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Short Communication: Detection and expression analysis of the bile salt hydrolase gene in *Pediococcus* and *Lactobacillus*

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Abstract. *Widodo, Nuratri Y, Sukarno AS, Ahyuningsih TD. 2020. Short Communication: Detection and expression analysis of the bile salt hydrolase gene in* Pediococcus *and* Lactobacillus*. Biodiversitas 21: 5901-5905.* Bile salt hydrolase (BSH) has been considered an important enzyme that is produced in response to bile stress. The *bsh* gene reportedly encodes BSH in lactic acid bacteria. Thus, the aim of this study was to detect and measure the changes in *bsh* gene expression in *Pediococcus pentosaceus* M103, *Lactobacillus casei* AP, *Lactobacillus casei* AG, and *Lactobacillus paracasei* M104. A BSH primer was used in amplifying the bacterial genomic DNA, and the obtained amplicon was then sequenced and analyzed in order to detect genes homologous to *bsh*. Changes in expression levels were measured in three concentrations of bile salts: 0.1%, 0.3%, and 0.5% (w/v). The BSH primer produced amplicons 850 to 950 kb in length. Amino acid sequences from the amplicons were determined to be similar to the product sequence of BSH. The level of expression of the *bsh* gene was altered in every concentration of bile salts in the growth medium with *bsh* genes from *P. pentosaceus* M103 were induced by bile salts at concentrations of 0.1% and 0.3% (w/v). We conclude that *P. pentosaceus* M103 may make suitable probiotic candidates that can tolerate conjugated bile acids.

Keywords: Bile salt hydrolase, BSH, *Lactobacillus*, *Pediococcus*, probiotic

INTRODUCTION

Probiotics are identified as living organisms that can be consumed in sufficient quantities to provide a beneficial effect on the host body. Probiotics from the lactic acid bacteria (LAB) have been incorporated into a wide variety of functional foods; this is to increase the nutritional value and health benefits of these foods. Results from in vitro and in vivo assays suggest that probiotics can be used to help lower cholesterol levels and prevent metabolic syndrome (Li et al. 2019; Andriani et al. 2020). To fully examine their health effects in the human body, LAB should be viable, present in high numbers, and be able to colonize the gastrointestinal (GI) tract (Nuraida et al. 2011; Maryati et al. 2016).

In the GI tract, LAB must be able to overcome harsh conditions, including the presence of bile. Bile acts as a detergent, playing a role in the emulsification and solubilization of lipids. Hence, the presence of lipophilic bile salts in the GI tract is able to disrupt cell membrane integrity, leading to cellular lysis. Thus, the ability to tolerate high concentrations of bile salts is considered to be an important trait of probiotics (Fontana et al. 2013; Ruiz et al. 2013).

Bile salt hydrolase (EC 3.5.1.24) is considered to be an enzyme responsible for the deconjugation of bile salts. It catalyzes the hydrolysis of the amide bond in the molecular structure of conjugated bile salts. The result is found less toxic, unconjugated bile acids and a glycine/taurine moiety. This compound is believed to be metabolized by microbes in the gut (De Boever et al. 2000). In many LAB, the synthesis of BSH is a natural response of the cell to the presence of conjugated bile acids.

Pediococcus and *Lactobacillus* have been identified as among the LAB genera with probiotic characteristics. *Lactobacillus* species, such as *L. casei* strain Shirota, *L. casei* strain Zhang (Wang et al. 2013), *L. rhamnosus* (Koskenniemi et al. 2011), *L. paracasei*, *L. plantarum* (Buntin et al. 2017), and *L. casei* strain AP (Afidah et al. 2019), have been shown to function as probiotics. Meanwhile, *P. pentosaceous* K23-3 is an example of a *Pediococcus* strain shown to have probiotic properties (Shin et al. 2008). Widodo et al. (2012) isolated *L. casei* AP and *L. casei* AG from the feces of Indonesian infants, and these strains were able to acidify milk during fermentation for 8 hours. Both *L. paracasei* M104 and *P. pentosaceus* M103 have previously been isolated from goat milk (Widodo et al. 2016) and both strains were able to acidify goat milk during fermentation. The ability of probiotics to tolerate bile salts has been associated with the activity of bile salt hydrolase (BSH); therefore, it is important to evaluate the presence and expression of the gene that encodes BSH. Thus, this study aimed to detect the homologs of the *bsh* gene in *P. pentosaceus* M103 and

L. paracasei M104 from goat milk and *L. casei* AP and *L. casei* AG from humans. The expression of the bacterial genes was evaluated in the presence of bile salts.

MATERIALS AND METHODS

Bacterial strains and culture conditions

In this study, four bacterial strains, *P. pentosaceus* M103, *L. paracasei* M104, *L. casei* AP, and *L. casei* AG, were obtained from Dr. Widodo at the Faculty of Animal Science, Universitas Gadjah Mada (UGM), Indonesia. Strains were propagated in a micro-aerobic environment in de-Man-Rogosa-Sharpe medium (Sigma-Aldrich Ltd, Jakarta Indonesia). Then, the cultures were incubated at 37°C for 12 h.

BSH primer and BSH-RT primer design

The primers were designed based on sequences obtained from the results of tblastn on the protein sequences of *L. casei* Zhang (EU599213.1) and *L. rhamnosus* (FM179322.1). The selected primers (bsh and bsh-rt) were from a region that flanked the *bsh* gene sequence. The bsh-rt primer and a GAPDH primer were used for RT-PCR analysis. The primers used in this study are listed in Table 1.

DNA isolation and PCR amplification

Bacterial genomic DNA was isolated using a Geneaid gSYNC™ DNA Extraction Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan), following the company's instruction manual. PCR amplification was performed in a 25-µL reaction volume, which was a combination of 12.5 µL KAPA Taq ReadyMix PCR Kit, 1 µL DNA template (>50 ng/µL), 1 µL of each bsh primer (10 pmol), and 9.5 µL nuclease-free water. Amplifications were carried out using an Applied Biosystems Thermal Cycler 2720 (Foster City, USA), with operational conditions as follows: initial denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s and extension at 72 °C for 1 min, followed by final extension at 72 °C for 10 min. Amplification products were then subjected to electrophoresis in 2% (w/v) agarose gel (Sigma-Aldrich Ltd, Jakarta, Indonesia) with ethidium bromide using TBE buffer solution.

Sequence analysis

Amplicons were purified using a Bigdye X-Terminator Purification kit (BDX); they were later sequenced using an Applied Biosystems 3500xL Genetic Analyzer (Applied Biosystems, Banten, Indonesia). Nucleotide sequences and predicted amino acid sequences of homologs of the *bsh* gene were then analyzed using BLAST and ClustalW. Sequences were aligned using the Molecular Evolutionary Genetics Analysis (MEGA) 5.05 to construct a phylogenetic tree.

Table 1. Primers used in this study

Primer name	Sequence (5′-3′)	Product length
bsh-r	CAAGGTGATATTTTCTGGGGCCG	1014 bp
bsh-f	ATGTGTTCTTCAATGACAATTAA	
bsh-rt-r	CCAGTACTGCGTGTAATCCGT	120 bp
bsh-rt-f	CCCTTCAATAGCAAAGACGGC	

Treatment in bile salts

Cells were sub-cultured twice in MRS media before treatment. The cultures were centrifuged for 30 s at 12,000 g in order to collect the cells. The bacterial cells were resuspended in a phosphate-buffered saline, and the resuspended cells (2% v/v) were then inoculated into MRS media containing bile salts. The strains were grown in MRS medium with 0.1%, 0.3%, or 0.5% (w/v) of bile salts. Cells were harvested after 4 h.

RNA isolation and cDNA synthesis

Total RNA was prepared using Zymo Research Direct-zol RNA Miniprep Plus (Tustin, USA). CDNA was synthesized using a Smobio ExcelRT™ Reverse Transcription Kit II (Hsinchu City, Taiwan), according to the manufacturer's protocol. Denaturation was performed by incubating the samples at 70°C for 5 min, cooling them on ice for 3 min, and finally by incubating them at room temperature for 10 min. First-strand cDNA synthesis was performed at 40°C for 50 min followed by termination at 85°C for 5 min.

Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR) analysis

QRT-PCR was performed in a Bio-Rad CFX96 Real-Time PCR System under the following conditions: enzyme activation at 95°C for 30 s, 45 cycles of denaturation at 95°C for 5 s, and annealing at 50°C for 5 s. Melt curve analysis was performed at 65°C for 5 s and 95°C for 5 s. QRT-PCR was performed in a 10-µL reaction volume, which consisted of 5 µL Bio-Rad SsoFast™ EvaGreen® Supermix (Hercules, USA), 1 µL cDNA, 1 µL of each BSH-RT primer, and 2 µL nuclease-free water. GAPDH was used as a reference gene for normalization.

RESULTS AND DISCUSSION

Amplification and sequence analysis of the homologous *bsh* gene

PCR amplification with a *bsh* primer was used in order to obtain a partial sequence of the *bsh* gene. A nucleotide sequence within the range of 745 to 927 bp was obtained from the sample (Figure 1). The lengths of the nucleotide sequences from *P. pentosaceus* M103, *L. casei* AP, *L. casei* AG, and *L. paracasei* M104 were determined to be 927, 893, 911, and 745 bp, respectively. The open reading frames (ORFs) of the sequences consisted of 296 amino acids and were identified as the predicted BSH protein. DNA sequencing revealed that the ORF began with an ATG start codon and ended with a TAG stop codon (data not shown).

The similarity of the sequences with those of other organisms was analyzed using BLAST. The nucleotide sequence of *P. pentosaceus* M104 was determined to have the highest identity (99.66%) with the conjugated *bsh* gene from *L. casei* Zhang (EU599213.1). *L. casei* AP and *L. casei* AG were also shown to have high sequence identity (98.88% and 99.56%, respectively) with the Ntn_hydrolase gene from *L. paracasei* LC2W (CP002616.1). The nucleotide sequence from *L. casei* AP also had a similarity of 75.17% to the choloylglycine hydrolase gene from *L. rhamnosus* GG (FM179322.1). *L. paracasei* M103 has also exhibited high nucleotide sequence identity of 100% with the linear amide C-N hydrolase gene from *L. paracasei* SRCM103299 (CP035563.1) (Oinonen and Rouvinen 2000). BSH has been identified to be a member of the choloylglycine hydrolase family and the Ntn hydrolase superfamily (Chae et al. 2013; Liang et al. 2018).

Phylogenetic tree analysis based on the sequence of homologous *bsh* genes is shown in Figure 2. *L. casei* AP and *L. casei* AG have shown close similarity. Interestingly, the sequence of the homologous *bsh* gene from *P. pentosaceus* M103 was found to be more closely related to that of *L. casei* LOCK919 than to its own genus. Meanwhile, *L. paracasei* M104 was more closely to be related *to L. paracasei* ATCC334.

The amino acid sequence of *P. pentosaceus* M104 had 98.65% sequence similarity with linear amide C-N hydrolase; it has been further identified as a choloylglycine hydrolase family protein of *L. paracasei* ATCC 25302 (EEI69212.1). The *L. casei* AP, *L. casei* AG, and *L. paracasei* M103 amino acid sequences had similarities of 98.25%, 99.3%, and 99.6%, respectively, with conjugated bile salt hydrolase-like protein from *L. casei* DSM 20011 = JCM 1134 (KRK15537.1).

Expression of the homologous bsh genes

Gene expression levels were observed and analyzed after bacterial cells were grown for 4 h in the medium containing 0.1%, 0.3%, or 0.5% (w/v) bile salts. The results showed that the expression levels of the *bsh* gene in all strains have shifted (Figure 3). In *L. casei* AG, the highest value was in the medium with 0.1% bile salts. This led to a 2.48 fold change in *bsh* gene expression levels. The expression levels decreased with the increased concentration of bile salts (0.3% and 0.5%). The highest expression of the *bsh* gene in *P. pentosaceus* M104 was 1.8-fold higher in media with 0.3% bile salts. Similarity of the decline expression patterns was shown in *P. pentosaceus* M104 towards the increase of bile salt concentration. *L. paracasei* M103 showed low expression levels (<0.35) at all concentrations of bile salts tested.

The expression of the *bsh* gene in *P. pentosaceus* M103, *L. casei* AP, *L. casei* AG, and *L. paracasei* M104 has not been induced by 0.5% bile salts in the growth medium (Figure 3). A concentration of 0.5% (w/v) bile salts resulted in up to a 4-fold down-regulation in the expression of *bsh* gene in all strains compared with its expression in the control (without bile salts). This result differs considerably from the finding of Zhang et al. (2009) who reported that *L. casei* strain Zhang exhibited a more than one fold change in expression when grown with 0.5% (w/v) bile salts.

The expression of the *bsh* gene in each bacterial sample was determined to be low at higher bile salts concentrations tested. This low expression might be a response to unconjugated bile salts, with conjugated bile salts causing a less deleterious impact on the cells than unconjugated bile salts. There are two major hypotheses of the impact of BSH. In some cases, unconjugated bile acids resulting from the BSH reaction can provide a more damaging environment to cells, whereas in other species, conjugated bile acids are more deleterious.

Free bile acids are more hydrophobic and can cross the cell wall more easily than conjugated bile salts. This might be dependent on the pKa value of the bile. A smaller pKa value has been reported to reduce the intracellular pH of the cell and disturb membrane integrity (Ruiz et al. 2013). Unconjugated bile acids often have a lower pKa value and tend to possess greater hydrophobic properties (Ridlon et al. 2016). It has been suggested that BSH produced by *Enterococcus*, *Bacteroides*, and *Clostridium perfringens* produces unconjugated bile salts with greater toxicity than conjugated bile salts (Shehata et al. 2016). Bacteria that are highly tolerant of conjugated bile salts are potential candidates for use as probiotics due to their higher likelihood of survival during transit in the GI tract.

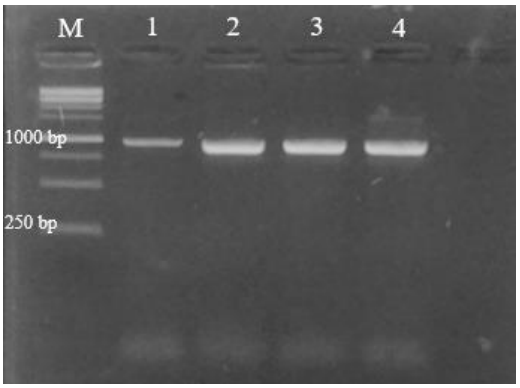

Figure 1. Visualization of amplicons of *Pediococcus pentosaceus* M103 (1), *Lactobacillus casei* AP (2), *L. casei* AG (3), and *L. paracasei* M104 (4) in 2% agarose gel

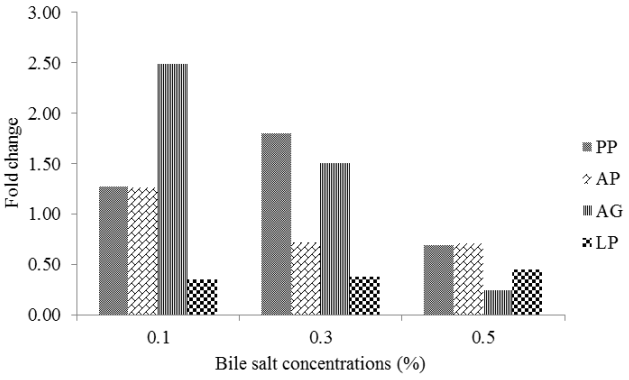

Figure 2. Expression levels of the *bsh* gene in *Pediococcus pentosaceus* M103 (PP), *Lactobacillus casei* AP (AP), *L. casei* AG (AG), and *L. paracasei* M104 (LP) grown in MRS media with different concentrations of bile salts: 0.1%, 0.3%, and 0.5% (w/v).

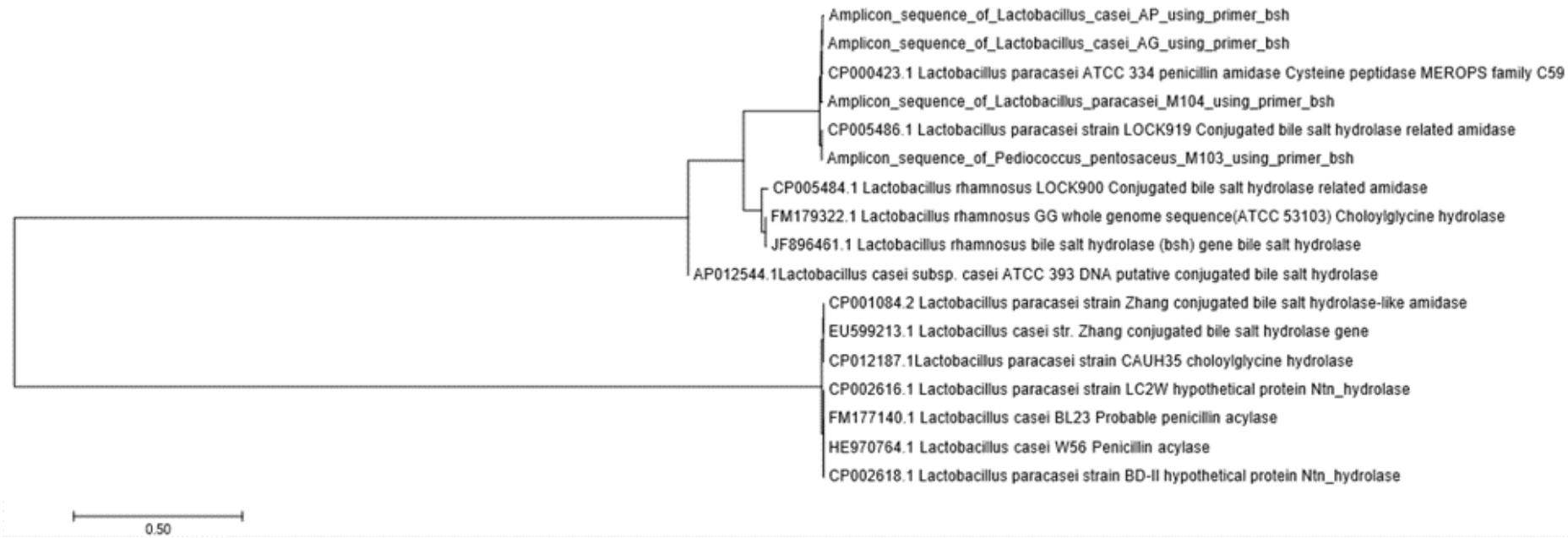

Figure 3. Phylogenetic tree of *Pediococcus pentosaceus* M103, *Lactobacillus casei* AP, *L. casei* AG, and *L. paracasei* M104

In conclusion, homologous *bsh* genes from *P. pentosaceus* M103 induced by bile salts at concentrations of 0.1% and 0.3% (w/v). This result might provide a clue to that *P. pentosaceus* M103 may make suitable probiotic candidates that can tolerate conjugated bile acids.

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