

Research article

Transformation and over expression of chromosomal bile salt hydrolase gene A (*bshA*) that transfer from transformer *Escherichia coli* to *Enterococcus faecalis*

Wafaa Sabri Mahood^{1*}, Atheer Ahmed Majeed¹

ABSTRACT

The clone *E. coli* MC1022 as replication host carrying plasmid pMG36e (4.5 kilobases) which confer erythromycin resistance was used in this study as a vector that have a clone of chromosomal bile salt hydrolase gene A (*bshA*) of *Lactobacillus acidophilus* Ar and prepare for transfer to *Streptococcus* species. A 801 bp bile salt hydrolase gene A fragment cloning for hypercholesteremic treatment in human blood by bacterial biotransformation when gene over expression occurrence. The recombinant plasmid pMG36e called pMG36/*bshA* vector was extracted from *E. coli* using ethanol 96% precipitation method. Natural transformation method was used to transform *Enterococcus faecalis*, which supplied by Symbiopharm company as probiotic supplement. Detection of cloning gene depend on erythromycin resistance character and over expression assay of bile salt hydrolase enzyme using specific activity of bile salt hydrolase enzyme in transformer strain of *E. Faecalis* and compared with wild type of *E. Faecalis*, the enzyme activity was increased from (397 to 607) U/mg.

Keywords: Bile salt Hydrolase A gene (*bshA*), *Enterococcus faecalis*, transformation, Probiotic

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INTRODUCTION

Enterococci are Gram positive cocci that often occur in pairs (diplococci) or short chains, *Enterococcus faecalis* formerly classified as part of the group D Streptococcus system. *E. faecalis* is a nonmotile microbe, it is catalase negative, usually facultative, anaerobic bacteria that grow in 6.5% NaCl, 40% bile salts, and 0.1% methylene blue milk and at pH 9.6. They grow at 10 and 45°C and can resist 30

min at 60 °C. *Enterococci* are important inhabitants of animal intestine and are widely used in probiotic products [1]. *E. faecalis* isolates as probiotics showed many desirable properties, it showed different susceptibilities to opsonic killing, suggesting that some of these isolates possess a capsule while other do not. This information may be helpful in assessing the safety of a given bacterial isolate and could



*Correspondence: Mahood.WS.wafa.sabry@yahoo.com

Department of Biology, College of Education – Ibn AL-Haytham University of Baghdad, Baghdad, Iraq.
Full list of author information is available at the end of the article.

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could detect likely enterococcal candidates for probiotic preparations [2]. *E. faecalis* isolated from a healthy adult showed the highest probiotic activity when a study showed microbiota acquisition in infancy is likely a determinant of early immune programming, subsequent infection, and allergy risk [3], other researcher showed that *E. faecalis* cause stimulate the Th1 immune response, downregulate pro-inflammatory cytokines (TNF- α) and upregulate anti-inflammatory cytokine (IL-10), does not express several virulence factors (haemolysin, gelatinase, lipase, Dnase, decarboxylase nor aggregation substance) and does not present antibiotic multiresistance [4,6]. Several strains of human pathogens or commensal bacteria including *E. faecalis* are able to inhibit *H. Pylori* [5]. All these properties make *E. Faecalis* candidate as probiotic microorganism especially some strains has been found to have a bile salt hydrolase, it hase the abilty to hypocholesterol. It also has been suggested that BSH activity should be a requirement in the selection of probiotic organisms with cholesterol lowering properties, since microorganisms that do not deconjugate bile salts do not appear to be able to remove cholesterol from the culture medium to any significant extent.

Bile salt hydrolase (BSH) is the enzyme responsible for bile salt deconjugation in the enterohepatic circulation. The BSH activity able to hydrolyze conjugated glycodeoxycholic acid and taurodeoxycholic acid, leading to the deconjugation of glyco- and tauro-bile acids [7]. Many mechanism were proposed to justify the varying effect exhepted by various strains of probiotic or type of prebioic but the results of hypocholesterolemic effect through in vitro and in vivo studies remain controversial [8,9]. The last information is expected that manipulation of the gut microbiota could be harnessed for preventative and therapeutic effect [10]. Other study decided to use gene manipulation to produce new genetic recombination for bacterial genes which responsible about cholesterol lowering in human blood and can be use such as hypercholesterolemia treatment replacement took high dosage from probiotic bacterial capsule [11].

The aim of this study is to transform *E. faecalis* and gene manipulation with bile salt hydrolase gene A (bshA) that has been carried by transformer *E. coli* to produce new strain recombination to be more efficient to hydrolysis bile salt expression as desirabl property in probiotic microorganism.

MATERIALS AND METHODS

Bacterial strain and plasmid

One isolate of *E. Faecalis* that supplied as probiotic supplement (symbioflora) for human treatment was using to transform with a pMG36e/ bsha vector which carried by transformer *E. coli* MC1022 cells. *E. coli* MC1022 wild type also used as (control), the properties of bacterial strains (Table 1). The research was approved by council of Biology Department, College of Education–lbn AL-Haytham, University of Baghdad. The bacterial strains were grown in nutrient broth media at 37°C for 18 h and activation of bacteria was done by streaking on media contain 100 μ g/ml Erythromycin for plasmid detection.

Table 1. Features of bacterial strains and vector

Bacterial strains and vector	Properties	Reference
<i>E. faecalis</i>	Probiotic bacteria (Sensitive for Erythromycin)	Symbiopharm Company
<i>E. coli</i> MC1022 carry vector of pMG36e/ bsha	Cloning and expression plasmid Replicon (rep), T7promoter, EmrR	KSU University
<i>E. coli</i> MC1022	Artificial bacteria	KSU University

Bile salt tolerance

The *E. faecalis* strain was treated with sodium salt of urodeoxycholic acid (SCA) using 0.2-0.5 % (wt/vol) concentration and incubated at 37 °C aerobically for 18 h to test bacterial growth bile salt tolerance.

Antibiotic tolerance

Bacterial strains of *E. faecalis* and transformer *E. coli* MC1022 in addition to *E. coli* MC1022 (control) were grown in nutrient broth media at 37 °C for 18 h after that re-cultured on nutrient agar contained 100 μ g/ml Erythromycin for plasmid detection and to test antibiotic tolerance.

Vector extraction

Single colony was picked from a plate of clones *E. coli* MC1022 carry vector of pMG36e / bsha with 100 μ g/ml erythromycin and incubated overnight at 37 °C in nutrient broth media. Washing cell from overnight culture and centrifuged at high speed for 15 min. The cells pellet was suspended in 1 ml solution [25 mM Tris.Cl (pH 8.0), 10 mM EDTA (pH 8.0)], and repeated the centrifugation twice. 150 μ l of solution I (3 M sodium acetate) was added for last cell pellet. The cell lysis process was done by sonication with a Soniprep 150 (USA) in ice-cold condition.

The sonicator was set to 16 micron amplitude for 5 min (1\2 min turn on and 1\2 min turn off). Cells suspension was centrifuged at 8,000 x g for 30 min, centrifuge at high speed for 5 min. The supernatant was removed carefully and put into new eppendorf tubes. Discard the old tubes and pellets. After that 2.5 volumes of ice cold 95% ethanol was added and mixed well to precipitate the DNA. Centrifuged at high speed for 5 min and liquid was removed, pellet was washed with 400 μ l of freezing ethanol 70%, the pellet was spun for 3 min. The supernatant was carefully removed and the pellet was allowed to air dry for 5-10 min. Pellet was suspended with 1 ml of TE [10 mM Tris.Cl (pH 7.5), 1 mM EDTA (pH 7.50)]. Extracted plasmid (vector) was measured in Nano drop spectronic device and store in the freezer at -20 °C for later uses [12].

Natural transformation assay

Preparation of competent cells

At 37 °C for overnight, *E. Faecalis* was incubated in shaking container contains 3 ml of Luria-Bertani (LB) medium. 0.5 ml of overnight culture was added to 50 ml of LB (prewarmed to 37 °C) in a 200-ml flask. The culture was incubated at 37 °C with shaking. Optical density of growth reached to 0.35-0.4 at 600 nm and chilled for highest transformation efficiency.

The culture was transferred to a sterile tube, and collected cells by centrifugation at 6,000 rpm for 8 min at 4 °C and the supernatant was discarded. The cells pellet was re-suspended in 20 ml of ice-cold 50 mM CaCl₂. Incubated the re-suspend cells on ice for 20 min. Collected cells by centrifugation at 6,000 rpm for 8 min at 4 °C and the supernatant was discarded. when the competent cells is store for long period as frozen stock, the cells was re-suspended in 2.5 ml of ice-cold 50 mM CaCl₂ containing 30% glycerol to save at -20 °C.

Transformation

The competent cells of *E. faecalis* on ice were thawed. Mixture of 100 microliters of the competent cells and 5 μ of plasmid solutions was added in TE and kept on ice for 30 min. Quick transferred mixture tube to 42 °C water bath for 1 min and then transfer into ice. One ml of LB was added and incubated at 37 °C for 30 min and inoculated on agar plate(s) containing 100 μg/ml of erythromycin [13].

BshA enzyme assay

The best wild and transformer *E. faecalis* isolate were grown into nutrient broth for bile salt hydrolase production which contained 0.5% Na-urohydroxycholic acid bile salt (substrate), 2.5% glucose (pH 5.8) to examine the effect of carbon sources on the enzyme production. A 250-ml flask containing 50 ml of the medium was inoculated with *E. faecalis* and incubated in shaker incubator (120 rpm) for 18 h at 37 °C. Control bacteria culture was prepared from wild type *E. faecalis* without bile salt.

The growth was centrifuged and the cell pellets were re-suspended in 1 ml of 0.01 M Na phosphate buffer pH 6,7 under ice-cold condition. Cells were washed twice and re-suspended with phosphate buffer containing 10 mM cysteine and 1M EDTA and adjusted the optical density at 600 for growth to 0.30-0.40. The cell lysis process used sonication with a Soniprep 150 (USA) in ice-cold condition. Cells suspension was centrifuged at 8,000 x g for 30 min, supernatant took for proteins concentration measurement in Bradford method, enzyme specific activity were performed by measuring the release of amino acids resulting from hydrolysis of the amide bond of bile salts.

The reaction mixture (200 μl) contained 100 μl cells and 100 μl 20 mmol l⁻¹ bile salts. Reaction mixtures were incubated at 37 °C. The enzymatic reaction was terminated at different times by the addition of an equal volume (200 μl) of 15% trichloroacetic acid (w/v) and precipitated proteins removed by centrifugation at 20000 rpm for 15 min. The amount of amino acids present in the supernatant fluid was measured. In brief, 680 μl 300 mmol l⁻¹ borate buffer, 1% SDS, pH 9.5, and 80 μl 0.3% (w/v) trinitrobenzenesulphonic acid (TNBS) were added to an 80 μl aliquot of supernatant fluid. The mixture was incubated in the dark at room temperature for 30 min. Color development was stopped by the addition of 800 μl 0.6 mmol l⁻¹ HCl. Amino acid concentrations were determined at 416 nm using a standard curve prepared with free taurine or glycine [14].

RESULTS

Bile salt tolerance

Tolerance to bile salt was done by reading bacterial culture optical density at 600 nm when substrate of 0.5% of bile

salt was added before bacterial growth and, it was 1.25 while after incubation of bacterial growth with 0.5% of bile salt for 18 h became 1.85.

Vector extraction and *E. faecalis* transformation

The ethanol 96% protocol for plasmid DNA extraction showed that plasmid DNA purity was 80% (1.7 nm) , it gave about 7-10 cell \ 100μl competent single colonies to each dish in selective media with 100 μl Erythromycin, the transformer *E. faecalis* carrying pMG36e/bshA vector was erythromycin resistance when overnight growth on nutrient agar was done.

Bile salt hydrolase enzyme assay

The data indicates that there is bshA over expression in *E. faecalis* pMG36e/bshA strain compared with *E. faecalis* wild type and no enzyme activity was seen for bshA gene function in bacterial growth in non-bile salt substrate broth media. **Table 2** shows increase of *E. faecalis* ability to hydrolysis of bile salt from (397 U/mg in non transformer *E. faecalis* to 606 U/mg). The results indicate that the transformer *E. faecalis* have ability to hydrolysis bile salt before transfer BSH gene as the enzyme activity was 397 U/mg.

Table 2. Comparison of over expression bshA enzyme activity between transformer *E. faecalis* and wild type *E. faecalis*

Samples	Activity (U/mg)
<i>E. faecalis</i> growth without bile salt	0
<i>E. faecalis</i> growth in 0.5% bile salt	397
Transformer <i>E. faecalis</i> growth in 0.5% bile salt	606

DISCUSSION

It has been speculated that *bsh genes* may have been acquired horizontally and BSH activity is important at some level for lactobacilli to colonize the lower gastrointestinal tract [15]. BSH present in all bifidobacterial strains and lactobacilli strains associated with the gastrointestinal environment, but *bsh genes* can potentially be acquired from these strains by other intestinal microorganisms (e.g., *L. monocytogenes*). Many hypotheses have been suggested explain the role of BSH, first, bacteria that are able to deconjugate bile salts may be able to use the amino acid released form hydrolysis as carbon, nitrogen, and energy sources, since glycine may be metabolized to ammonia and carbon dioxide and taurine may be metabolized to ammonia, carbon dioxide, and sulfate [16]. Second, the alteration of membrane characteristic, it has been proposed that BSHs facilitate incorporation of cholesterol bile into bacterial membranes [17]. Third, decrease the toxicity of conjugated bile acids for bacteria. Compared with their conjugated counterparts, deconjugated bile acids have decreased solubility and diminished detergent activity and thus may be less toxic to bacteria in the intestine [18]. Bile salt deconjugation includes the production of secondary bile salts, which have been linked to various intestinal diseases, such as the formation of gallstones and colon cancer [19]. Furthermore cholesterol super-saturation in bile is necessary for cholesterol gallstones to form, but not all people

with supersaturated bile form gallstones. Obviously, other overt mechanisms are required for ultimate formation of gallstones, therefore BSH activity may be considered as an important colonization factor, proposed the mechanism based on the ability of certain probiotic lactobacilli, *Streptococcus* and bifidobacteria to deconjugate bile acids enzymatically, increasing their rates of excretion. Cholesterol, being a precursor of bile acids, converts its molecules to bile acids replacing those lost during excretion leading to a reduction in serum cholesterol. This mechanism could be operated in the control of serum cholesterol levels by conversion of deconjugated bile acids into secondary bile acids by colonic microbes. The use of such orally applied microorganisms (probiotics) is a major aim of the concept of functional biodrugs [20].

Ability to remove cholesterol using bsh gene is responsible for convert LDL cholesterol to healthy case, these genes highly expression in transformer *E. coli* probiotic strain used settler vector specific express in *Streptococcus* strains as *E. faecalis* probiotic strain produce new recombination change bsh gene function in these probiotic strains such as biodrugs to hypercholesterolemia. The results of this study indicates high expression of bile salt hydrolase gene in the transformer *E. faecalis* as there was increase in enzyme activity from 397 to 606, the bile salt tolerance was increase from 1.25 to 1.85 this refers to the ability of *E. faecalis* to survive in bile salt environment and indicate to desirable property in intestinal microflora as probiotic bacteria.using this new recombinant strain of probiotic *E. faecalis* may lead to hypocholesterol and prevent the forming of gallstone. It could be suggest increasing ability of BSH in *E. faecalis* is important character when using it as probiotic microorganism; it will be aid in the control of serum cholesterol levels.

Hypercholesterolemia (elevated blood cholesterol levels) which is considered a major risk factor for the development of coronary heart disease, and although pharmacologic agents they are available to treat this condition often suboptimal and expensive and can have unwanted side effects [21]. Oral administration of probiotics has been shown to significantly reduce cholesterol levels by as much as 22 to 33% or prevent elevated cholesterol levels in mice fed a fat-enriched diet. These cholesterol-lowering effects can be partially ascribed to BSH activity (other possible mechanisms include assimilation of cholesterol by the bacteria, binding of cholesterol to the bacterial cell walls, or physiological actions of the end products of short chain fatty acid fermentation [16].

The consumption of probiotics is gaining popularity especially in the maintenance of health and prevention of disease. In particular, the role of probiotics as a hypocholesterolemia agent has been explored extensively. Progress has been made in the recent years on the selection, identification and characterization of strains that actually fulfill the criteria of true probiotic microorganisms and that are able to exert cholesterol reducing effects in vitro [22].

In conclusion, this study used expression vector as pMG36e specific for streptococcus spp to transformation *E. faecalis* strain of probiotic supplement. The results showed difference values of specific activity for *bshA* enzyme (397 – 606) U/ mg between wild type *E. faecalis* and transformer *E. faecalis* with *bshA* gene, the data of this study indicates

the successfully of recombinant *E. faecalis* isolate to produce high level of the enzyme. It has ability to hydrolysis the substrate of bile salt as desirable property, and this strain can be use in advance studies to explorer if it can be use as biodrug to hypocholesterol and prevent the formation of gallstone.

Conflict of interest

The authors declare that they have no conflict of interests.

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Author affiliation

1. *Department of Biology, College of Education –Ibn AL-Haytham, University of Baghdad, Baghdad, Iraq.*
2. *Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.*

