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Title: Deciphering Probiotic Effects of *Sulfitobacter pontiacus* Strain BL28 on the Host Sea Cucumber *Apostichopus japonicus*

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Author Contributions

Conceptualization: SM, YS and TS; Research and Data analysis: RK, TN, AR, SM and TS;

Writing - original draft preparation: TN, AR, SM and TS; Writing - review and editing: all;

Resources: YS and TS. All authors contributed to the article and approved the submitted version.

Running title

Deciphering probiotic effects of *S. pontiacus* BL28 on the host sea cucumber

Abstract

Recently, sea cucumber has been an increasingly important marine invertebrate in the fields of EcoDevo and fisheries studies. After the discovery of *Sulfitobacter pontiacus* strain BL28, which is a growth promoting probiotic bacteria isolated from blastula of *Apostichopus japonicus*, host-microbes interaction has become a major new focus of study to be unveiled. Previous genome assessments of the strain BL28 hypothesized involvements of cobalamin/B12 biosynthesis, type IV secretion system, polyhydroxybutyrate (PHB) production and sulfate metabolism for host growth promotions. Here, we performed *A. japonicus* host transcriptome after five weeks of supplementation with *S. pontiacus* BL28. Unexpectedly, the host transcriptome revealed significant gene regulations of the BL28 supplementation on the host lipid and chondroitin metabolisms and transcriptionally “rest” status of heat shock protein genes un-expressed. Physiological and environmental crossover by the *S. pontiacus* BL28 supplementation may trigger stress-free growth promotion in the host sea cucumber. These symbiotics-type probiotics could help herald the future eco-friendly aquaculture of sea cucumber.

Keywords: Probiotics, *Sulfitobacter pontiacus*, Sea cucumber, *Apostichopus japonicus*, Growth promotion, Transcriptome

Introduction

Sea cucumber, a member of echinoderms, have attracted growing attention in ecological developmental biology (EcoDevo) research because of their unique biological features, such as their ability to regenerate the entire gut and associated nervous system, the presence of a mineralized skeleton during the larval stage and the evolution of their immune systems [1]. Currently, at least, a total of 27 sea cucumber genome assemblies have been deposited in the public database [e.g. [2]] and RNA-seq could be a key technology to elucidate key genes related to gut regeneration, immune system, environmental stress and other biological processes [1].

Sea cucumbers are also an important fisheries resource. Due to their slow growth and overfishing, however, wild resources are now under threat [3-5]. In particular, *Apostichopus japonicus* is the primary species in fisheries in Northeast Asian countries, and has seen significant development in

aquaculture, particularly in countries like Japan and China. Global total production of *A. japonicus* in 2022 was around 262,543 metric tonnes (MTs), including 248,508 MTs from aquaculture in China and 5,100 MTs of *A. japonicus* captured in Japan (FishStatJ, 2025, FAO, Global Capture Production, Fisheries and Aquaculture). The production in China exceeded 90% of the total production. The value of whole industry chain in China was reached to \$9 billion [6]. However, we have still face problems in production instability, slow growth, growth variations, disease and so on [3-5]. To address these issues, probiotics with growth-promoting effects in sea cucumber juveniles have been explored, and *S. pontiacus* strain BL28 was recently discovered during pioneer microbiome assessments of early-stage *A. japonicus* specimens [4-5,7]. After feeding with the BL28 supplemented diets, slight (1-2%) increases of *Sulfitobacter* abundance in fecal microbiota of sea cucumber juveniles indicated successful administrations of the bacterial strain into the gut of the host sea cucumber juveniles [5]. Microbiological assessments of BL28 including genome analyses and electron microscopic observation hypothesized that involvements of cobalamin/B12 biosynthesis, type IV secretion system, polyhydroxybutyrate (PHB) production and sulfate metabolism could be related to the growth promotion in sea cucumber juveniles [5].

However, the biological mechanism of how *S. pontiacus* BL28 promotes the growth in sea cucumber juveniles remains unclear. Here, we set up individual sea cucumber host transcriptome based on Nanopore RNA-seq technology and performed host sea cucumber transcriptome analyses after supplementation with the growth-promoting probiotics of *S. pontiacus* BL28 aiming to reveal the biological interactions between the host marine echinoderms and the pioneer microbes.

Materials and Methods

Probiotics Trials of Sea Cucumber Juveniles

Juvenile sea cucumber were collected in October 2023, in Taisei, Hokkaido, Japan, and transferred to the laboratory. After acclimation of those juveniles to laboratory conditions at 18°C, five juveniles were randomly distributed in a 1 L aquarium [5]. The aquarium was equipped with an inlet and outlet filter, respectively, of 0.22 µm PES filter (Hawach Scientific, Shaanxi Province, China) avoiding cross contamination by aquarium exhaust (Fig. S1). *S. pontiacus* BL28 was cultured in Marine Broth (Difco, Becton Dickinson, Franklin Lake, USA) at 20°C and after washing the bacterial cell pellets, live bacterial cells were supplemented with a commercial feed obtained from Hokkaido Aquaculture Promotion Corporation, Japan. In brief, the experimental diet was prepared by adding 0.05 g of powder feed to 1 mL of artificial seawater or BL28 suspension at a final concentration of 10⁴ CFU/mL according to Yu *et al.* (2023) [5]. Both a control

with no BL28 supplementation group and the BL28 supplemented diet group were set in three aquaria, respectively, and fed at 18°C with a salinity of 3.3-3.5% using natural seawater collected at the sea cucumber farm under dark conditions. Over five weeks, feeding and cleaning were performed twice weekly (Fig. 1) [5]. After the fourth week, the body length of each sea cucumber was measured after anesthetization. At the end of the fifth week after 1.5 hours of final feeding without cleaning, each juvenile was gently taken from an aquarium using bamboo tweezers and immediately homogenized using a BioMasher II (Nippi, Tokyo, Japan) in 750 µL of TRIzol LS solution. After addition of 250 µL of Nuclease-free water, homogenized samples were immediately frozen at -80°C before RNA extraction. The whole body of the juvenile was used because if too small, organs and/or tissues could not be separated accurately thus eliminating any other biological transcriptome noises due to handling.

RNA Extraction and Purification

After thawing the sea cucumber samples, total RNA was extracted and purified using TRIzol LS Reagent followed by DNA removal using RQ1 RNase-Free DNase (Promega, Madison, WI, USA) and further purification using RNeasy PowerClean Pro Cleanup Kit (Qiagen, Venlo, Netherlands). Three individuals from the BL28 treated group and the control group, respectively, were used in this study. In both groups, the three individuals with the highest specific growth rates (SGRs) were sequenced (Fig. 1). In preliminary experiments, the use of total RNA and Nanopore PCR-cDNA Barcoding Sequence kit was able to provide enough quality and sequence length, and sequence reads were properly mapped to reference *A. japonicus* genome sequences, therefore mRNA preparation protocol was not used for further RNA-seq. We also faced difficulties in keeping mRNA amounts for quantitative reverse transcription (qRT)-PCR validation after RNA-seq, so we decided to assess host transcriptomic response only based on Nanopore PCR-cDNA data set because of the very similar theory of qRT-PCR and PCR-cDNA approach (RT and transcript amount comparison among groups) to compare transcripts between the two groups.

Sea Cucumber Host RNA-Seq and the Downstream Analyses

To obtain longer sequences efficiently from individual juveniles of *A. japonicus*, PCR-cDNA Barcoding Sequence kit SQK-PCB109 (Oxford Nanopore Technologies, Oxford, UK) was used for the host RNA-seq to decipher the effects of *Sulfitobacter* probiotics feeding on the host. The library was prepared according to the standard protocol provided by Nanopore company. The libraries were loaded into an R9.4 flow cell (FLO-MIN 106) and a 72-hour sequencing run was performed using MinION Mk1C platform with MinKNOW 23.07.12 software. Basecalled data

was generated using the high-accuracy model, 450 bps. Read quality controls, reference genome mapping to *A. japonicus* genome (GCA_002754855.1), feature count output, and primary individual transcriptomic profiling were performed using Dusselpore [8] with minor modifications. For listing differential expressed genes (DEGs), edgeR 4.0.16 on R 4.3.3 platform with the QLF test was used. For Gene Ontology (GO) enrichment, a web-based ShinyGo 0.8 which includes the *A. japonicus* genome data set [9] was used. DEGs list and these related isoforms were evaluated using the KEGG and AlphaFold database connected to UniProtKB to confirm protein structures and functions [10-11]. Integrate genome view (IGV) was used to visualize genome mapping data [12].

Results and Discussion

RNA-Seq Statistics

BL28 probiotics reproducibly affected the growth of *A. japonicus* juveniles (Fig. 1) [5]. Transcriptome reads with around 5 Gb raw sequences from the three individuals with the highest SGR were successfully generated (Fig. S2). The number of reads ranged from 3.9 to 6.3 million with an average length from 527 to 844 bp (Fig. S2A). The average quality values (Phred score) were around 20. After quality control and aligning to the *A. japonicus* genome using minimap2 [13], 49% to 66% mapping ratios were observed (Fig. S2B) allowing for downstream analysis of host transcriptomic regulation by BL28 supplementation. QC and read mapping were automatically and efficiently performed using a modified Dusselpore pipeline [8].

Host Transcriptome Profiling

The Dusselpore implements a primary gene expression pipeline that lists up top 30 genes with high expression variation [8]. The primary heatmap showed these genes with high variation in each of the juvenile *A. japonicus* treatment groups (Fig. S2C). This gene list included genes with low statistical significance, therefore the DEG list with $p < 0.05$ and $q < 0.05$ was further updated based on QLF test implemented in edgeR program and finally 12 and 30 up-regulated genes in BL28 treatment group and control group were listed, respectively (Table 1). Unfortunately, genes responsible for transcriptional factors in *A. japonicus* have not been accurately identified yet, so we evaluated up- and down-regulated genes compared to normalized read counts of house-keeping genes, which have recently been re-evaluated using human data such as genes responsible for glucose-6-phosphate isomerase, proteosome subunit beta type-4 and 2 [14], gene-by-gene. Finally, all genes in the DEG list were evaluated to be up-regulated genes in each group below average counts (Table 1). The DEG list was unlikely to be identical to the gene list assessed by high variation (Table 1 and Fig. S2C), however, 7 and 15 genes in the DEG list overlapped with the Dusselpore top 30 in each group, respectively (Table 1).

Up-regulated DEGs in the BL28 treated group included genes annotated to putative isocitrate

dehydrogenase (BSL78_28981), barrier to autointegration factor (BSL78_07950), histone H1-beta (BSL78_26408), putative carbohydrate sulfotransferase 11 (BSL78_22910), putative phospholipase B1, membrane-associated (BSL78_19058), putative pancreatic triacylglycerol lipase (BSL78_18401) and proprotein convertase subtilisin/kexin type 9 (BSL78_25086). However, further primary to tertiary protein structure comparison to the related human gene products revealed that putative carbohydrate sulfotransferase 11 (BSL78_22910) could be related to human genes (Fig. S3A). BSL78_19058 is likely to encode a part of phospholipase B1 gene as Q6P1J6 as a structural template, with BSL78_19057 and 19059 genes (Fig. S3B). A gene annotated to isocitrate dehydrogenase (IDH) (BSL78_28981) only encodes C-terminal half region of human cytosolic NADP-IDH (O75874), in particular, the “big domain” retaining NADP binding sites of Arg314 and His315 in O75874 [15] and an adjacent gene (BSL78_28982) is likely to encode N-terminal half region in completing entire IDH (Fig. S3C).

Those of DEGs in the control group included genes annotated to putative SAM pointed domain-containing Ets transcription factor-like (BSL78_17428), putative deleted in malignant brain tumors 1 protein (BSL78_17698), putative SPRY domain-containing SOCS box protein 4 (BSL78_08486), complement component C3 (BSL78_27279), putative collagen alpha-1(I) chain (BSL78_22043), α -amylase (BSL78_04898), heat shock protein 70 (BSL78_15519, BSL78_02893, BSL78_02917, BSL78_15518, BSL78_02892), matrix metalloproteinase 16 (BSL78_23900), putative sodium-dependent proline transporter-like (BSL78_15313), heat shock protein 26 (BSL78_13663, BSL78_08844, BSL78_13662, BSL78_13664), putative thioredoxin-like (BSL78_24942) and kazal-type proteinase inhibitor (BSL78_12786). However, further protein structure comparison to the related human gene products also revealed that putative SPRY domain-containing SOCS box protein 4 (BSL78_08486), α -amylase (BSL78_04898) and putative thioredoxin-like (BSL78_24942) could be related to human gene products (Fig. S3EF). The gene annotated to complement component C3 (BSL78_27279) is likely to encode whole complement C3 with BSL78_27282, which is located adjacent to BSL78_27279 (Fig. S3D). Those DEGs were unlikely to be expressed in BL28 treated sea cucumber juveniles, in particular, heat shock protein genes, C-region of complement component C3 gene and the amylase gene were transcribed at low levels. More interestingly, five of eight genes (63%) in Hsp70 isoforms and three of five genes (60%) in Hsp26 isoforms were likely to be “rest” status in those gene expressions (Tables 1 and S1-S2) [15].

Not only major genes responsible for triggering signaling pathway but also pluripotency factors were unlisted in DEGs (Table 1) [17].

GO Enrichment and Host Transcriptome Interpretation

GO enrichment also supported the DEGs profiling (Fig. 2). In BL28, treated sea cucumber, lipid/tricarboxylic acid and chondroitin metabolisms were enriched as major biological process

(BP) (Fig. 2 AB). Activities for chondroitin and lipid metabolisms in the sea cucumber host were also enriched in the BL28 treatment by metabolic function (MF) enrichment (Fig. 2 CD). Chondroitin metabolic activity was predicted to function in Golgi apparatus membrane and phospholipase B activity was also predicted in brush border membrane.

In the control sea cucumber, protein refolding, chaperone-mediated protein folding, cellular response to heat and response to hypoxia were enriched as BP (Fig. 2. EF). Enrichment in MF also supported those biological processes (Fig. 2. GH). In general, experimental bacterial infections or supplementation of microbes caused immune system stimulation in the host organisms [18]. However, no significant signs in regulating host sea cucumber immune pathways were detected by mixed administration approach with both long (five weeks) and short (1.5 hours) of BL28 administration.

Carbohydrate sulfotransferase 11 performs a role in chondroitin sulphate biosynthesis catalyzing the transfer of sulfate from 3'-phospho-adenosine 5'-phosphosulfate to position 4 of the N-acetylgalactosamine residues of chondroitin [19]. Fucosylated chondroitin sulfate is the major glycosaminoglycan in sea cucumber and is covalently attached to collagen fibrils consisting of supramolecular organization shaping globular dots filling the gap region in the fibrils [20]. Chondroitin sulfate is the second major component of connective tissue in sea cucumber [20]. As glycosaminoglycan including chondroitin sulfate has roles in remodeling tissues, up-regulation of BSL78_22910 might be related to the growth promotion triggered by BL28 supplementation. Phospholipase B1 and proprotein convertase subtilisin/kexin type 9 (PCSK9) perform roles in lipid metabolism, in particular, PCSK9 plays a major role in regulating blood levels of low-density lipoprotein cholesterol (LDL-C) binding LDL-receptor in preventing recycling and promoting degradation in human [21]. IDHs serve diverse biological functions in energy production and in cellular defense against oxidative damage [15]. Conformational change during the human IDH activity interacting between the "big" and "small" domains is proposed [15]. As the BSL78_28981 product only encodes the "big" domain retaining possible NADP bidding sites, the function of the gene products might be related to NADP recycling. Up-regulation of genes responsible for chondroitin sulfate and lipid metabolism and a glyoxylate cycle in BL28 supplemented juveniles might indicate host physiological signs in the growth promotion by ingesting energy richer diets supplemented by the *Sulfitobacter* bacterium.

Notably, in BL28-supplemented juveniles, most of the Hsp70 and Hsp26 isoform genes were unlikely to be up-regulated (Tables 1 and S1-S2). As Hsp70 proteins are known to chaperone the refolding of denatured peptides to minimize proteolytic degradation as a part of an eukaryotic conserved phenomenon referred to as the heat shock response function, the physiological stress which could include elevated temperatures, mechanical damage, hypoxia, lowered pH and reactive oxygen species generation, may also promote protein unfolding and *hsp70* gene expression in

humans and other animals [16,22]. In response to heat stress, after global intron splicing and cap-dependent translation become impaired, heat shock factor 1 (HSF1) becomes activated via homotrimerization, gain in phosphorylative status via calcium/calmodulin-dependent protein kinase II (CAMKII) and transactivation upon binding to the heat shock element (HSE) promoter and thus vigorous increase in *hsp70* mRNA transcriptional rate after activated HSF1 promotes RNA polymerase II (Pol-II) escape from pretranscriptional initiation complex (PIC) [16]. Conversely, at “rest” state, global intron splicing and cap-dependent translation proceed at basal rates in the skeletal myofiber, HSF1 activity is negatively regulated in both the nucleus and cytoplasm via constitutive phosphorylation and via sequestration by Hsp70, Hsp90, and other binding proteins, and thus HSF1 sustains transactivational inhibition, causing Pol-II to remain in a promoter-paused state suppressing downstream *hsp70* mRNA and protein expression [16]. In juveniles fed with BL28 supplemented diets, no significant transcriptional regulations of any genes responsible for the HSF1, kinases, Pol-II and other related proteins for heat shock response were recorded, but the “rest” state in the aspect of Hsp70 gene transcriptional regulation was considered (Tables 1 and S1-S2).

Hsp70 gene down-regulation and growth promotion after probiotics administration have been observed in various fish species such as yellow perch [22] and the false percula clownfish [23]. More interestingly, the mixed *Bacillus* species as probiotics in cultured yellow perch minimized the host stress responses that exhibited a decrease in cortisol, Hsp70 signaling and oxidative stress-related markers, and an increase in the growth-related factor (Igf1) after hypoxia and air-exposure stress [22]. Cortisol is the primary corticosteroid hormone in fish and its levels are increased in response to various stressors by supplying energy through glycogen, lipids and protein catabolism for metabolic adaptation [22,24]. Both decreases in cortisol level and Hsp70 gene expression are also reported in the fish *Pangasianodon hypophthalmus* after stresses of ammonia pollution and high temperature and administration of manganese nanoparticles (Mn-NPs), which is known to be a micronutrient [25]. Cortisol levels have also been assessed in sea cucumber *Cucumaria frondosa* [24], but the entire gene set responsible for cortisol biosynthesis is unlikely to be found on the *A. japonicus* genome, and thus, unfortunately, transcriptional regulations in cortisol biosynthesis of *A. japonicus* were not fully evaluated in this study. However, considering up- and down-regulated DEGs including lipid and energy metabolism and Hsp70, respectively, of sea cucumber juveniles after administration of the *S. pontiacus* BL28, the probiotics treatments might affect optimal nutritional levels in the host sea cucumber juveniles.

Recently, complement protein C3 has been synthesized by intestinal cells and secreted into the lumen in a laboratory mouse model and human cohorts [26]. Surprisingly, fecal C3 levels varied depending on gut microbial compositions, and using a single-cell RNA-seq approach, intestinal C3 is largely expressed by stromal cells located in the subepithelial compartment of the intestine under homeostasis [26]. In this study, we observed that significant gene expression changes in

BSL78_27279 encoding the N-terminal part of complement component C3 in between the juveniles administered with BL28 and the control animals (Table 1), which indicates microbial composition dependent regulation in the complement component C3 might also be present in sea cucumber, a member of Deuterostomia. As the C3 levels were increased by pathogen administrations in a mouse model [26], the *S. pontiacus* strain BL28 was unlikely to be recognized as a pathogen by sea cucumber hosts. We need further understanding as to how the gut complement system is regulated in sea cucumber and how the microbial composition dependent regulation of the complement system is conserved in Deuterostomia animals in both mammals and sea cucumber.

S. pontiacus strains were originally isolated from H₂S-O₂ interface of the Black Sea [27]. Recently, anaerobic thiosulfate oxidation of this group of bacteria has been described [28]. A set of genes for thiosulfate oxidation was found on the genome of BL28. Moreover, *S. pontiacus* was identified to be a key bacterial species to prevent *Emiliana huxleyi* death from the pathogen *Phaeobacter inhibens* using a synthetic microbial community, which shows a novel algal-bacterial interaction [29]. No clues suggesting the *S. pontiacus* protection have never been identified [29], *S. pontiacus* might have potential in maintaining the microbial community in aquatic ecosystems. In addition, re-assessment of pangenome among *S. pontiacus* strains revealed the presence of multicopper manganese oxidase gene. As related gene products are responsible for biogenic manganese oxide nanoparticle formation [30], *S. pontiacus* might perform roles in ecosystem control and micronutrient supply for host sea cucumber, and also create stress-free growth. We suggest that BL28 is capable of creating a stable stress-free relationship between host sea cucumber and the probiotic *Sulfitobacter* bacterium in both nutritional and environmental aspects.

Conclusion

The host sea cucumber transcriptome after supplementation of the *S. pontiacus* strain BL28 suggests that the BL28 supplementation leads to physiologically and environmentally stress-free status in the host *A. japonicus* juveniles under experimental aquarium conditions, in particular, fulfilling the nutritional levels of the host animals. We did not find any significant clues that cobalamin/B12 biosynthesis, type IV secretion system, PHB production and sulfate metabolism as BL28 factors triggered the host growth transcriptomic responses. Considering the isolation source of strain BL28, which is a member of pioneer microbiome of *A. japonicus*, we speculated that symbiotic associations between the host and bacterium could be established. Further studies to identify microbial factors triggering early events occurring in the host sea cucumber are also needed to elucidate how the bacterium affects the host echinoderms in the field of EcoDevo. Those results also might contribute to developing symbiosis-based probiotics treatments.

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332

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Table 1. DEG list of the host transcriptome based on QLF test. Orange background: up regulated genes in BL28 diet fed group, blue: up regulated in control diet fed group. Pink: re-evaluated house-keeping gene (in human) for evaluation of up and down regulated genes in this sea cucumber host transcriptome study.

Figure legends

Fig. 1. Design of BL28 feeding trials and specific growth rate of sea cucumber juveniles used for host RNA-seq. (A) Feeding trials: after one week acclimation, five weeks feeding trials with and without BL28 were performed. Orange polygon: cleaning and feeding with control diets. Purple polygon: cleaning and feeding with BL28 supplemented diets. *The last feeding without cleaning before host RNA preparation was at 1.5 hours. (B) The specific growth rate (SGR) of sea cucumber juvenile. Bayesian statistics supports that SGR fed with BL28 was higher than those of control group ($n=3$).

Fig. 2. GO enrichment of DEGs in the host transcriptome. (A) & (B): BP, up in BL28, (C) & (D): MF, up in BL28, (E) & (F): BP, up in control, (G) & (H): MF up in control.

Supplementary figures

Fig. S1. A closed aquarium for probiotics supplementation trials of the sea cucumber used in this study. At air inlet and exhaust, 0.22 μ m filter was connected to prevent cross contamination.

Disinfect green plastic mesh scaffolds were put in this aquarium to support free movements of sea cucumber juveniles.

Fig. S2. The Dusselpore outputs for the sea cucumber host transcriptome. (A) Raw reads statistics of the host RNA-Seq. (B) QC and mapping statistics of the host RNA-seq. (C) Heatmap of top30 genes which the expression varied in sea cucumber individuals by BL28 supplementation.

Fig. S3. Predicted protein structure comparison between sea cucumber and related human gene product. Blue sea cucumber transcriptome DEGs products.

Supplementary tables

Table S1. Normalized read counts per million for HSP70 isoform genes.

Table S2. Normalized read counts per million for HSP26 isoform genes.