8000 Hz sound significantly enhanced biomass and growth rate of *Escherichia coli*. Also, 2000 Hz is believed to be one of the major frequency components in the natural world<sup>20</sup>, and this frequency was also found in the present study to be capable of altering pigment production in the test bacterium. However, response of the test bacterium to sonic stimulation, with respect to growth and pigment production, did not fit any particular pattern with increasing frequency. Gu *et al.*<sup>21</sup> reported the response of bacteria to sonic stimulation to be nonlinear and showing frequency and intensity peculiarities.

Analysis of the gene expression data of *C. violaceum* culture exposed to 300 Hz sound revealed that one of the top 20 upregulated genes was a member of the LuxR family of transcriptional regulators. In total, two such LuxR family transcriptional regulators were found to be upregulated. Violacein synthesis in the sound-stimulated culture was found to increase by 1.52-fold. As violacein synthesis in this organism is under the control of QS, we expected differential expression of the genes which are part of the QS circuit of *C. violaceum*. Though upregulation was observed for the LuxR analogue (i.e. CviR), it was not observed for AHL synthase. This indicates that there may not be any enhanced signal production in sonic-stimulated culture. Intriguingly, a hypothetical gene CV\_2441 probably coding for diene lactone hydrolase was upregulated, which is an AHL-degrading enzyme<sup>22</sup> whose effect is likely to be quorum quenching. The gene CV\_3413 coding for an acyl carrier protein was found to be 2.59-fold upregulated. Acyl carrier protein has been considered as the key component of bacterial fatty acid synthesis. A notable feature of the fatty acid synthesis pathway is that all of its intermediates are covalently bound, acyl carrier protein (ACP), which a small, acidic and extremely soluble protein. However, none of the genes involved in fatty acid synthesis was found to be upregulated. A good amount of ACP flux was diverted in the sound-stimulated culture towards polyketide synthesis, as polyketide synthase (CV\_4293) was also upregulated. ACP is the central cofactor protein for fatty acid synthesis, which supplies acyl chains for lipid A and lipolic acid synthesis, as well as QS, toxin and bioluminescence activation. Furthermore, ACPs or PCPs (peptidyl carrier proteins) are also utilized in polyketide and non-ribosomal peptide synthesis, which produce important secondary metabolites<sup>23</sup>.

External sonic stimulation can be considered a sort of stress for the test bacterium. Sound waves travel through



**Figure 2.** Effect of various sonic frequencies on growth and violacein production of *Chromobacterium violaceum*. Bacterial growth was measured as OD<sub>764</sub>; optical density of violacein was measured at 585 nm and violacein unit was calculated as the ratio OD<sub>585</sub>/OD<sub>764</sub> (an indication of violacein production per unit of growth). \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001. Control, Culture receiving no sonic stimulation; Experimental, Culture receiving sonic stimulation.



**Figure 3.** Function-wise categorization of the significantly upregulated genes.

the liquid medium containing bacteria as mechanical vibrations. These vibrations are likely to increase the oscillations among the microbial population by promoting probabilistic collision between the intracellular molecules, thereby increasing the magnitude of the systemic noise. Inside the cells, intracellular processes inevitably involve collisions between molecules such as DNA mRNA and proteins<sup>24</sup>, and sonic treatment is likely to enhance the impact of such collisions. In this situation, the stress response machinery of the organism may be expected to be upregulated. One such stress adaptation protein disulphide oxidase CV\_3998 (coded by dsbA) was found to be upregulated in this study by 1.91-fold. An alteration in the activity of Dsb proteins can cause accumulation of unfolded proteins, which may trigger activation of extracytoplasmic stress factor  $\sigma E$ <sup>25</sup>. In our sound-stimulated culture the gene rpoE coding for this RNA polymerase sigma-E factor was 2.5-fold upregulated. A variety of stresses have been mentioned in the literature to induce the RpoE response in Gram-negative bacteria, which may culminate in accumulation of misfolded envelope proteins<sup>26</sup>. Audible sound was speculated

to trigger secondary oxidative stress in *E. coli*<sup>21</sup>. Analysis of stressed *C. violaceum* proteome by Castro *et al.*<sup>27</sup> indicated that stress can trigger changes in the expression of *C. violaceum* receptors, transporters and proteins associated with the biosynthetic pathways, energy production and molecule recycling. This observation matches well with the differential expression data presented in this study. They reported differential expression of the genes coding for Rhs, ATP synthase  $\beta$ -subunit protein, and ribosomal protein subunits L1, L3, L5 and L6 in stressed *C. violaceum* culture. In our sonic-stimulated culture too, ATP synthase subunit delta (atpH) was upregulated, and so were the genes coding for ribosomal protein subunits L3, L5 and L6 and Rhs. The L3 subunit may be more useful to the bacterium under stressful conditions, which was upregulated in our experimental culture by 2.6-fold. Similarly, a total of seven Rhs-like proteins were upregulated in our experimental culture. Among other genes whose differential upregulation can be taken as an indication of stress, one (CV\_1222) is that probably coding for an aldehyde dehydrogenase, which was upregulated by 3.56-fold. Upregulation of aldehyde dehydrogenases has

been considered as a stress response in bacteria put under chemical and environmental stress<sup>28</sup>. Oxidative stress built up from the reactive oxygen species (ROS) may trigger lipid peroxidation resulting in the formation of a variety of aldehydes, whose toxicity can be mitigated by the aldehyde dehydrogenases.

The ability to cope with such environmental stress is believed to stem from a plethora of specific transporters present in this bacterium<sup>29</sup>. We found a good number (nearly 30) of proteins involved in transport and/or secretion to be significantly upregulated. Many of them may assist in the rapid uptake of nutrients by the sonic-stressed cells followed by their faster utilization. For example, a three-fold upregulated glucose 6-phosphate dehydrogenase (G6PD; CV\_0145), and 3.16-fold upregulation of nucleotide sugar epimerase can be taken as an indication of enhanced glucose metabolism through the pentose phosphate pathway. Interestingly, these enzyme activities are essential for later formation of erythrose-4-phosphate (E4P), which is the starting point of synthesis of the aromatic amino acids. One of these aromatic amino acids, tryptophan, is the precursor of violacein synthesis<sup>30</sup>. *C. violaceum* can convert a larger amount of glucose to aromatic amino acids compared to other organisms, owing to its lack of a complete hexose monophosphate pathway (HMP). HMP-defective mutants have been shown to produce a higher amount of E4P, which is the limiting substrate for tryptophan biosynthesis. Tryptophan hyper-producing *Corynebacterium glutamicum* was shown to possess a modified pentose phosphate pathway<sup>31</sup>.

The reaction catalysed by the overexpressed G6PD generates reducing power in form of NADPH. Biosynthesis of violacein needs NADPH for conversion of L-tryptophan in cell-free extracts; the NADPH binding site(s) in VioA, VioC and VioB may be responsible for this requirement<sup>32,33</sup>. Requirement for NADPH has been demonstrated for at least two enzymes (VioD and VioC) participating in violacein biosynthesis<sup>15</sup>, and thus increased availability of NADPH stemming from overexpressed G6PD can have a direct positive impact on violacein biosynthesis.

Among the genes of the *vio* operon, the one coding for polyketide synthase (i.e. *vio B*) was found to be upregulated by 3.92-fold, which is a rate-limiting enzyme of the violacein synthesis pathway<sup>15,30</sup>. VioB is a large, multi-functional enzyme speculated to catalyse the 1,2-indole shift of tryptophan and the condensation reaction to generate the violacein-pyrrole ring<sup>33</sup>. Another enzyme, 5-aminolevulinic synthase (CV\_0803), which was two-fold upregulated, is involved in the pathway leading to the formation of the natural tetrapyrrole pigments.

In this study, cell yield was found to increase up to a minor extent only, whereas violacein production was enhanced at a much higher magnitude. This suggests that violacein production is not directly related to (or regu-

lated by) cell density. Literature review reveals that QS signals may not be the sole control mechanism in *C. violaceum* for violacein production. The production can be suppressed even in densely populated cell cultures, grown in high glucose and oxygen levels<sup>30</sup>. Thus, violacein production can also be considered to be dependent on type of the carbon source and its concentration in the culture medium, indicating possibility of a control mechanism similar to that mediated by cyclic AMP.

In total, four kinases (CV\_0024, CV\_3542, CV\_4059, CV\_1789) were found to be upregulated, indicating enhanced phosphorylation. It may be noted here that protein kinases play a vital role in the regulation of enzyme activities within a cell<sup>34</sup>. Multiple genes (including nine *Sec* genes, and two type-III secretion proteins) associated with secretion were found to be upregulated. It indicates a notable increase in the secretion activities of the sound-stimulated culture. This fact may be looked in the context of a large number (45) of upregulated genes coding for ribosomal proteins, which can be taken as an indication of enhanced protein synthesis. Higher protein synthesis may cause the cell to secrete more protein content to the exterior. The ability of audible sound signal to evoke the level of total protein content in *E. coli* has been reported by Gu *et al.*<sup>21</sup>. Total protein mass from stressed *C. violaceum* cultures was reported to be higher in a proteomic analysis by Castro *et al.*<sup>27</sup>. Hence we may consider of acoustic stimulation as a sort of stress to the exposed bacterial culture.

In total, five genes (*fliP*, *fliC*, *flag* (two), *cheY*) associated with chemotaxis/motility/flagella were found to be upregulated in sound-stimulated culture, indicating an increased mobility of *C. violaceum* cells. Chemotaxis is believed to help in the survival of cells. One of the essential chemotaxis-associated genes, *cheY* (upregulated 2.54-fold) in its phosphorylated form diffuses through the cytoplasm and attaches to the flagellar motor switch. This bound CheY ~ P acts as an allosteric regulator governing the rotations of the flagellar motor. Enhanced membrane permeability and faster glucose uptake in the sound-stimulated culture can be thought to interfere with the chemosensory response, and they may also represent a source of noise. This noise can affect intracellular processes having a significant effect on cell motility. As the Che proteins of the bacteria are expressed by specific genes, noise in gene expression can have a notable effect on the chemotactic motility. Noise associated with gene expression can be of intrinsic or extrinsic origin. Cell-intrinsic noise can arise from fluctuations in cell-specific factors, viz. metabolite (e.g. E4P, violacein) concentrations. Internally driven fluctuations can be propagated from one cell to another. Faster nutrient uptake in the sound-stimulated culture is likely to cause a rapid change in extracellular concentration of chemoattractants, making the bacteria more responsive to these changes<sup>7</sup>. Intracellular

noise in bacterial cells may be present at the level of transcription or translation. Translational noise has been shown to have an influence on gene expression<sup>35</sup>, and this noise is believed to be at a higher level in sound-stimulated culture owing to increased protein synthesis. Sonic-stimulated culture is characterized by an increased chatter (quick fluctuations in the rotation of the flagellar motors).

One major aim of this study was to analyse the effect of sonic stimulation on QS of the bacterial population. QS is a mechanism to regulate population behaviour. The collective cellular response may be related to the impact of noise. The behavioural variability of a cell population can be regulated by the slope of a histidine kinase activation curve<sup>36</sup>. In the sound-stimulated culture, a total of four histidine kinase associated genes were found to be upregulated, of which two were two-component sensors coding for histidine kinase. Phosphorylation of a histidine residue occurs in response to detection of an extracellular signal, to initiate a change in cell state or activity<sup>37</sup>. In the context of the present study, this extracellular or environmental change can be considered to be the sound wave. The remaining two upregulated genes are regulatory proteins involved in auto-phosphorylation of histidine kinase, of which CheY is a chemotaxis regulator protein and the other is LuxR family transcriptional regulator.

Overall biosynthesis, metabolism and enzyme activities seem to be upregulated in the sound-receiving culture. Higher metabolism and higher enzyme activity will increase the demand for energy, and hence these cells may be expected to produce and consume more ATP than control cells. This is in line with the observed upregulation (1.97-fold) of ATP synthase subunit delta (atpH) and GTPase Ogb (2.29-fold) involved in ATP synthesis (i.e. energy availability) and GTP hydrolysis (i.e. energy release) respectively. The most effective sound signal found in this study was 300 Hz. A nearby frequency of 340 Hz could drive ATP formation in bovine F1/F0 ATPase function in sub-mitochondrial particles<sup>38</sup>. Sound is considered to be capable of altering ATP rotation or proton gradients across membrane, provided that other transmembrane transporters and enzymes are also susceptible to sonic vibrations<sup>3</sup>. It should be noted here that ten (including five porins) transport-associated genes were upregulated in our experimental culture.

A liquid growth medium disturbed by the travel of sound waves through it can be considered to offer a high level of external (environmental) noise to the cells growing in it, and this external noise inevitably exerts an impact on the ligand-binding dynamics of the bacterial population in question. Optimum levels of noise can generate more efficient chemotaxis, which seems to be the case here, as indicated by upregulation of chemotaxis/motility-related genes. Conventionally, the performance of a system is measured by the signal-to-noise ratio. However, this ratio may not be a fitting metric for bio-

logical systems. It has been suggested that if the objective is to assess the effect of noise on one or more biological functions, it is more useful to quantify variations in those very functions<sup>39</sup>, e.g. cell yield and violacein concentration measured in this study. We have clearly demonstrated a notable degree of differential gene expression in sound-treated bacterial culture in this study. Fluctuations in the gene expression level, rates of metabolic reactions, and the titre of participating reactants and products can contribute to a rise in intrinsic noise<sup>40,41</sup>.

## Conclusion

Though the effect of sonic vibration on yeast metabolome has been reported earlier<sup>3</sup>, to the best of our knowledge, there are no previous reports describing the differential gene expression in bacteria exposed to sonic stimulation. We did not perform experimental validation for various alterations in bacterial metabolism, motility, stress response, etc. suggested by all the differently expressed genes. However, this study has demonstrated the capacity of audible sound signals to affect bacterial growth and QS-regulated pigment production. Differential upregulation of a large number (342) of genes (4.63% of the *C. violaceum* genome) was found to occur in the sound-stimulated culture. In the bacterial culture receiving sonic treatment, particularly the genes associated with protein synthesis, chemotaxis, motility, various enzyme activities, E4P synthesis, transcriptional regulators and histidine kinase were upregulated. The gene expression profile of this sound-stimulated culture can be considered to be associated with violacein overproduction. This information can be useful for strain improvement of *C. violaceum* for enhanced production of the bioactive metabolite violacein. Such studies providing clear demonstration of the ability of sonic waves to affect microbial metabolism can provide new perspectives at the interface of acoustics, microbiology, biophysics and biochemistry. In the long term, such studies can pave the way for development of sonic stimulation as a tool for manipulating microbial growth and metabolism.

**Conflict of interest:** Authors declare that they have no conflict of interest.

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