# Response of *Endozoicomonas montiporae* to heat stress and coral host lysates

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## Abstract

*Endozoicomonas,* a core bacterial group in corals, may also be a coral symbiont. *Endozoicomonas* communities often decrease rapidly in corals under heat stress. However, how the bacteria respond to changes in temperature and coral host during heat stress is unknown. Here, we employed the cultivable, dominant species *E. montiporae* as a working organism to explore how *Endozoicomonas* responds to heat stress. We designed two experiments to clarify the extent to which *E. montiporae* is influenced by temperature and coral host. We detected differentially expressed protein (DEP) profiles in this bacterium at 31°C and 33°C compared to 25°C by tandem mass tags-based quantitative proteome analysis. Fifty DEPs, including many heat shock proteins, were detected when the temperature changed. The expression of antioxidant defense proteins and key pyruvate synthase proteins decreased, suggesting that *E. montiporae* were in a physiology of stress at 33°C. Furthermore, some proteins were differentially expressed because of the heat-stress-treated coral lysate specifically, suggesting that not only heat but also heat-induced host factors can affect the protein expression of the bacterium. This study provides an in-depth analysis of how the molecular mechanisms of *Endozoicomonas* are affected by heat stress and coral host.

## Introduction

Temperature is a crucial factor governing coral physiology [1]. When the seawater temperature increases, heat stress impacts not only the coral host but also all of the coral microbial partners in the coral holobiont [2]. The most typical example is that coral-algae symbiosis can be dissociated by high temperature [3, 4]. Along with symbiotic algae, the impact of heat stress on the coral bacteria has recently received increasing attention due to these bacteria's indispensable ecological contribution to coral hosts [5-7].

Bacteria in the coral holobiont are highly sensitive to heat stress. For instance, Ziegler et al. [8] demonstrated that coral bacteria were significantly affected by 20 h of incubation. Similarly, Lee et al. [9] showed rapid changes in the coral bacteria between coral mucus and tissue within a day. Moreover, Shiu et al. [10] even showed that the coral bacterial community quickly changed from normal to pathogenic- or stress-associated microbiota within 12 h of a short-term heat stress experiment, whereas the density and photosynthetic efficiency (Fv/Fm) of *Symbiodiniaceae* remained unchanged.

Although heat stress usually elicits a clear shift in bacterial community, we still do not know how coral bacteria respond to heat stress, or their underlying biochemical or molecular mechanisms. This is because it is difficult to detect the response of a specific bacterial group in corals, as many studies have shown that coral-associated bacterial communities are diverse and dynamic in various corals with environmental variations. Hence there is a need to establish a bacterial manipulable model to explain in-depth physiological and biochemical characteristics that change when bacteria are exposed to specific biotic or abiotic factors. A similar idea was also proposed recently by van Oppen and Blackall [11]: using pure bacterial cultures for molecular approaches could provide insights into the in-depth, molecular mechanisms of coral bacteria and their interactive relationships with their coral host under a specific environmental condition, such as heat stress. Gao [12] used a cultivable coral pathogenic bacterium, *Vibrio coralliilyticus*, to investigate transcriptional responses when bacteria interact with coral mucus, and furthermore to elucidate a potential behavior and infection mode of the *V. coralliilyticus* that helps explain the pathogenesis of *V. coralliilyticus* and coral disease. However, there is no coral-health associated bacterial working model available for such an advanced study.

To establish the working model, the first need is to find an appropriate coral bacterium strain that may represent specific functionally important or dominant bacteria in the coral microbiota. Of the vast number of coral bacteria, the genus *Endozoicomonas* (*Gammaproteobacteria*, *Oceanospirillales*, *Hahellaceae* or *Endozoicomonaceae*) is the best studied and is recognized to be a functionally important, dominant bacterium in the core microbiome of various Scleractinian corals, such as common species of *Acropora*, *Stylophora*, *Pocillopora* and *Porites* [13-16]. *Endozoicomonas* has long been suggested to be a potential bacterial symbiont [2, 17]. Hence, it is a suitable bacterium for establishing such a working model. To date, there are only three cultivable *Endozoicomonas* species isolated from Scleractinian corals: *E. montiporae*, *E. acroporae* Acr-14$^{T,}$ and *E. coralli* [18-20]. Of them, only *E. montiporae* (*E. montiporae* CL-33$^{T}$) has had its complete genome sequenced. The genomic features also indicate that *E. montiporae* is a facultative symbiont [21, 22].

Furthermore, in Lee et al. [9], *E. montiporae* prevailed in *Acropora* sp., and the former's relative abundance decreased rapidly in the latter—from 80% to less than 20%—when the temperature was increased to 31°C and 33°C. A shift in the associated bacterial community (the *E. montiporae* was the second most dominant in the community) happened in the coral mucus when the sugar composition of coral mucus was altered by heat stress [23]. Based on these tight relationships between coral host and this bacterium, we believed that *E. montiporae* is the most suitable model microorganism for developing a working model to investigate the in-depth molecular physiological response of coral bacteria under heat stress [10].

In this study, we used *E. montiporae* as a model organism and detected differentially expressed protein profiles of this bacterium *in vitro* at two high temperatures, 31°C, and 33°C, based on the previous study [9]. Furthermore, we assumed that some of the heat stress-induced responses in the bacterium can be affected not only directly by temperature but also indirectly by the host factors, also induced by heat stress. Thus, the differentially expressed protein profiles of the bacteria were also detected by co-incubating with coral lysates after high temperature treatments. We found that temperature was the main factor explaining the changes in most proteins of *E. montiporae*. However, some proteins were affected by factors of

107 temperature-induced coral lysates. Further discussion is provided on the molecular
108 response of the specific coral bacterium under heat stress and influence from heat-
109 induced coral hosts.

## Materials and Methods

### Bacteria culture inoculation

*E. montiporae* CL-33$^T$ was cultivated in an MMBv4 medium at 25°C on a shaker with 200 rpm as previously described [21].

### Experimental design for heat stress experiment

The initial *E. montiporae* CL-33$^T$ culture was grown at 25°C to an optical density at 600 nm (OD) of 0.6, and then diluted 1/200 in total of nine Falcon centrifuge tubes with 20 mL fresh MMBv4 medium [21]. Nine bottles were incubated at 25°C, 31°C and 33°C (n=3 for each temperature) (Fig. 1A). Bacterial growth was monitored by a spectrophotometer, measuring the OD value after 16, 24 and 48 h from each tube. A 1 mL subsample from each cultural bottle was centrifuged at 9,000 *g* at 4°C. After discarding the supernatant, the pellet was washed with 1 mL 1X phosphate-buffered saline (PBS) three times. A total of 27 sample tubes were flash-frozen using liquid nitrogen immediately and stored at -80°C until total protein extraction.

### Experimental designing for coral lysate experiment

To test the protein profiles of *E. montiporae* co-incubating with coral lysates, we prepared and placed coral lysates into dialysis tubing (D9777, 14 kDa for molecular weight cutoff; Sigma, Aldrich, USA) and co-incubated the dialysis tubing in the bacterial culture at a specific temperature. The details of the experiment are described in the following paragraphs.

For the dialysis tubing preparation, the tubing was cut into 25-cm long pieces, boiled in the washing solution (1 mM EDTA, 0.2% w/v sodium bicarbonate) for 10 mins and washed three times with sterilized water. The treated tubing was then moved into another glass bottle with boiled-EDTA solution (1 mM, pH=8.0) for 10 mins. The tubing was stored at 4°C until use.

To prepare the coral lysate, nubbins of the coral *Acropora* spp. (about 3 cm long for each) were collected from one colony from the northeast coast of Taiwan (25°02'N, 121°98'E). Coral nubbins (n=12) were acclimated in a 30-liter aquarium filled with filtered nature seawater for two weeks at 25°C. The temperature of the water baths was maintained by Hailea 150A aquarium chillers. Light intensity was about 150-μ mol photons $m^{-2}$ $s^{-1}$ under 12/12 h light and dark cycles.

The treatment tank was raised by 1°C per day for 9 days from 25°C to 33°C. The

control tank was maintained at 25°C throughout the experiment (Fig. 1B). HOBO temperature loggers were placed in the tanks to monitor the water temperature. Coral mucus and tissue were collected from three randomly chosen coral nubbins on Days 1 and 9 from the two tanks. The detailed method for coral mucus and tissue collection is described below.

First, the nubbins were rinsed with artificial seawater (ASW). Then, the mucus was "milked" from each nubbin and collected in a 5 mL tube. Later, we sprayed the tissues (with some mucus) from the same nubbin with ASW. In the laminar hood, we transferred mucus (1 mL) and tissue (1 mL) into two separate dialysis tubings. These dialysis tubings were placed into two 50 mL Falcon tubes containing 15 mL bacteria cultures, separately. A total of 12 samples (mucus: n=6, tissue: n=6) from the treatment and control tanks were incubated at 25°C at 200 rpm on Day 1 (Fig. 1B). Another dialysis bag, which contained ASW (1 mL), was put into another 15 mL bacteria culture with three replicates, which acted as controls (25°C and 200 rpm). After six hours of incubation, all the bacteria culture from each tube were collected into several 2 mL Eppendorf tubes and centrifuged at 9000 x *g* for 10 mins. The supernatant was discarded, and the pellet was stored at -80°C until total protein extraction. Bacteria growth (1 mL) was monitored by measuring the OD values at the same time.

On the ninth day, coral mucus and tissue were collected using the same approach described above—except that the sample tubes, which had the coral extract (mucus or tissue) from the treatment tank, were now at 33°C. Three of the ASW sample tubes were incubated at 33°C as well. For the control tank, those sample tubes (containing mucus, tissue or ASW) were incubated at 25°C. Thus, there were a total of 18 samples on the ninth day. After six hours, all of the 15 mL bacteria cultures were collected from the Falcon tubes and the OD value was measured.

**Protein preparation and LC-Mass Spectrometry Method**

Total protein was extracted using ExtractPRO Protein Extraction Reagent (VisualProtein, Energenesis Biomedical, Taiwan) following the manufacturer's instructions. The protein pellet was firstly suspended in 8 M Urea in 50 mM Tris-HCl (pH 8.0) for the extraction.

Protease digestion and labeling before LC-MS analysis were performed as described in Lan et al. [24]. Protein concentrations were measured using a Pierce 660

nm protein Assay kit (Thermo Fisher Scientific, Rockford, USA). Proteins (100 µg) from each sample were reduced in 10 mM dithiothreitol for 1 h at 37°C and alkylated in 50 mM iodoacetamide at room temperature for 30 min in the dark. The protein solution was then diluted with 4 M urea with 50 mM Tris-Cl, pH 8.5 and digested with 250 units/ml of Benzonase (Sigma-Aldrich, St. Louis, USA) at room temperature for 2 h, followed by lysyl endopeptidase (Wako, Japan) digestion (1∶200 (w/w)) at room temperature for 4 h. The protein solution was further diluted down to less than 2 M urea with 50 mM Tris-Cl (pH 8) and incubated with 2 µg of modified trypsin (w/w, 1∶50) (Promega, Madison, WI, USA) at 37°C overnight. This protease digested solution was acidified with 10% trifluoroacetic acid, desalted using an Oasis HLB cartridge (Waters) and dried with a SpeedVac (Thermo Fisher Scientific, Rockford, USA).

After protein digestion, the peptide mixture was labeled with TMT 10-plex and 11-plex isobaric tandem mass tags (catalog no. 90110 and A34808, Thermo Fisher Scientific, Rockford, USA) following the manufacturer's instruction. The labeled peptide samples were then pooled and lyophilized in a vacuum concentrator. The final dried pellet was re-dissolved in 10 of 3% ACN / 0.1% formic acid and its protein profile analyzed by LC-MS/MS (Orbitrap Fusion™ Tribrid™ Mass Spectrometer).

**Data analysis**

Peptide and protein identification were performed using the Proteome Discoverer software (v2.2, Thermo Fisher Scientific) with SEQUEST search engine [25, 26]. Protein identification used 10 ppm and a fragment ion mass tolerance of 0.02 Da. The q-value threshold of 0.01 was used to filter the peptides. In addition, the minimal required peptide length was set to two peptides per protein. Only the proteins that were exhibited in all three replicate samples were kept.

The identified proteins were subjected to Gene Ontology (GO) annotation using the analytical system of Generic Gene Ontology Term Finder (http://go.princeton.edu/cgi-bin/GO Term Finder). The biological pathways of the proteins were acquired from the KEGG Pathway database (http://www.genome.ad.jp/kegg/) coupled with UniProtKB annotation (UniProtKB database, http://www.uniprot.org/). Protein profiles were analyzed for statistical significance analysis with the Proteome Discoverer software. Proteins with a $\log_2$ fold change ($\log_2$FC) <1.5 (or >1.5) and $p < 0.05$ were considered significantly different in

the protein quantification (one-way ANOVA). Furthermore, significantly expressed proteins were clustered into different function categories using the Cluster of Orthologous Groups of proteins (COG) database (https://www.ncbi.nlm.nih.gov/COG). Hierarchical clustering analysis and nMDS analysis were used to visualize the pairwise dissimilarity in protein composition between different samples after transformation (Log (x+1)) by Primer 6 (PRIMER-E, Lutton, Lvybridge, United Kingdom) [27].

In the coral lysate experiment, comparisons among or within the treatment and control experiments revealed the proteins that were differentially produced across the bacteria that were affected by four different factors (coral lysate, tank, temperature and time) (Fig. 1C). (1) The differentially produced proteins of bacteria between the artificial seawater and mucus (or tissue) represented the effect of coral lysate in 25°C or 33°C treatment experiments (25M/25S, 25T/25S, 33M/33S, and 33T/33S). (2) Comparisons between control and treatment experiments on the first day were represented as tank effects (25M/C1M and 25T/C1T). (3) The differentially produced proteins of bacteria between the control and treatment experiments on the last day represented as temperature effects (33M/C2M, 33T/C2T, and 33S/C2S). (4) Time also appeared to have an effect, with the first and last day of the control experiments yielding different proteins (C2M/C1M, C2T/C1T, and C2S/25S).

## Results

### Growth curve of *E. montiporae* in heat stress experiments

To monitor the growth conditions of *E. montiporae* under thermal stress and determine the sampling time for the second experiment (the coral lysate experiment), we estimated the OD values of *E. montiporae* during different growth phases in the three temperatures treatments. The maximum OD value of *E. montiporae* was 1.28 at 25°C and 1.2 at 31°C after 36 hours. However, the growth curve at 33°C was lower than those of the other two temperatures, especially after 24 hours of incubation (Fig. 2A); therefore, *E. montiporae* grew slower at 33°C than at the other two temperatures.

### An overview of protein profiles in heat stress experiments

A total of 1,477,327 unique spectra and 33,841 unique peptides were detected by the LC-MS/MS, and 2,017 unique proteins were identified across the 24 samples. Approximately 41% of the 4,910 predicted open reading frames in the genome of *E. montiporae* CL-33$^{T}$ were identified from all 24 samples. Most proteins are functionally related to the categories in the cellular metabolic process, nitrogen compound metabolic process, and primary metabolic process according to GO (Suppl. Fig. S1). Noteworthily, 59 proteins were annotated in GO as responding to stress—e.g., chaperone proteins and catalase (Suppl. Fig. S1).

Results of hierarchical clustering and nMDS analyses showed that the protein profiles of the *E. montiporae* grown for 48 hours were clearly distinct from the profiles of those grown for 16 and 24 hours (Fig. 2B and C). Comparing within the same incubation time, the protein profiles from 25°C were different from those at 31°C and 33°C (Fig. 2B). Bacteria grown for 16 hours at 25°C were especially separate from other samples.

### Significantly expressed proteins in the heat stress experiments

The number of differentially expressed proteins (DEPs) decreased with increasing incubation time (Fig. 2D, Data set 1). At 16 hours, the number of DEPs in the culture at 33°C (79 proteins) was more than the culture at 31°C (68 proteins). After 24 hours, both *E. montiporae* cultured at 31°C and 33°C showed about 43 to 51 DEPs. In the time course, up-expression proteins were detected less and less in *E. montiporae* cultured at 31°C and 33°C (Fig. 2F). Furthermore, the up-expression proteins in *E. montiporae* cultured at 33°C were less than that at 31°C at every time point. In contrast, *E. montiporae* cultured at 33°C had more down-expression proteins

than *E. montiporae* cultured at 31°C. Sixty-six of the total 154 DEPs were annotated by COGs (Suppl. Table. S1). Except for proteins with unknown function, most of the COG annotated-proteins were in the metabolism category (43.52%).

A heatmap (*z*-score transformed) was made to detect expression patterns of the DEPs in the metabolism category (Fig. 2E). Cluster I shows down-expression proteins after 16 hours at 31°C and 33°C, respectively. Those down-expression proteins are mainly involved in the oxidation-reduction process and pyruvate metabolism. Cluster II shows the up-expression proteins after 16 hours at 31°C and 33°C. These up-expression proteins were involved in the oxidation-reduction process, pyruvate metabolism, glycerolipid metabolism, carbohydrate metabolism, transport, and others. Furthermore, Cluster III shows down-expression proteins after 48 hours, especially the culture at 33°C. These proteins are mainly involved in steroid degradation and ABC transport.

We categorized the proteins related to heat shock-related responses into two groups: (1) heat shock protein and co-chaperone proteins, and (2) antioxidant defense proteins (Suppl. Table. S2). The first group of proteins were up-expressed after 16 hours at 31°C and 33°C. In particular, the small heat shock protein IbpA ($log_2$FC 2.81) , chaperone protein ClpB ($log_2$FC 1.76), lon protease ($log_2$FC 1.72), and periplasmic serine endoprotease DegP ($log_2$FC 1.93) were significantly up-expressed after 16 hours at 33°C when tricorn protease was significantly down-expressed after 48 hours. In the category of antioxidant defense proteins, catalase proteins were down-expressed after 24 and 48 hours in the *E. montiporae* culture at 33°C ($log_2$FC: -2.08 and -2.1).

**Physiological condition of coral and *E. montiporae* in the coral lysate experiment**

The average PAM values decreased from 0.7 to 0.6 following an increase in the water temperature in the experimental tank (Suppl. Fig. S2) (coral color card E3). However, the average PAM value in the control tank was consistent at 0.67 throughout the experiment. In addition, the color of the coral nubbins remained dark brown in the control tank (Suppl. Fig. S2) (coral color card E5).

The average OD values of bacterial cultures changed from 0.5 to 0.9 during incubation (6 hours in total), indicating that the bacterial growth stayed in the exponential phase throughout the experimental time (Suppl. Fig. S3).

**An overview of the protein profiles in the coral lysate experiment**

A total of 2,950,378 spectra and 33,582 unique peptides were detected. Among them, 1,901 unique proteins were identified across all samples, accounting for 38.7% of the 4,910 predicted open reading frames in the *E. montiporae* CL-33$^T$ genome. Most proteins were involved in organic substance metabolic processes, cellular metabolic processes, primary metabolic processes and nitrogen compound metabolic processes (Suppl. Fig. S5).

In the nMDS analysis, one replicate (25M_1) from the mucus treatment at 25°C was not clustered with the other two; it was deemed an outlier (Suppl. Fig. S4A) and removed from subsequent analyses (Suppl. Fig. S4B). Clustering samples were clearly separated by temperature (33°C and 25°C) (Fig. 3A). Furthermore, the samples from the first day in the control tank (C1M and C1T) and treatment tank (25M, 25T and 25S) were clustered together (Fig. 3A).

**DEPs in the coral lysate experiment**

Comparing the effects of coral lysate and temperature, a high number of DEPs (approximately 50) were detected based on changes in temperature, and only a few (seven) were detected to have been caused by the coral lysate (Fig. 3C, Data set 2). Furthermore, most of the DEPs in temperature effect were down-expressed (Fig. 3D). Five proteins, involved in two categories of bacterial heat stress response, were highly up- or down-expressed during the 6 hours of incubation (Suppl. Table S3): the small heat shock protein IbpA, lon protease, catalase, aldehyde dehydrogenase and 4-hydroxyphenylacetate 3-monooxygenase oxygenase component.

COG analysis was performed for all of the DEPs in the metabolism process category (Suppl. Table S4). In those proteins, the *z*-score heatmap displayed 18 down-expressed proteins related to steroid degradation in mucus and tissue treatments (Fig. 3E). Moreover, there were also eight down-expressed proteins involved in pyruvate metabolism (Fig. 3E).

To gain more insights into the potentially important cellular mechanisms of bacteria-coral host interactions, proteins that underwent significant changes but had a $\log_2FC < 1.5$ were also discussed. Hence, if we consider all of the differential expression proteins ($p < 0.05$, regardless of their $\log_2FC$), 10 to 70 proteins were influenced by the coral lysate effect (Fig. 4A, Suppl. Table S5). Furthermore, many differential expression proteins were down-expressed in the tissue treatment at 25°C (25T/25S), but up-expressed in the tissue treatment at 33°C (33T/33S) (Fig. 4B). In

addition, most of the $log_2FC$ of differential expression proteins were <1 in the tissue treatments at 25°C and 33°C (Fig. 4C). Noteworthily, 14 differential expression proteins were detected in the mucus treatment at 25°C (25M/25S) and had a $log_2FC$ > 1; these 14 proteins were involved in the signal recognition particle pathway and toxin-antitoxin system (Table 1).

The tank and time effects were very low because most proteins maintained a similar protein abundance ($log_2FC$ < 1.5; Suppl. Fig. S6 and S7).

## Discussion

*Endozoicomonas* is a core bacterial group in corals that plays a role in coral health, and often decreases rapidly in corals under heat stress [5, 6, 10, 28]. However, how these bacteria respond to changes in temperature and coral hosts during heat stress were unknown until now. This study is the first to provide insights into the physiological changes of *E. montiporae* to heat stress and host factors using a proteomic approach. We designed two experiments to clarify how much temperature and the coral host influence the bacterium, and how bacterial metabolism is affected by both factors. Our study shows that temperature is the main factor affecting protein abundance in the bacterium, though the coral host also has some influence. More details are given in the following paragraphs.

### Impact of high temperature on the protein profiles of *E. montiporae*

Temperature was the main factor in this study affecting changes in the protein profiles of *E. montiporae*. Previous studies have shown that *Endozoicomonas* are highly sensitive to temperature changes in various corals [5, 9, 10, 17, 29]. Most recent studies also show the same phenomenon—e.g., at 32°C, *Endozoicomonas* populations' relative abundances rapidly halved in the coral *Pocillopora damicornis* within 48 h [30]. In the coral *Stylophora pistillata*, the resident *Endozoicomonas* population quickly decreased at 32°C and 34.5°C over 6 hours [28].

However, what exactly caused these reductions in *Endozoicomonas* is still mostly unknown. There are some possible reasons for the phenomenon. One is that heat stress made it difficult for the bacteria to live and grow in their host corals, and the bacteria consequently left for other habitats. Or other bacteria outcompeted the *Endozoicomonas*. Our molecular study clearly shows that a stressful physiological status is one of the major factors that causes *Endozoicomonas* populations to decline in corals. At 33°C, those crucial metabolisms such as the proteins for central metabolisms were mostly down-expressed in this study. In other words, *Endozoicomonas* physiology—including growth—was clearly influenced by heat stress. Protein expression changed starkly for a number of proteins, such as heat-shock proteins, stress-associated reactive oxygen species, and other important metabolism-related proteins. We elaborate on each of these below.

The heat shock-related response is one of the fundamental responses that most bacteria have to heat stress [31]. How heat shock proteins (HSPs) help bacteria adapt and survive in thermal stress environments had been widely explored by previous studies [32-36]. In this study, a number of HSPs and co-chaperones were significantly increased in *E. montiporae* in the two heat stress experiments; for example, small heat shock protein IbpA, chaperone protein ClpB, lon protease, and periplasmic serine endoprotease DegP were all up-expressed after 16 hours at 33°C ($log_2$FC values were about 2). Previous studies suggested that those proteins contribute to the resolution of protein aggregates to confer superior heat tolerance [37-39]. In particular, we found that IbpA and lon protease were quickly expressed during the 6 h of incubation at 33°C in the coral lysate experiment (meaning $log_2$FC of IbpA was 2.36 and lon protease was 1.16). The major consequences of increasing temperature for cells are protein unfolding and aggregation, with concomitant loss of function [38]. Those unfolding or aggregated proteins could be degraded and cleaned by both IbpA and Lon proteases under heat stress [40]. Thus, *E. montiporae* responded quickly to the expression of HSPs to prevent the irreversible aggregation or misfolding of proteins in the early stages of the heat stress treatment, especially at 33°C.

Reactive oxygen species (ROS) are crucial to the coral holobiont, and they are tightly associated with coral physiology under heat stress and bleaching conditions [41, 42]. Removing ROS is critical for corals to maintain their homeostasis during stress. In this study, we also detected a rapid change in the proteins that are responsible for ROS-related metabolisms. For example, catalases are mainly responsible for removing ROS in many bacterial cells. Similarly, *E. montiporae* expressed more catalases ($Log_2$FC: 1.91) and other antioxidant defense proteins (i.e. hydroperoxy fatty acid reductase) at 31°C that would facilitate the bacterium to remove extra ROS from cells and prevent further damages by ROS. However, the expression of those proteins was decreased at 33°C, suggesting that the bacterium's physiology was not functioning properly. The growth curve of the bacterial culture at 33°C also plateaued earlier than the other two temperature treatments, also indicating that the bacteria grew incompetently at 33°C. Additionally, we also noticed that energy-related metabolism under heat stress in the coral lysate experiment was significantly decreased—such as the key proteins, phosphoenolpyruvate synthase and

399 phosphoenolpyruvate carboxykinase—also suggesting that the bacteria had poor
400 cellular physiology at 33°C.
401 The temperature effect also greatly reduced ecotin levels in *E. montiporae*
402 (meaning of $\log_2$FC was -2.51). Ecotin, which is usually located in periplasmic space
403 of many bacteria, is both a serine protease inhibitor and functionally related to
404 bacterial defenses against bacteriophages and other bacteria [43-46]. A recent study
405 found that ecotin plays a role in the defense of *Escherichia coli* against attacks from
406 *Vibrio cholera* [47]. The decrease in ecotin levels in *E. montiporae* at high
407 temperature might weaken the bacterium's defenses against other bacteria.
408 Intriguingly, Lee et al. [9] found that the *E. montiporae* population decreased at high
409 temperatures while *Vibrio* spp. increased rapidly under the same conditions. Perhaps
410 the phenomenon of these opposing changes in *E. montiporae* and *Vibrio* spp. was
411 partially the result of *E. montiporae* becoming more defenseless at the high
412 temperature, allowing *Vibrio* spp. to colonize the coral tissues. Myint et al. [47] also
413 showed that an *E. coli* ecotin mutant became more susceptible to *V. cholera* attack
414 than did the wild type strain. However, the relationship between ecotin and the
415 defense mechanism in *E. montiporae* requires mutation experiments to prove.
416
417 **Effects of heat stress-induced coral lysate on *E. montiporae***
418 In addition to temperature, we found that host factors changed a number of
419 proteins in the co-incubation experiment with coral lysates, suggesting that bacterial
420 physiological responses to heat stress are constituted by both temperature and host
421 factors. Under stressful conditions, coral host factors also affected or influenced the
422 bacterium. Some intriguing proteins are discussed as follows.
423 First, N,N'-diacetylchitobiose-specific EIIA component (ChbA) of *E. montiporae*
424 was up-expressed with a high value of $\log_2$FC (i.e., 1.29) in the thermal stressed-coral
425 mucus samples. ChbA protein participates in transporting cellobiose and belongs to
426 the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS system), a
427 major carbohydrate active transport system in bacteria [48]. Cellobiose is a sugar
428 component of cellulose and the major composition of algal cells [49, 50]. A recent
429 report shows that *Symbiodiniaceae* increased in coral mucus after incubating at 31°C
430 [51], suggesting that the cellobiose concentration increases in the mucus at higher
431 temperatures. In our study, the increase in ChbA levels in *E. montiporae* could be

induced to transport increasing amounts of cellobiose. Moreover, high ChbA expression might also help in the removal of unwanted algal components in coral host by the PTS system, as suggested by Neave et al. [22], who discovered many genes of the PTS system encoded in *Endozoicomonas* genomes.

Second, the proteins involved in steroid degradation in *E. montiporae* had more down-expression in the mucus and tissue treatments than in the seawater treatment under heat stress (Fig 3E). For example, the $\log_2$FCs of 3-oxosteroid 1-dehydrogenase were -2.01 and -2.3 in mucus and tissue treatments, but -1.69 in the seawater treatment. This implies that the thermal-stressed coral lysate had additional effects on the expression of proteins during the heat stress. Why did the temperature effect cause the down-expression of proteins that were involved in steroid degradation in *E. montiporae* under heat stress? The steroid uptake mechanisms in bacteria have remained unclear because of high physiological and structural differences among bacterial groups [52]. A previous study, however, demonstrated that down-expression of steroid metabolism processes in the coral *Acropora* spp. and *Symbiodiniaceae* during the thermal stress [53]. Hence, we speculate that the phenomenon that the proteins involved in steroid degradation in *E. montiporae* decreased could be caused by the lower concentration of steroids in the coral lysate after the heat stress treatment. This hypothesis, however, requires more experimentation to prove.

It is worth addressing that fewer proteins underwent significant changes in the coral lysate effect than the temperature effect in the coral lysate experiment. This could be partially due to a limitation in the experiment—that the dialysis tubing with a pore size of 14 kDa used to load coral lysate only allowed small molecules to pass into the bacterial culture. Molecules such as glucose, vitamin B12, inorganic salt, and cytochrome C can pass through the tubing, but larger molecules—such as liposome and starch—could not. Thus, the effect of host factors on *E. montiporae* are likely to be underestimated.

**Three protein groups in *E. montiporae* were more active only with unheated coral mucus**

Coral mucus composition is dynamic of changes in host physiology and environmental factors. For example, Lee et al [9] demonstrated that there was a shift in the proportion of different sugar components in coral mucus after heat stress.

Interestingly, we found that *E. montiporae* showed significant changes in protein expressions, and that $log_2$FC values were higher than 1 when the bacteria were co-incubated with mucus at 25°C compared with the seawater samples (Table 1). Most of the proteins were involved in three types of protein groups: signal recognition particle pathway, type II toxin-antitoxin system and V-type ATPase. These significantly changed proteins were presented only with unheated coral mucus rather than heat stress induced-coral mucus. This indicates that specific factors in coral mucus must change during heat stress and affect the expression of those proteins in *E. montiporae*. In other words, those highly expressed protein groups are likely to be functionally related to coral mucus under regular conditions.

The first group comprised six significantly changed proteins involved in the signal recognition particle pathway (SRP). SRP is essential for delivering the nascent protein to properly localize the inner membrane and mediate secretory proteins [54, 55]. SRP depletion may cause the accumulation of mis-localized proteins in bacteria [56]. Many reports showed various evidence that the SPR pathway plays important roles in membrane integrity, cell physiology, and viability [57-60]. In this study, high expression of the SRP is specifically associated with coral mucus, suggesting that the SRP might be functionally crucial for *E. montiporae* living in or passing coral mucus during colonization.

The second group is two highly expressed proteins, HigB and HigA, also called Type II toxin-antitoxin (TA) system, which is present in many bacteria [61, 62]. They are a pair of proteins composed of a stable toxin and its cognate antitoxin protein. Under normal growth conditions, they form a tight and nontoxic complex. When the antitoxin is degraded by proteases under environmental stress, cell fundamental functions such as growth, gene regulation and biofilm formation are impaired [63, 64]. Hence, the high expression of HigB and HigA also indicates that *E. montiporae* was in a non-stressful condition. Moreover, the TA system is also functionally related to defense in bacteria. For example, Sberro et al. [65] demonstrated that the TA system in *E. coli* provides resistance against the T7 phage. Our proteomic results suggest that the up-expression of the TA system would be helpful to *E. montiporae* in defense.

The third group is V-type ATPase. The $log_2$FC of V-type ATPase subunit E in *E. montiporae* increased to 1.63.V-type ATPase is a membrane-embedded protein

complex [66]. V-type ATPase was found not only in eukaryotes but also archaea and bacteria, including *Thermus thermophilus*, *Enterococcues hirae, Lactobacillu plantarum*, and *Clostridium fervidus* [67-71]. V-type ATPase in bacteria have multiple functions, including proton pump, ion pump and ATP synthesis [72-75]. Moreover, V-type ATPase also plays a role in maintaining physiological homeostasis for bacteria [76-78]. Although the function of V-type ATPase is still unclear in *E. montiporae*, highly expressed V-type ATPase in *E. montiporae* serves as potential evidence of a specific response that is functionally related to coral mucus in coral-*E. montoporae* interactions.

## Conclusion

To establish a working model for the in-depth investigation of bacteria-coral interactions, this study used the cultivable, dominant coral-associated bacterium *E. montiporae* as a model organism to dissect changes in proteomic profiles of the bacteria, responding to heat stress and heat stressed-coral lysate. We successfully detected a number of pivotal proteins related to the heat-stress physiology of *E. montiporae* that provides new research directions in the molecular microbiology and microbial ecology of *E. montiporae* in the coral holobiont. This is one of few primary works exploring the molecular mechanisms underlying physiological changes in coral symbiotic bacteria, and we anticipate that this study will inspire more molecular studies on coral microbes that will better elucidate the detailed coral-bacteria interactions.

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**Conflict of Interest**

The authors declare that they have no conflict of interest.

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**Figure legend:**

**Fig.1 Experimental designs and setup. (A)** Experiment to identify differentially expressed proteins when the bacterial culture (*Endozoicomonas montiporae* CL-33$^T$) was exposed to different heat stresses. **(B)** Setup for coral lysate experiment, where coral mucus and tissue were incubated with cultures of *E. montiporae* CL-33$^T$ for 9 days, with temperature increased by 1°C per day (25°C to 33°C). **(C)** Overview of the different factors analyzed throughout the coral lysate experiment. * denotes artificial sea water.

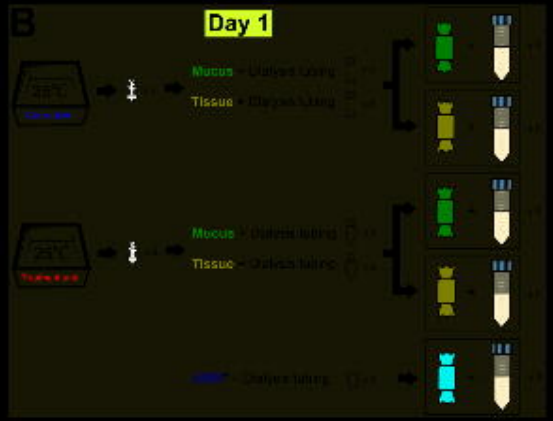
**Fig.2 Growth pattern and quantitative proteomics during heat stress**. (**A)** Growth curves of *Endozoicomonas montiporae* CL-33$^T$ at 25°C, 31°C, and 33°C in heat stress experiments. The quantitative proteomics analysis of heat stress experiments **(B–F).** (**B)** Hierarchical clustering and **(C)** nMDS ordination represent the similarity between the protein profiles from different samples. **(D)** The number of significantly changed proteins at each incubation time point at 31°C and 33°C. **(E)** Hierarchical clustering based heatmap visualization of the metabolism-related (from COG functional

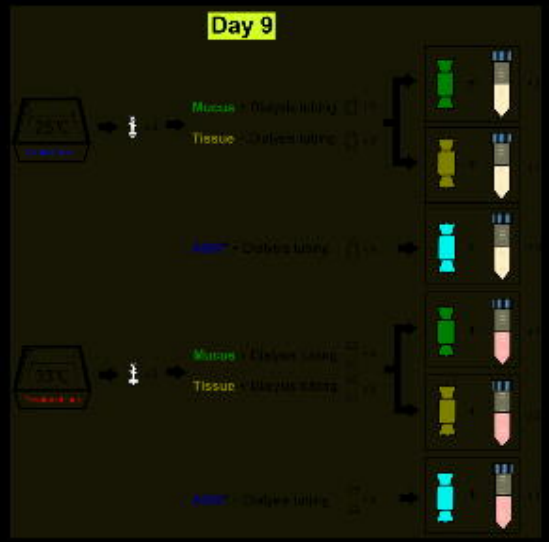
852 categories) proteins that showed significantly changes in any one of the samples. The
853 heatmap displays the relative protein abundance (*z*-score transformed) from low
854 (blue) to high (yellow) per row. The columns represent three time points (16, 24, and
855 48 h) at two incubation temperatures (31°C and 33°C). The significantly changed
856 proteins were marked by a white circle. The right side of the image show the protein
857 functions by category. Numbers in brackets indicate the number of proteins when
858 there are multiple proteins in one category. **(F)** The number of proteins up-expression
859 and down-expression in each incubation time at 31°C and 33°C.
860
861 **Fig.3 Quantitative proteomics analysis of coral lysate incubated with bacterial**
862 **cultures at different temperatures**. **(A)** Hierarchical clustering and **(B)** nMDS
863 ordination of different samples similarities. **(C)** The number of significantly changed
864 proteins that were affected by coral lysate (25M/25S, 25T/25S, 33M/33S, and
865 33T/33S) and temperature (33M/C2M, 33T/C2T, and 33S/C2S). **(D)** The number of
866 proteins with increased and decreased expression in each comparison. **(E)**
867 Hierarchical clustering heatmap visualization of the metabolism-related (from COG
868 functional categories) proteins that have significantly changed in any of the three
869 treatments (33M/C2M, 33T/C2T, and 33S/C2S). The heatmap displays the relative
870 protein abundance (*z*-score transformed) from low (blue) to high (yellow) per row.
871 The columns names (33M, 33T, and 33S) represent three treatments. Relative protein
872 abundance (*z*-score transformed) is graded from low (blue) to high (yellow) in each
873 row. The bracketed numbers represent the number of proteins if there are multiple
874 proteins in a category. The significantly changed proteins are marked by a white
875 circle.
876
877 **Fig.4 The quantitative proteomics analysis comparing the effects of different**
878 **coral lysates (25M/25S, 25T/25S, 33M/33S, and 33T/33S).** (**A)** Bar plots on counts
879 of significantly changed proteins. (**B)** The numbers of proteins with increased (black)
880 and decreased (grey) expression. (**C)** The distributions of statistical significance (-
881 $\log_{10}$ (*p*-values)) and magnitude of change ($\text{Log}_2$ fold change) for all the proteins in

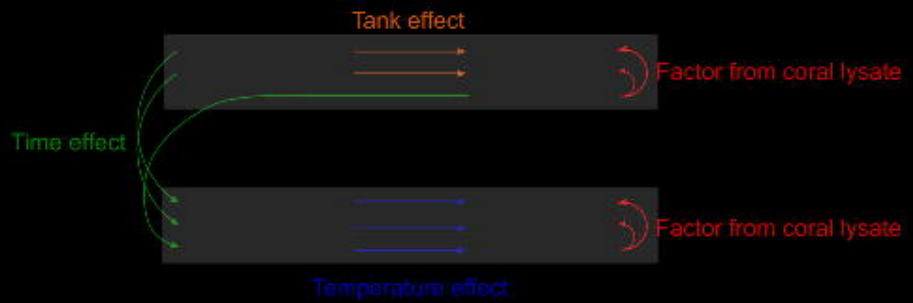
four different comparisons represented by volcano plots. The proteins for which there was no significant difference are marked in gray circles. Significantly changed proteins are marked in different colors based on different levels of $\log_2$ fold change. (red: $\log_2$ fold change > 1.5, orange: $\log_2$ fold change > 1, and green: $\log_2$ fold change < 1).

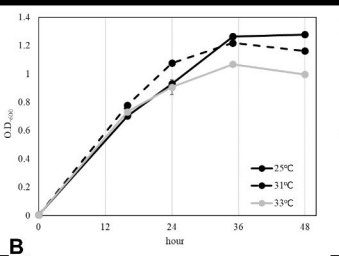
Table 1. List of significantly changed proteins ( $p$-value < 0.05) which have $\log_2$ fold change >1 from the coral lysate comparisons. - : no significantly ( $p$-value > 0.05) or the $\log_2$ fold change <1.

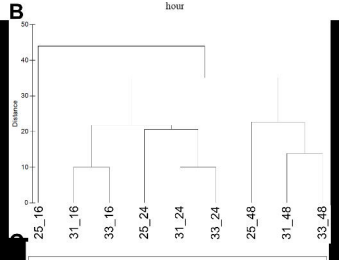
| | $Log_2$ fold change | | | |
|---|---|---|---|---|
| Protein name | 25M/25S | 25T/25S | 33M/33S | 33T/33S |
| 30S ribosomal protein S20 | 2 | - | - | - |
| 50S ribosomal protein L20 | 1.73 | - | - | - |
| 50S ribosomal protein L16 | 1.64 | - | - | - |
| 30S ribosomal protein S2 | 1.22 | - | - | - |
| 50S ribosomal protein L5 | 1.2 | - | - | - |
| Signal recognition particle protein | 1.46 | - | - | - |
| V-type proton ATPase subunit E | 1.63 | - | - | - |
| mRNA interferase HigB | 1.56 | - | - | - |
| Antitoxin HigA-2 | 1.42 | - | - | - |
| Na(+)-translocating NADH-quinone reductase subunit C | 1.17 | - | - | - |
| Response regulator protein TodT | 1.14 | - | - | - |
| hypothetical protein | 2.48 | - | - | - |
| hypothetical protein | 2.21 | - | - | - |
| hypothetical protein | 1.43 | - | - | - |
| Long-chain-fatty-acid--CoA ligase FadD15 | | -1.26 | - | - |
| PTS system N,N'-diacetylchitobiose-specific EIIA component | - | - | 1.29 | - |
| 3-isopropylmalate dehydrogenase | - | - | 1.16 | - |
| 50S ribosomal protein L20 | - | - | - | 1.2 |
| hypothetical protein | - | - | - | 1.25 |
| hypothetical protein | - | - | - | 1.08 |
| hypothetical protein | - | - | - | 1.05 |

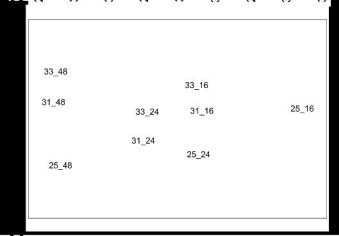

B
Day 1
Mucus
Tissue

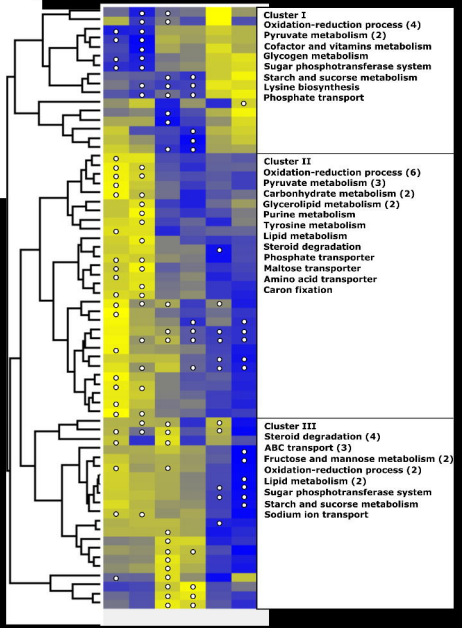

Day 9
Mucus
Tissue

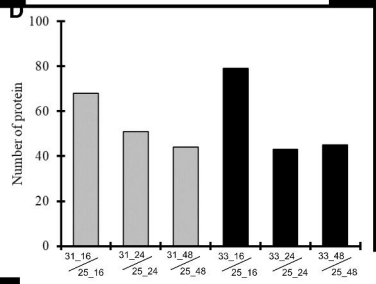

Tank effect
Factor from coral lysate
Time effect
Factor from coral lysate
Temperature effect

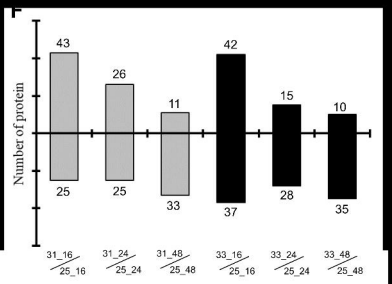

1.4
1.2
1
0.8
0.6
0.4
0.2
0
O.D.600
0
12
24
36
48
hour
25ºC
31ºC
33ºC

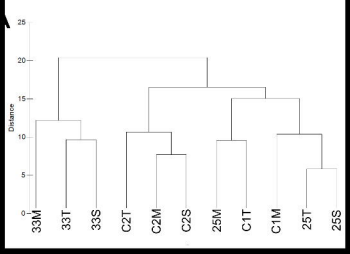

B
50
40
30
20
10
0
Distance
25_16
31_16
33_16
25_24
31_24
33_24
25_48
31_48
33_48

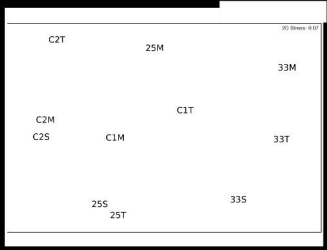

33_48
31_48
33_16
33_24
31_16
25_16
31_24
25_24
25_48

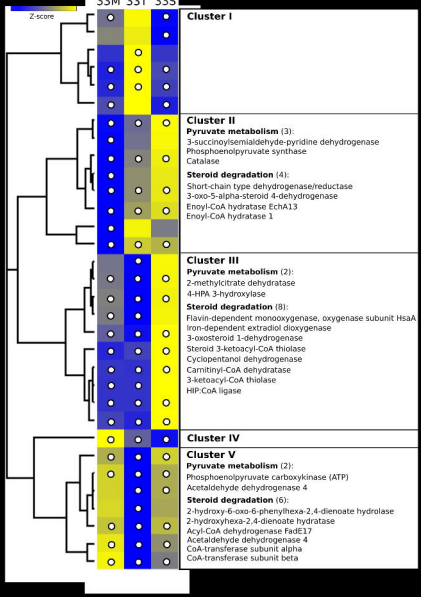

Cluster I
Oxidation-reduction process (4)
Pyruvate metabolism (2)
Cofactor and vitamins metabolism
Glycogen metabolism
Sugar phosphotransferase system
Starch and sucrose metabolism
Lysine biosynthesis
Phosphate transport
Cluster II
Oxidation-reduction process (6)
Pyruvate metabolism (3)
Carbonhydrate metabolism (2)
Glycerolipid metabolism (2)
Purine metabolism
Tyrosine metabolism
Lipid metabolism
Steroid degradation
Phosphate transporter
Maltose transporter
Amino acid transporter
Caron fixation
Cluster III
Steroid degradation (4)
ABC transport (3)
Fructose and mannose metabolism (2)
Oxidation-reduction process (2)
Lipid metabolism (2)
Sugar phosphotransferase system
Starch and sucrose metabolism
Sodium ion transport

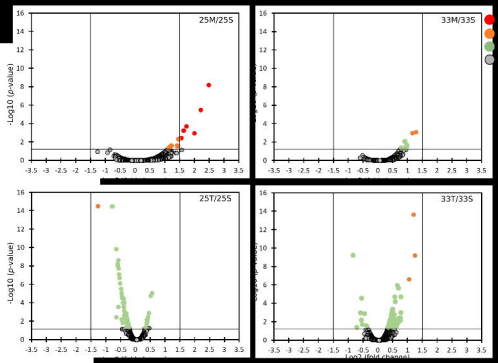

D
100
80
60
40
20
0
Number of protein
31_16/25_16
31_24/25_24
31_48/25_48
33_16/25_16
33_24/25_24
33_48/25_48


F
Number of protein
43
26
11
42
15
10
25
25
33
37
28
35
31_16/25_16
31_24/25_24
31_48/25_48
33_16/25_16
33_24/25_24
33_48/25_48


Distance
25
20
15
10
5
0
33M
33T
33S
C2T
C2M
C2S
25M
C1T
C1M
25T
25S


2D Stress: 0.07
C2T
25M
33M
C1T
C2M
C2S
C1M
33T
25S
25T
33S


33M
33T
33S
Z-score
Cluster I
Cluster II
Pyruvate metabolism (3):
3-succinoylsemialdehyde-pyridine dehydrogenase
Phosphoenolpyruvate synthase
Catalase
Steroid degradation (4):
Short-chain type dehydrogenase/reductase
3-oxo-5-alpha-steroid 4-dehydrogenase
Enoyl-CoA hydratase EchA13
Enoyl-CoA hydratase 1
Cluster III
Pyruvate metabolism (2):
2-methylcitrate dehydratase
4-HPA 3-hydroxylase
Steroid degradation (8):
Flavin-dependent monooxygenase, oxygenase subunit HsaA
Iron-dependent extradiol dioxygenase
3-oxosteroid 1-dehydrogenase
Steroid 3-ketoacyl-CoA thiolase
Cyclopentanol dehydrogenase
Carnitinyl-CoA dehydratase
3-ketoacyl-CoA thiolase
HIP:CoA ligase
Cluster IV
Cluster V
Pyruvate metabolism (2):
Phosphoenolpyruvate carboxykinase (ATP)
Acetaldehyde dehydrogenase 4
Steroid degradation (6):
2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase
2-hydroxyhexa-2,4-dienoate hydratase
Acyl-CoA dehydrogenase FadE17
Acetaldehyde dehydrogenase 4
CoA-transferase subunit alpha
CoA-transferase subunit beta


25M/25S
33M/33S
25T/25S
33T/33S
-Log10 (p-value)
Log2 (fold change)