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ENVIRONMENTAL BIOTECHNOLOGY

Differential gene expression in *Escherichia coli* during aerosolization from liquid suspension

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Abstract

Comparative transcriptome analysis was used to determine the differentially expressed genes in *Escherichia coli* during aerosolization from liquid suspension. Isogenic mutant studies were then used to examine the potential part played by some of these genes in bacterial survival in the air. Bioaerosols were sampled after 3 min of nebulization, which aerosolized the bacteria from the liquid suspension to an aerosol chamber (A0), and after further 30 min of airborne suspension in the chamber (A30). Bacteria at A0 showed 65 differentially expressed genes (30 downregulated and 35 upregulated) as compared to the original bacteria in the nebulizer. Droplet evaporation models predicted a drop in temperature in the bioaerosols, which coincides with the change in the expression of cold shock protein genes—*cspB* and *cspG* in the bacteria. The most notable group of differentially expressed genes was sorbitol transport and metabolism genes (*srlABDEMR*). Other genes associated with osmotic stress, nutrient limitation, DNA damage, and other stresses were differentially expressed in the bacteria at A0. After further airborne suspension, one gene (*ypfM*, which encodes a hypothetical protein with unknown function) was downregulated in the bacteria at A30 as compared to those at A0. Finally, isogenic mutants with either the *dps* or *srlA* gene deleted (both genes were upregulated at A0) had lower survival than the parental strain, which is a sign of their potential ability to protect the bacteria in the air.

Keywords Airborne bacteria · RNA-seq · Stress response · Transcriptome analysis

Introduction

In the prediction of airborne transmission and dispersal of pathogens from respiratory droplets, certain models have been proposed to theoretically describe the physical change in the droplets during evaporation (Gralton et al. 2011; Lighthart and Kim 1989; Liu et al. 2017; Parienta et al. 2011). Although a number of studies applied different approaches and parameters in constructing the models, a prediction which has often been denoted is that water in the newly formed bacterial droplet will evaporate within milliseconds before reaching equilibrium to form a stable droplet nucleus in the air (Liu et al. 2017; Parienta et al. 2011). During evaporation, temperature, solute content, and other environmental conditions of the droplet may change depending on various factors such as nebulization conditions (e.g., nebulization methods, composition of the bacterial suspension, and temperature), air temperature, and relative humidity (RH) (Liu et al. 2017; Parienta et al. 2011). Previously, Krumins et al. (2014) reported an increase in rRNA gene expression in an airborne bacterium, *Sphingomonas aerolata*, in response to different volatile organic compounds as growth substrates. However, before the bacteria adapt to such environmental stimulus, they first need to face the rapid and diverse physical changes in the droplet immediately after aerosolization. How the bacteria regulate their gene expression in

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response to such changes, and whether some of these newly expressed genes are important to the survival of the bacteria, represent knowledge that is critical to understanding the behavior of bacteria in aerosolization.

Transcriptome analysis reveals the genes that are actively expressed at any given condition and time. Therefore, it has been applied in the study of early stress-dependent gene expression such as osmostress (Weber and Jung 2002) and pH (Tucker et al. 2002) in bacteria analysis. Moreover, this method could help to screen and deduce the potential stimuli and stresses experienced by the bacteria due to the change in the external environment by tracing specific stress-inducible and stress-associated genes (Etchegaray et al. 1996; Yim and Villarejo 1992; White-Ziegler et al. 2008). It also shows the potential adaptation and survival response of the bacteria in the new environment (Cheung et al. 2003; Empadinhas and da Costa 2008; Higgins et al. 1988; Pilizota and Shaevitz 2012; Yamanaka et al. 1998). In the study of the airborne transmission and control of bacterial aerosols, this information is valuable because identifying the environmental parameters and their mechanisms in inactivating the bacteria and how the bacteria defend themselves and adapt to the new environment could help to predict the transmission risk of different bacteria in various environments and inspire researchers to develop new infection control technology.

Prior theoretical models have predicted dramatic physical changes in bioaerosols during aerosolization, but no study has yet examined whether or not the environmental changes could trigger a new gene expression profile, and, if so, which gene expressed may affect the survival of the aerosolized bacteria. In this study, *Escherichia coli*, a bacterium commonly used in transcriptome and bioaerosol studies, was selected as a model bacterium to investigate the biological response of the aerosolized bacteria (Cox and Baldwin 1967; Higgins et al. 1988; Ng et al. 2017; Pilizota and Shaevitz 2012; White-Ziegler et al. 2008). The bacteria were nebulized in a chamber, and transcriptome sequencing (RNA-seq) was performed to determine the differentially expressed genes (DEGs) in the bacteria. Finally, isogenic mutants with a target gene deleted were tested to determine the functional role of the genes in bacterial survival.

Materials and method

Bacterial strain and experimental setup

E. coli BW25113 was purchased from Coli Genetic Stock Center (CGSC, Yale University, New Haven, USA). Fresh cultures of the bacteria were grown to a stationary phase in Luria Bertani medium (Affymetrix Inc., Santa Clara, USA) in a shaker-incubator at 37 °C and 150 rpm. The bacterial cells were harvested by centrifugation at 3000×*g* for 7 min, then washed three times with phosphate buffer saline (PBS, pH 7.4) and, finally, re-suspended in the same buffer to eliminate the presence of any unknown metabolite or condition in the bacterial culture due to the bacterial growth.

The bacterial solution was transferred to a six-jet Collison nebulizer (BGI Inc., Waltham, USA) for nebulization at 20 psi for 3 min. The aerosols generated were purged and suspended in a cylindrical chamber (diameter × height: 65 cm × 90 cm; volume: 300 L) (Fig. S1 in the supplementary materials shows the schematic diagram of the aerosolization setup.) The temperature inside the chamber was 20 ± 2 °C; the same as the laboratory temperature. The RH of the chamber was adjusted to between 80 and 90% by spraying sterile water into it. This RH condition optimized the amount and quality of the RNA extracted from the aerosolized bacteria. (RNA quality data shown in Fig. S2 in supplementary materials.) The temperature and RH of the chamber were monitored using a digital hygrometer.

Before nebulization, the bacteria suspended in the nebulizer were defined as N0. After 3 min of nebulization, the bacterial suspension was called N3. Upon completing the nebulization process, the age of bacterial aerosols in the chamber was set as time zero, A0. The bioaerosols further suspended in the air for 30 min were named A30. The initial state of bacteria at N0 and change in the mRNA level of the bacteria at N3 were measured. For N0 and N3 samples, a 0.3-mL aliquot was pipetted into 0.6 mL of RNAprotect bacteria reagent. To collect the A0 and A30 airborne *E. coli* for RNA analysis, the bacterial cells were sampled on a 0.22-μm mixed cellulose ester filter (Advantech, Tokyo, Japan) at a flow rate of 28 L/min for 3 min (Ng et al. 2017). Immediately after sampling, the filter was placed into a 4.5-mL PBS and RNAprotect bacteria reagent mixture (Qiagen, Hilden, Germany) at a volume ratio of 1:2 and vigorously shaken in a vortex for 30 s to elute the bacteria and stabilize the RNA.

All the samples (N0, N3, A0, A3) in the RNA-protect mixture were incubated for 10 min at room temperature. The samples were then centrifuged for 10 min at 5000×*g*. The supernatant was discarded and the cell pellet was used for RNA extraction. The aerosolization experiments were conducted in triplicates for comparative transcriptome analysis (Auer and Doerge 2010). Besides transcriptome analysis, the survival of bacteria in different phases was also determined using the same methods described in the isogenic mutant studies below (Fig. S3 in supplementary materials).

RNA extraction and RNA sequencing

Bacterial RNA was extracted using a RNeasy® Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The concentration of the extracted RNA samples was determined using a Nanodrop system (NanoDrop, Madison, USA), and the integrity of the RNA was examined by the RNA integrity number (RIN) using an Agilent 2100 bioanalyzer (Agilent, Santa Clara, USA). All samples with RIN values greater than 8.0 were used for the library construction. A

single-end read, each of 50 bp read-length, was sequenced on the BGISEQ-500 sequencer (BGI Co. Ltd., Shenzhen, China) which was performed by BGI Co. Ltd. Sequencing data were submitted to the GenBank SRA archive with the BioProject ID: PRJNA356202 and SRP accession SRP094916.

Bioinformatics analysis

The scope of the study was to differentiate the change in the bacterial RNA expression in three phases: (1) nebulization (N0 to N3)—bacteria before and after nebulization, (2) aerosolization (N3 to A0)—bacteria in liquid suspension and aerosolized bacteria, and (3) airborne suspension (A0 to A30)—aerosolized bacteria before and after 30 min of airborne suspension. At least 20 Mb clean reads were obtained for each sample (Table S1 in supplementary materials) and then mapped to *E. coli* str. K-12 MG1655 genome (NCBI accession: NC_00913.3) using HISAT/Bowtie2 tool (Kim et al. 2015; Langmead et al. 2009). Lengths higher than 80% of each read were required to map to the reference sequence for them to be considered a mapped read (Table S1 in supplementary materials). Biological replicates within each treatment groups demonstrate > 0.85 correlations (Spearman rank), indicating high reproducibility of replicates.

Next, the gene expression level was quantified by the RSEM software package (Li and Dewey 2011). The RNA expression of *E. coli* in the three different phases: (N0 vs N3), (N3 vs A0), and (A0 vs A30) were compared pairwise using the NOISeq method to determine the DEGs (Tarazona et al. 2011). The DEGs were determined based on the criteria set in the NOISeq method as |fold change| > 2 and probability > 0.8 (Tarazona et al. 2011). The DEGs were subjected to annotation analysis of gene ontology (GO) and the GO functional classification using WEGO software (Kanehisa et al. 2008; Ye et al. 2006). Quantitative polymerase chain reaction (qPCR) analysis was used to validate the differential gene expression of selected genes, *cspG*, *ompF*, and *osmY* (method shown in supplementary materials—gene expression by qPCR). Our data showed that the results of transcriptome sequencing and qPCR were well-matched (Table S2 in supplementary materials).

Airborne survival of the isogenic mutants

Isogenic mutants were obtained from the same supplier as the parental strain (Table 1). They were grown and treated in the same way as the parental strain described above. After the same bioaerosol experiment, airborne bacteria were sampled on a filter and then eluted from the filter with PBS. The culturable and DNA counts of the bacteria were determined by the plate-count method with tryptone soy agar incubated at 37 °C for 48 h and qPCR, respectively. The DNA counts were used to normalize the culturable counts data to account for the physical loss of the bacteria during experimentation (described in our previous study, see Ng et al. 2017).

The QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was used to extract the DNA following the manufacturer's protocol. The concentration of the extracted DNA samples was determined using *uidA* gene primers, which are specific to *E. coli* (Fram and Obst 2003). QuantiNova™ SYBR® Green PCR Kit (Qiagen, Hilden, Germany) with the forward primer 784 (5′-GTG TGA TAT CTA CCC GCT TCG C-3′) and the reverse primer 866 (5′-AGA ACG GTT TGT GGT TAA TCA GGA-3′) was used.

The thermocycling program of the Applied Biosystems StepOne RT-PCR System (Applied Biosystems, Walthan, USA) was set with an initial activation cycle at 95 °C for 2 min, followed by 40 × 5 s cycles of denaturation at 95 °C and combined annealing/extension at 60 °C for 10 s. *E. coli* BW25113 was used to set a standard calibration curve. Log reduction in the bacterial survival was calculated by comparing the normalized culturable counts at the A0 and A30 sampling points. Mutants with a higher log reduction than the parental strain show the importance of the deleted gene to the bacterial survival in the air. One-way analysis of variance (ANOVA) with Duncan's post hoc test (SPSS v. 23, SPSS, Armonk, USA) was applied to analyze the difference between means. A P value lower than 0.05 ($P < 0.05$) was regarded as statistically significant between the means.

Droplet evaporation models

The theoretical changes in the droplet diameter, temperature, concentration of salt, and *E. coli* volume fraction were calculated using the models proposed in Parienta et al. (2011) (equations and calculation described in Models, Eqs. S1–4 and Tables S3 and S4 in supplementary materials). Due to the lack of detailed information of the physical parameters, including the mechanical, material, and thermal properties of the bacteria, the rod shape of the bacteria was not explicitly described in the current model, but rather was considered by the empirical coefficients using an aerodynamic diameter approach. The initial droplet size generated by the nebulizer was assumed to be 2.0 μm in diameter based on the manufacturer's data (BGI, Waltham, USA). The aerodynamic diameter of the culturable bioaerosols (1.5 μm) was measured using a six-stage viable Andersen sampler (Lai et al. 2004). The recorded initial bacterial solution temperature of 23.4 °C, air temperature of 20.0 °C, and RH of either 80 or 90% were used in the calculation. The change in the temperature of the bacterial solution after nebulization was measured in order to compare with the model result.

Results

Droplet evaporation models

Based on the above assumptions, the temperature of the droplet would fall to 17.5 and 18.8 °C at 80 and 90% RH,

Table 1 The genetic information of the bacteria used in the present study

Strain name	GCSC number	Deleted gene	Mutation function
BW25113	7636	None	Not applicable
JW0797-1	8844	*dps*	Fe-binding and storage protein; stress-inducible; protect DNA damage
JW1653-1	9407	*cfa*	Cyclopropane fatty acyl phospholipid synthase, SAM-dependent
JW3474-5	10,574	*slp*	Outer membrane lipoprotein
JW2676-1	10,111	*srlR*	Sorbitol-inducible *srl* operon transcriptional repressor
JW5429-2	11,385	*srlA*	Glucitol/sorbitol-specific enzyme IIC component of PTS
JW0912-1	8925	*ompF*	Outer membrane porin 1a

respectively, in a short period of time due to droplet evaporation (Fig. 1a, b). After the water content of the droplet had completely evaporated to an equilibrium level and the droplet mass was stabilized, the temperature of the droplet nucleus was predicted to increase to the air temperature level of 20 °C. The continuous nebulization and evaporation of the bacterial solution in the nebulizer when the liquid stream breaks down into aerosols also reduced the temperature of the bacterial solution creating a cooler condition for the bacteria. The measured temperature range of 17.8–18.0 °C in the bacterial solution after nebulization was similar to the model prediction of from 17.5 to 18.8 °C. Droplet evaporation also led to an increasing concentration of salt and bacteria volume fraction (i.e., potentially higher osmotic pressure and dehydration stress to the bacteria) (Fig. 1c, d).

Changes in the gene expression in the aerosolization phase

All phases were compared back to the preceding phase, e.g., N3 to N0, A0 to N3, and A30 to A0. The numbers of DEGs identified in the nebulization, aerosolization, and airborne suspension phases were as follows: 7 (3 downregulated and 4 upregulated), 65 (30 downregulated and 35 upregulated), and 1 (downregulated), respectively (Tables 2, 3, and 4). The results indicate that the major changes in transcription levels occurred in the aerosolization phase. The greatest decline in bacterial survival was observed in this phase too (Fig. S3 in supplementary materials). This demonstrates that the aerosolization process could induce a high level of stress to the bacteria.

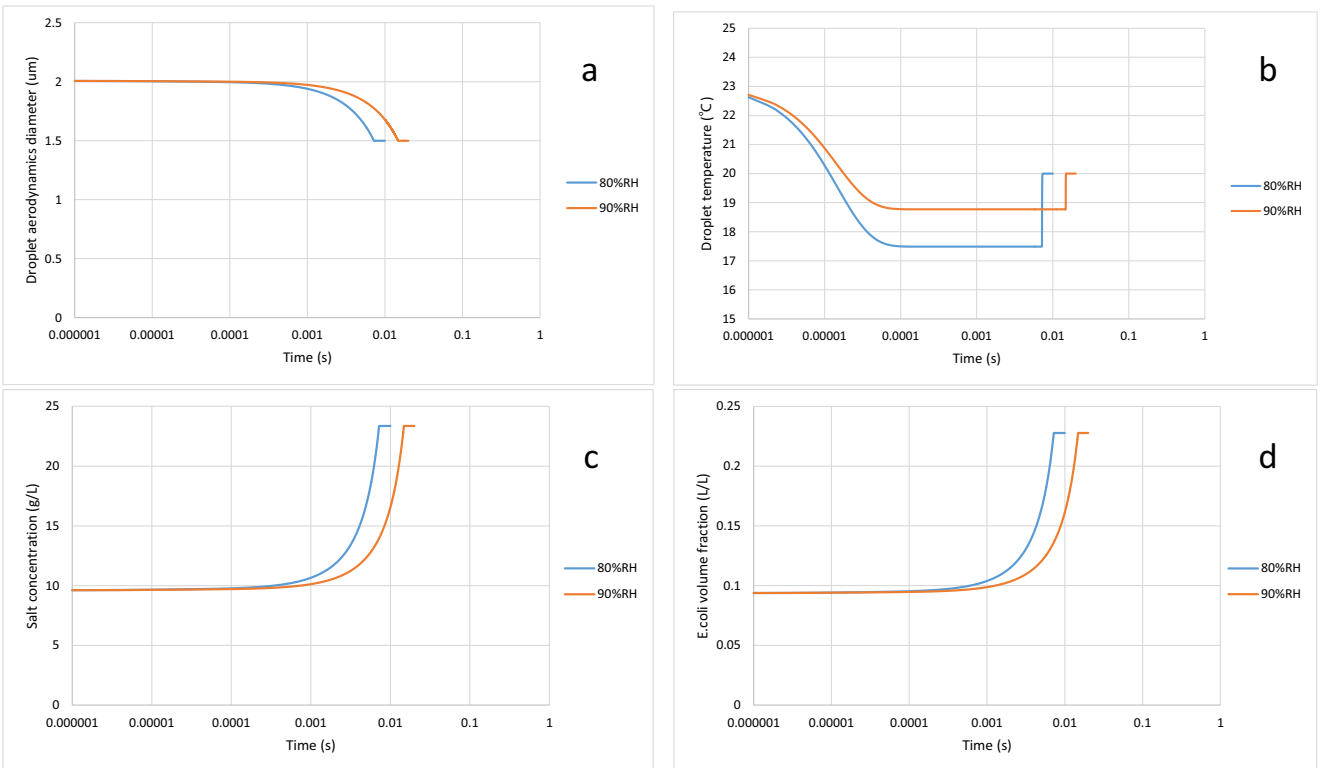

Fig. 1 Change in droplet **a** diameter, **b** temperature, **c** salt concentration, and **d** *E. coli* volume fraction at 80 and 90% RH

Table 2 DEGs identified in the nebulization phase

Gene	Fold change	Gene description
yjcH	− 2.20	DUF485 family inner membrane protein
acs	− 2.15	Acetyl-CoA synthetase
trpC	− 2.01	Indole-3-glycerolphosphate synthetase and *N*-(5-phosphoribosyl)anthranilate isomerase
aroG	2.04	3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, phenylalanine repressible
ydfK	2.58	Cold shock protein, function unknown, Qin prophage
cspB	2.60	Qin prophage; cold shock protein
cspG	6.75	Cold shock protein homolog, cold-inducible

Responses in the nebulization phase

The DEGs identified in the nebulization phase cover four GO terms (cellular process, metabolic process, response to stimulus, and single-organism process) and highlight the pathway—metabolism (energy, carbohydrate, and amino acid) in the KEGG pathway analysis (Fig. 2, details of the GO annotation and KEGG pathway listed in Tables S5 and S6 in supplementary materials, respectively). Among the genes in these metabolic pathways, *aroG* was upregulated, while *trpC*

Table 3 DEGs identified (downregulated) in the aerosolization phase

Gene	Fold change	Gene description
cspG	− 10.83	Cold shock protein homolog, cold-inducible
ygeY	− 8.34	Putative peptidase
hyuA	− 7.80	D-stereo-specific phenylhydantoinase
xdhD	− 7.42	Putative hypoxanthine oxidase, molybdopterin-binding/Fe-S binding
flu	− 7.33	CP4–44 prophage; antigen 43 (Ag43) phase-variable biofilm CP4–44 prophage; antigen 43 (Ag43) phase-variable biofilm formation autotransporter formation autotransporter
ssnA	− 6.94	Putative chlorohydrolase/aminohydrolase
ygfM	− 6.32	Putative oxidoreductase
mokA	− 6.23	Pseudo
ynfN	− 5.75	Qin prophage; cold shock-induced protein
ygeX	− 5.41	2,3-diaminopropionate ammonia lyase, PLP-dependent
fimC	− 5.10	Periplasmic chaperone
proV	− 4.13	Glycine betaine/proline ABC transporter periplasmic binding protein
cspI	− 4.12	Qin prophage; cold shock protein
cspB	− 3.95	Qin prophage; cold shock protein
fimA	− 3.79	Major type 1 subunit fimbrin (pilin)
fimF	− 3.64	Minor component of type 1 fimbriae
fimI	− 3.61	Fimbrial protein involved in type 1 pilus biosynthesis
ydfK	−3.46	Cold shock protein, function unknown, Qin prophage
ygaY	− 3.06	Pseudo
ttcC	− 2.85	Pseudo
pspA	− 2.74	Regulatory protein for phage shock protein operon
yjcC	− 2.56	Putative membrane-anchored cyclic-di-GMP phosphodiesterase
ompF	− 2.51	Outer membrane porin 1a
puuA	− 2.50	Glutamate—putrescine ligase
tdcE	− 2.49	Pyruvate formate-lyase 4/2-ketobutyrate formate-lyase
ibpB	− 2.25	Heat shock chaperone
soxS	− 2.22	Superoxide response regulon transcriptional activator; autoregulator
ilvB	− 2.21	Acetolactate synthase 2 large subunit
pspB	− 2.20	*psp* operon transcription co-activator
ilvN	− 2.05	Acetolactate synthase 1 small subunit

Table 4 DEGs identified (upregulated) in the aerosolization phase

Gene	Fold change	Gene description
uspB	2.07	Universal stress (ethanol tolerance) protein B
ylaC	2.09	DUF1449 family inner membrane protein
osmY	2.09	Salt-inducible putative ABC transporter periplasmic binding protein
yeaQ	2.09	UPF0410 family protein
cbpA	2.09	DnaK co-chaperone; curved DNA-binding protein
ecnB	2.09	Entericidin B membrane lipoprotein
ybjP	2.12	Lipoprotein
glgS	2.14	Motility and biofilm regulator (surface composition regulator); glycogen synthesis control protein
yceK	2.15	Outer membrane integrity lipoprotein
purE	2.16	N5-carboxyaminoimidazole ribonucleotide mutase
yqjE	2.16	DUF1469 family inner membrane protein
uspG	2.17	Universal stress protein UP12
xanP	2.19	Xanthine permease
yqjK	2.19	Uncharacterized protein
yqjD	2.20	Membrane-anchored ribosome-binding protein
cfa	2.21	Cyclopropane fatty acyl phospholipid synthase, SAM-dependent
purM	2.21	Phosphoribosylaminoimidazole synthetase
yghA	2.21	Putative oxidoreductase
ymdF	2.21	KGG family protein
yegP	2.36	UPF0339 family protein
ytjA	2.38	Uncharacterized protein
cbpM	2.43	Modulator of CbpA co-chaperone
dps	2.51	Fe-binding and storage protein; stress-inducible DNA-binding
purT	2.56	Phosphoribosylglycinamide formyltransferase 2 (glycinamide ribonucleotide transformylase)
gadE	2.72	*gad* regulon transcriptional activator
ygaU	2.77	Uncharacterized protein
slp	2.82	Outer membrane lipoprotein
msyB	2.85	Multicopy suppressor of *secY* and *secA*
yncL	3.15	Stress-induced small inner membrane enterobacterial protein
srlR	3.22	Sorbitol-inducible *srl* operon transcriptional repressor
srlM	4.25	Sorbitol-responsive *srl* operon transcriptional activator
srlD	4.61	Sorbitol-6-phosphate dehydrogenase
srlA	5.00	Glucitol/sorbitol-specific enzyme IIC component of PTS
srlB	5.04	Glucitol/sorbitol-specific enzyme IIA component of PTS
srlE	5.65	Glucitol/sorbitol-specific enzyme IIB component of PTS

and *acs* were downregulated (Table 2). The genes annotated in response to stimulus were *acs* and *cspBG*. The *acs* gene encodes acetyl-CoA synthetase, which catalyzes acetate into acetyl-CoA. Its downregulation may lead to a reduction in carbohydrate and energy metabolism and is a sign of a response to nutrient limitation. The gene *trpC* encodes the tryptophan biosynthesis protein, which is important for tryptophan biosynthesis. The *aroG* gene encodes 3-deoxy-*D*-arabino-heptulosonate-7-phosphate synthase, which is responsible for the biosynthesis of chorismate, a precursor for the biosynthesis of tryptophan, phenylalanine, tyrosine, and other aromatic compounds.

Two cold shock genes, *cspB and cspG*, and a cold shock protein gene, *ydfK*, were upregulated during nebulization. This demonstrates that the fall in the temperature of the bacterial solution due to droplet evaporation stimulates a cold-stress response in the bacteria during nebulization.

Responses in the aerosolization and airborne suspension phase

A wider range of GO terms and gene function groups in the KEGG pathway was revealed in the aerosolization phase (Fig. 3; details of the GO annotation and KEGG pathway

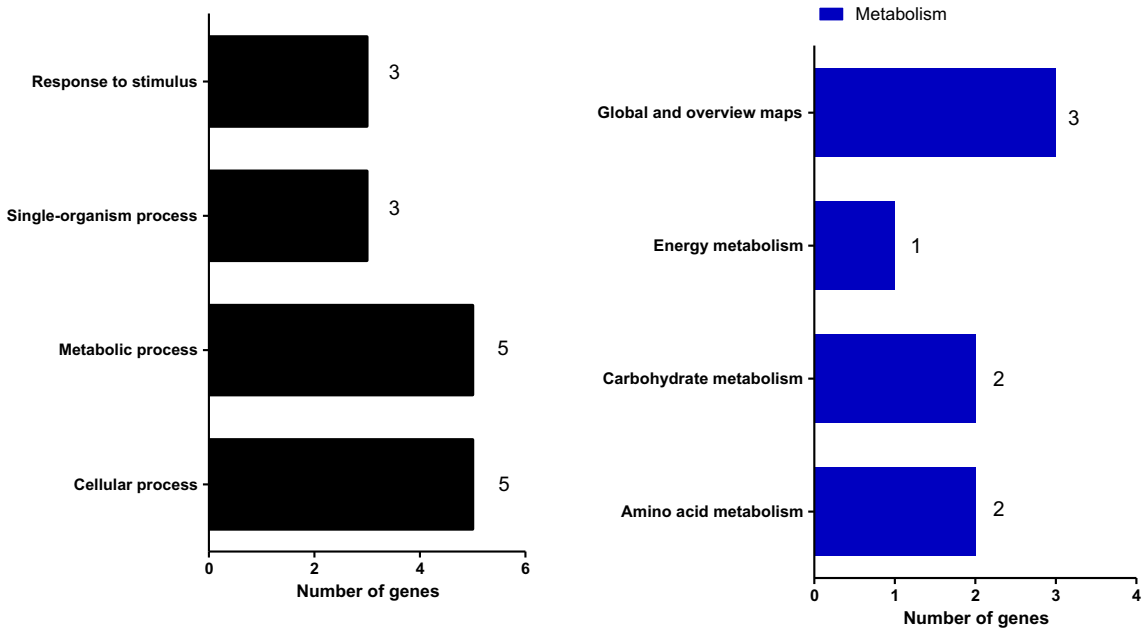

Fig. 2 Gene ontology of the DEGs identified in nebulization phase and the distribution by function according to KEGG pathway

listed in Tables S7 and S8 in supplementary materials, respectively). According to the GO analysis, a large number of stress-responsive genes ($n = 11$) and stimulus-responsive genes ($n = 13$) were regulated in bacteria and responded to the aerosolization. For instance, *cspBGI* (cold stress) were downregulated in 3.95-fold (Table 3). Osmotic change was associated with *osmY* (osmotically inducible protein Y), *ompF* (osmoregulated outer membrane protein F), and *proV* (for glycine betaine/proline transport). Other genes induced by stress and stimuli include *dps* (DNA-binding proteins from starved cells), *pspAB* (phage shock proteins), *ibpB* (small heat shock protein), and *uspG* (universal stress protein).

Genes associated with membranes (e.g., various membrane-bound proteins and membrane structures) were also

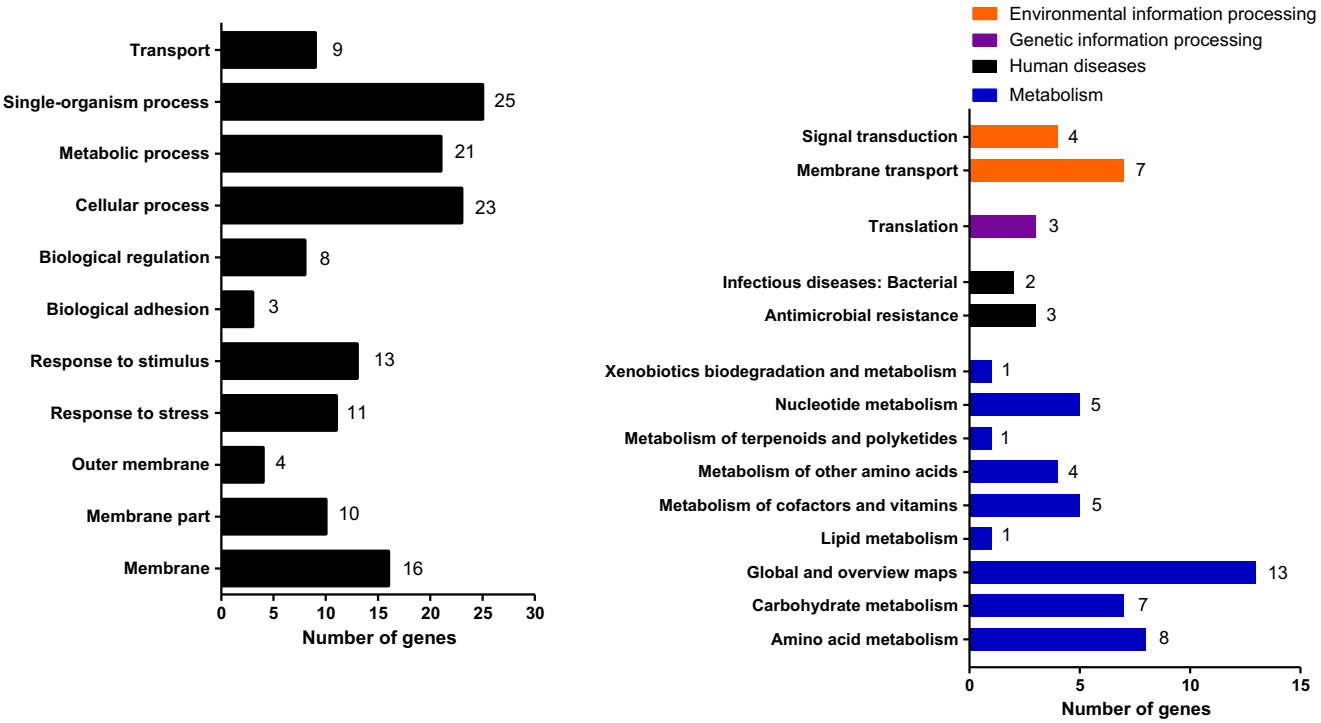

Fig. 3 Gene ontology of the DEGs identified in aerosolization phase and the distribution by function according to KEGG pathway

differentially expressed in the air. In the outer membrane, *flu* (which controls colony from variation and autoaggregation), *ompF*, and *ibpB* were downregulated, while *slp* (outer membrane starvation lipoprotein) was upregulated. Other than these genes associated with the membrane, *srlAER* and *xanP*, associated with sorbitol and xanthine permease and *ecnB*, *uspB*, and *ygjD*, related to cell regulation in the stationary phase, were all upregulated (Table 4). Another group of DEGs, *fimACFI* in the type 1 fimbriae gene cluster, which controls biological adhesion, was completely downregulated.

In the KEGG pathway, many metabolic pathways were affected in the aerosolized bacteria in addition to the amino acid and carbohydrate metabolism such as nucleotide metabolism (*purEFMT* upregulated) and metabolism of cofactors and vitamins, which further reflect nutrient limitation. The most notable carbohydrate metabolism was the sorbitol metabolism, in which *srlABDE* were upregulated (*srlMR* were also upregulated; a complete regulon of SrlR: *srlA* > *srlE* > *srlB* > *srlD* > *srlM* > *srlR* > *gutQ*).

After 30 min of airborne suspension, only one gene, *ypfM*, which encodes a hypothetical protein with unknown function, was downregulated. This result marks a complete change in the transcriptional signature of the bacteria at the time we took the first bioaerosol sample (A0), and this change was still applicable when the bacteria responded to the air environment after 30-min suspension (A30).

Isogenic mutant studies

In order to examine whether the DEGs are important for bacterial survival in the air, some of these genes were selected in the isogenic mutant studies to compare their survival with the parental strain. Among these mutants (*ompF*, *dps*, *cfa*, *slp*, and *srlA*), the *dps* and *srlA* mutants had lower survival rates, when compared to the parental strain under aerosolization (Fig. 4). This result points to the induction of *dps* and *srlA* in the aerosolized bacteria as being important for bacterial survival.

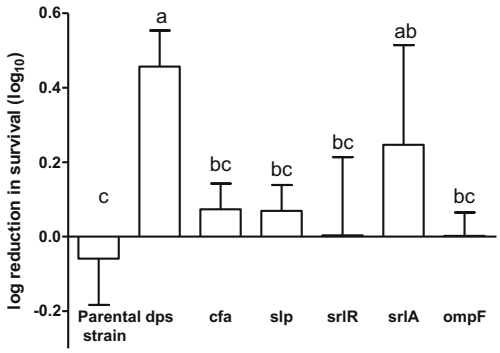

Fig. 4 The log reduction in survival of different *E. coli* mutants and the parental strain after airborne suspension for 30 min at > 80% RH. Error bars represent the standard deviation of replicates ($N = 3$). The mean of log reduction in survival was statistically analyzed by one-way ANOVA. Grouping was performed using post hoc test Duncan analysis

Discussion

DEGs and predicted physical changes in the aerosols

Temperature

Various stresses and mechanisms have been proposed that could damage or even inactivate aerosolized bacteria, e.g., dehydration, oxidation, osmotic stress, and membrane damage (Thomas et al. 2011; Zhen et al. 2014). This current study investigated the early and subsequent gene expression profile of the bacteria once they are aerosolized so that we could get more information about the environmental conditions that bacteria either sense or respond to in practice. Although there are numerous uncertainties in modeling the physical environment of the bioaerosols, the model established here offered some insights into the potential changes in the bioaerosol environment. According to our model calculation, a quick change in the physical environment of the droplet was predicted within 0.01 s. During the 3-min nebulization, the temperature of the bacterial solution decreased from 23.4 to about 18.0 °C. White-Ziegler et al. (2008) reported an increase in cold shock gene expression in *E. coli* at temperatures lower than 23 °C which supports the observation that the bacteria encountered cold stress continuously even though their temperature level increased to 20 °C after the droplet mass stabilized in the air.

Under a low-temperature environment during nebulization (such as when temperature dropped to 17.8 °C), an upregulation of cold-stress responding genes was expected. However, when the aerosolized bacteria reached the air temperature of 20.0 °C, some of these cold-stress genes were repressed. Out of the nine paralogs (*cspA-I*) of the CspA family in *E. coli*, *cspBG* were upregulated by the bacteria in the nebulizer, yet *cspBGI* were downregulated by the aerosolized bacteria. This result is reasonable according to knowledge gleaned from Yamanaka et al. (1998) who mentioned that *cspABG* were known to be induced by cold stress but that the function of *cspBG* may be different from *cspA* because they were induced in a narrower range of low temperature than *cspA*. Further support for this study's findings can be found in Etchegaray et al. (1996) where it is shown that *cspA* expression was observed during temperature shifts from 37 to 30 °C, while CspB protein production occurred when the temperature dropped to below 20 °C; a maximum induction appeared at 15 °C. This explains our observation that the expression of *cspB* was regulated in the bacteria but not *cspA*. Later, Uppal and Jawali (2015) reported that *cspBGI* were induced by cold stress and under the regulation of a cyclic AMP receptor protein (CRP). Noting that *cspI* is highly homologous to *cspG* and that CRP is a global regulator for sugar metabolism, their research also found that *cspA* can be regulated selectively by CRP during growth at 15 °C but

not at 37 °C. The regulation of *cspBGI* reflects a response of the aerosolized bacteria to the current range (17.8–20.0 °C) of temperature change.

The cold shock proteins of the CspA family have a high aromatic amino acid content, e.g., CspBG proteins have eight aromatic residues (six Phe, one Tyr, and one Trp) (Yamanaka et al. 1998). The upregulation of *aroG* for the biosynthesis of chorismate, the aromatic amino acid precursor, and the downregulation of *trpC* for the biosynthesis of tryptophan as shown in our data may be linked to the production of the Csp proteins. The regulation of *cspBG* in bacteria corresponds well with the measured and modeled temperature of the bacterial solution. These results justified that the aerosolized bacteria also have the ability to regulate their genes in response to environmental stimuli in the air within a short period of time as bacteria in other environments (Weber and Jung 2002; Tucker et al. 2002).

Salt content

In osmotic stress studies with *E. coli*, 0.3 M NaCl was commonly used to induce osmotic stress in the experiment (Higgins et al. 1988). The model predicted the quantitative level of solutes in the droplet nuclei; NaCl concentration increased from about 0.14 M in PBS in the liquid suspension to 0.33 M in the droplet nuclei. This implies that the osmotic change in the droplet nuclei is adequate to induce osmotic stress in *E. coli* but not to inactivate it. A number of genes that are important for the osmotic stress response or adaptation were regulated by the aerosolized bacteria, including *ompF* and *osmY* (Weber and Jung 2002; Yim and Villarejo 1992). This result complies with the physical model whereby the osmotic potential increases in the droplet nuclei. The protein *osmY* encodes a periplasmic protein which is induced at a hyperosmotic state and helps protect *E. coli* from hyperosmotic stress (Weber and Jung 2002). The gene *ompF* is repressed in high osmolarity (Weber and Jung 2002). The change in the transcription level of osmoregulatory genes, such as the upregulation of *dps*, *osmY*, and downregulation of *ompF* as reported by Weber and Jung (2002) in his osmotic stress-dependent gene expression study with *E. coli*, supports our deduction that the aerosolized bacteria experienced a high osmotic stress.

The regulation of some genes, though not directly related to osmo-protection, also supports the importance of and adaption to osmotic stress. For example, *ecnB* was activated in the stationary phase under high osmolarity conditions (Bishop et al. 1998). Repression of *fimA* expression by increased osmolarity was reported by Schwan et al. (2002). Reduction in metabolism occurs when *E. coli* faces salt stress (Cheung et al. 2003). The response of *E. coli* to osmotic shock can be completed within several minutes as additionally shown in Pilizota and Shaevitz's study (2012). This osmotic shock and the corresponding gene expression profile are likely to be maintained in the bioaerosol during further airborne suspension (Dunklin and Puck 1984).

Although some potential osmotic stress-related genes were regulated in the aerosolized bacteria, we could not find the same regulation of the genes linking to the synthesis and uptake of regular compatible solutes in *E. coli* such as glycine betaine (*bet*), proline (*pro*), and trehalose (*ots*). Uptake and synthesis of compatible solutes are critical for the cell to balance the external high osmotic potential. In contrast, *proV*, a glycine betaine/proline ABC transporter periplasmic binding protein was downregulated. Previous bioaerosol studies reported the loss of K^+, also a compatible solute, due to cell membrane damage, which may cause cell death during aerosolization (Dunklin and Puck 1984). Membrane damage may affect the uptake and retention of the solutes. Moreover, due to the nutrient and metabolic limitations as shown in the downregulation of several metabolic processes, the production of the solutes by the bacteria may be challenging.

Potential roles of sorbitol

Interestingly, the only potentially compatible solute indicated in this study was sorbitol, a polyol, but its role as an osmoprotectant or osmolyte in *E. coli* has not been reported. The only bacterium that is known to synthesize sorbitol as an osmoprotectant under high osmotic pressure is *Zymomonas mobilis*. This bacterium is able to produce sorbitol from fructose via a glucose-fructose oxidoreductase pathway in the periplasm (Empadinhas and da Costa 2008). In yeasts and other organisms, sorbitol is produced through the reduction of glucose by aldose reductase in the cytosol (Welsh 2000).

Sorbitol also acts as a carbon source for bacteria when other carbon sources are limited. External sorbitol can be transported into the bacterial cell through substrate-specific phosphoenolpyruvate-dependent sugar phosphotransferase systems (PTS). The enzyme II complex composed of SrlA, SrlB, and SrlE (their corresponding genes were upregulated in this study) is involved in sorbitol transport. In addition, the PTS phosphorylates the incoming sorbitol and translocates the phosphorylated sorbitol (*D*-sorbitol 6-phosphate) across the cell membrane. The SrlD, sorbitol-6-phosphate 2-dehydrogenase (*srlD*-upregulated in this study, catalyzes D-sorbitol 6-phosphate to D-fructose 6-phosphate with NAD^+, which is transferred to NADH.

Sorbitol, even if present in the bacterial culture, is not generally consumed by *E. coli* because of the carbon catabolic repression (CCR) by other preferable high metabolic ranking sugars, like glucose. When the supply of these high-ranking carbon sources becomes limited, the signal molecule cyclic AMP (cAMP) level increases and binds to the global regulator

CRP, which further activates the alternative carbon metabolic system (Aidelberg et al. 2014). Since the bacteria were washed to remove the culture medium and then suspended in PBS without any added sorbitol, the expression of all the *srl* genes was not expected. Kornberg (2001) concluded that permeases for sorbitol (SrlA) can transport fructose under high fructose levels. The expression of *srl* genes is associated with the global regulator CRP-cAMP, which also plays a role in the cold-stress regulation, as explained above. An increase in CRP-cAMP levels also suppresses the formation of type 1 Fimbriae (*fim*) (*fimACFI*—downregulated in this study) (Müller et al. 2009).

Other environmental conditions

Other than the cold and osmotic stress, as shown by the expression of *cfa*, *yeaQ*, *slp*, *osmY*, *cbpA*, and *gadE*, acid stress might also occur in the bacteria (Tucker et al. 2002). However, a clear RNA profile for oxidative stress response was not found. In our previous studies, we found that isogenic mutants with the deletion of either the *oxyR* or *soxR* gene survived at the same level as the parental strain at high RH conditions, supporting the lack of oxidative stress in the current study (Ng et al. 2017). Other studies also examined the potential environmental conditions on bacteria survival in the air (Bishop et al. 1998; Dunklin and Puck 1984; Cox and Baldwin 1967; Hess 1965).

Functional role of some DEGs in bacterial survival

The genes *ompF*, *dps*, *cfa*, *slp*, and *srlA* were selected in this study to examine whether these genes regulated in the aerosolized bacteria are important to survival. The gene *ompF* was downregulated in the bacteria. Its role is to allow passive diffusion of small molecules across the outer membrane so it was likely that its mutant would show no difference in survival when compared to the parental strain. The *dps* gene was upregulated, and it is an important protein to physically protect DNA from various sources of damage. As shown in our results, *purEFMT* were upregulated implying that DNA damage may be critical. The *cfa* gene transfers the unsaturated fatty acids to cyclopropane fatty acids to increase the membrane fluidity, which is a rapid way to strengthen the cell membrane against acid stress and increased physical pressure stress without the need for fatty acid biosynthesis. However, since unsaturated fatty acids can also be converted to saturated fatty acids by *cti* to decrease the membrane fluidity as a means for solvent stress protection, the survival difference of the mutant as distinct from the parental strain helps to understand the type of stress that acted on the membrane (Zhang and Rock 2008). The *slp* gene plays a role in stabilizing the outer membrane during the carbon starvation and stationary phases and is CRP-cAMP-independent. The *slp* mutant helps to examine the importance of this general outer membrane stress response to the survival of airborne bacteria.

The results show that only *dps* and *srlA* mutants had a higher log reduction in survival than the parental strain. This implies that these genes are important in protecting the aerosolized bacteria. Khil and Camerini-Otero (2002) identified over 1000 genes that are involved in the DNA damage response of *E. coli* with mitomycin C, a DNA-damaging agent. Although the gene expression data could not be compared directly because of the different conditions causing the DNA damage, their study also demonstrated an upregulation of *dps* and *srlAB* genes following the DNA treatment. However, the roles of these genes in the DNA damage response in their study were not clarified. Interestingly, 29 out of about 30 unlinked genes classified under the SOS response appeared in their study but none of them were differentially expressed in our study here (Khil and Camerini-Otero 2002).

Implications of the study and further holistic physiological analysis

This study showed that aerosolized bacteria can sense and respond to the stimuli in the air. These stimuli experienced by the bacteria may include cold, osmotic, nutrient limitation, and DNA and membrane damage stresses. Although a different RNA-seq profile was shown in the aerosolized bacteria as compared to the bacteria in liquid suspension, the total number of DEGs revealed was limited in contrast to the bacterial gene response in other conditions. We deduce that aerosolized bacteria encounter multiple stresses, and the airborne state may limit their stress response ability. This study implies that airborne bacteria such as airborne bacterial pathogens (e.g., *Mycobacterium tuberculosis*) or other bacteria that perform various environmental processes can also be studied using this methodology. This new research approach could contribute to our understanding of the survival and environmental interaction of the airborne bacteria, and so the control and prediction of the bacteria in different environments. Although DEGs were shown in this study, further holistic physiological analysis is essential to prove that these genes play a functional role in protecting the bacteria against different stresses. The results from this comparative transcriptome analysis can facilitate further physiological analysis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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