Anchorless cell surface proteins function as laminin-binding adhesins in *Lactobacillus rhamnosus* FSMM22

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ABSTRACT

Anchorless cell surface proteins (CSPs) were extracted with 1 M lithium chloride solution from *Lactobacillus rhamnosus* FSMM22. Loss of the anchorless CSPs resulted in a two-fold decrease in FSMM22 cells bound to a constitutive extracellular matrix glycoprotein, laminin, *in vitro*. DNA-binding protein HU, glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, and 30S ribosomal protein S19 (RpsS) were identified by mass spectrometry in the extract as laminin-binding adhesins. Among the four proteins, RpsS was immunohistochemically confirmed to exist on the cell surface. Our findings strongly suggest that anchorless CSPs can enhance bacterial adhesion to the host.

Introduction

To achieve situational attachment/detachment to various adhesion sites on the host in response to changes in the surrounding environment, commensal and pathogenic bacteria use several different types of cell surface proteins (CSPs). For example, pilus adhesins (Lebeer et al. 2012) are cell wall binding proteins that are strongly anchored to the bacterial cell wall (covalently bound through the action of sortases, e.g. LPXTG proteins, or through non-covalent interactions). Another example is anchorless proteins that associate weakly or moderately with the bacterial cell wall, such as moonlighting proteins, which show multiple functions at different cellular localisation (Jeffery 1999; Kainulainen and Korhonen 2014). However, the full composition of CSPs has not yet been determined for any bacterial species, owing mainly to the wide variety and complexity of CSPs and their counterparts.

Lactobacillus rhamnosus strains FSMM15 and FSMM22 were previously isolated from fermented mare's milk as potential probiotics (Shi et al. 2012). These strains showed similar adhesion for porcine colonic mucin compared to *Lactobacillus rhamnosus* GG ATCC 53103 (LGG). Moreover, compared to FSMM15, FSMM22 showed about a 100-fold increase in the number of bacterial cells bound to the laminin (Shi et al. 2012). Therefore, these two strains have the potential to serve as a model for investigating the roles of CSPs in binding to laminin. A recent study by Nishiyama et al. (2015) revealed that an anchored CSP, mucus-binding factor, was important for the binding of FSMM22 to porcine colonic mucin and to some glycoproteins that compose the host's extracellular matrix protein (ECM), including laminin. To identify a variety of CSPs in FSMM15 and FSMM22 and focus on their binding properties to laminin, 1 M lithium chloride (LiCl) solution, which is commonly used for the extraction of anchorless CSPs (Rojas et al. 2002), was used in this study.

Materials and Methods

Bacterial cell culture

LGG was purchased from the American Type Culture Collection (Manassas, VA, USA). Single colonies of *L. rhamnosus* FSMM15 and FSMM22 from our culture collection and LGG were statically pre-cultured in 15 mL of de Man-Rogosa-Sharpe (MRS) broth (Oxoid, Basingstoke, UK) for 20 h at 37°C. For the main culture, 0.4 to 1% of bacterial suspensions were inoculated into 250 mL of MRS broth and incubated under anaerobic conditions using AnaeroPack Kenki (Mitsubishi Gas Chemical, Tokyo, Japan). After incubation for 20 h at 37°C, cells were pelleted by centrifugation at 3,000 × *g* for 15 min at 4°C, washed twice with phosphate buffered saline (PBS), and used in the following experiments.

CSP extraction and their effects on the laminin-adhesion properties of FSMM15 and FSMM22

CSPs were extracted from bacterial cells by suspension in either 1 M LiCl solution or PBS for 1 h

at 4°C with agitation. Then, the suspension was centrifuged at 8,000 × *g* for 30 min at 4°C, and the supernatant was filtered through a nitrocellulose membrane (0.2-μm pore size, Advantec, Japan). The filtrate was concentrated using Centriprep YM-3 (Merck Millipore, Billerica, MA, USA), dialyzed against PBS with a 1-kDa molecular weight cut-off membrane (GE Healthcare, Chicago, IL, USA) at 4°C overnight, freeze dried, and kept at –30°C until use. Protein concentration was estimated spectrophotometrically at 280 nm under the assumption of $E^{1\%}_{1\mathrm{cm}} = 10$.

To evaluate the effects of CSP removal on the laminin-binding properties of the FSMM strains, the bacterial cell number was determined before and after the extraction as previously described (Nishiyama et al. 2015), with a modification that bacterial cells were harvested at the stationary phase.

Inhibition enzyme-linked immunosorbent assay (ELISA)

Inhibition ELISA was performed to detect laminin-binding proteins (LBPs) in the CSPs. Approximately 2.5 μg of mouse laminin-111 (BD Biosciences, Bedford, MA, USA) was dissolved in 1 mL of 0.25 M carbonate-bicarbonate buffer (pH 9.6), and a 100-μL aliquot was added per well of a 96-well Maxisorp plate (Thermo Fisher Scientific, Waltham, MA, USA) and incubated overnight at 4°C. Unbound laminin was removed by washing with 0.1% Tween-20 in PBS (PBS-T). To prevent unspecific binding of CSPs, wells were treated with 200 μL of 1% bovine serum albumin (BSA) in PBS at 37°C for 2 h. Each of the lyophilized CSPs obtained from FSMM15 and FSMM22 was reconstituted in 500 μL of 0.1% BSA in PBS; then the 100-μL aliquot was added and allowed to bind to laminin at 37°C for 2 h. As a control, 100 μL of 0.1% BSA solution was used. Unbound CSPs were removed by washing with PBS-T; next, 100 μL of chicken polyclonal anti-laminin antibody (Abcam, Cambridge, UK; diluted 1:20,000 with 1% BSA in PBS) was added and incubated at 37°C for 2 h. After removal of unbound anti-laminin antibody, 100 μL of goat anti-chicken IgY conjugated-horseradish peroxidase (Abcam; diluted 1:10,000 with 1% BSA in PBS) was added and incubated for 1 h at room temperature (RT). The titre was measured at 492 nm using a Multiskan FC microplate photometer (Thermo Fisher Scientific).

Isolation and identification of LBPs

LBPs were isolated from CSPs according to Muñoz-Provencio et al. (2011) with modifications. Immobilization of laminin and the CSP binding reaction were performed as described above. In brief, CSPs bound to the immobilized laminin were recovered with 60 μL of 1% (w/v) sodium dodecyl sulfate (SDS) solution by incubation at RT for 2 h with agitation. The SDS solution was thoroughly dried and the CSPs were recovered with 25 μL of Laemmli buffer (Laemmli 1970), denatured at 95°C for 5 min, and then subjected to 12.5% SDS polyacrylamide gel electrophoresis (PAGE). A precision plus protein dual color standard (Bio-Rad Laboratories, Hercules, CA, USA) was used as a protein size marker. Protein bands were visualized using the Dodeca silver staining kit (Bio-Rad Laboratories)

according to the manufacturer's instruction and were then manually excised. Destaining of the gel pieces, in-gel digestion of the proteins, and protein identification using a mass spectrometer were performed as previously reported (Senda et al. 2011).

Immunohistochemical staining

A rabbit polyclonal antibody was prepared against a custom-made synthetic peptide for the N-terminal 19 amino acid sequence, MGRSLKKGPFADAHLLKKI, of RpsS (GenBank ID: BAI42919.1). A biotinylated anti-rabbit IgG raised in goats was purchased from Vector laboratories (Burlingame, CA, USA). Dead cells were stained with 10 μg mL^{-1} propidium iodide in PBS before fixation. Harvested cells were washed with PBS and fixed in 4% paraformaldehyde in PBS at RT for 30 min. After incubation, cells were washed with PBS, and then incubated with 400 μg mL^{-1} lysozyme in PBS at 37°C for 30 min to partially degrade the cell wall. Then, cells were washed with PBS, and one drop of the cell suspension was spotted onto a glass slide. After the solvent dried, cells were washed with distilled water. To detect total cells, 4′,6-diamidino-2-phenylindole (DAPI) staining was performed, applying 10 μg ml^{-1} DAPI in PBS at RT for 5 min. For immunohistochemical staining of RpsS, cells were incubated with 0.3% H_2O_2 in methanol at RT for 30 min to eliminate endogenous peroxidase activity and were also incubated with 3% normal goat serum at RT for 30 min to block non-specific reactions. After removal of the goat serum, cells were incubated with anti-RpsS antibodies (1:50 in dilution buffer) at RT for 2 h. After this incubation, cells were washed with PBS and then incubated with the biotinylated anti-rabbit IgG (7.5 μg mL^{-1} in dilution buffer) at RT for 1 h. After washing with distilled water, cells were incubated with an avidin:biotinylated enzyme complex (Vector laboratories) at RT for 30 min. For colour development, cells were incubated with PBS containing 0.02% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.006% H_2O_2.

The effects of artificial gastric and intestinal fluid treatment on the presence of cell-surface RpsS were examined according to Fernadez et al. (2003). Approximately 10^{10} cells were incubated in 10 mL of artificial gastric juice (125 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$, 3 g L^{-1} pepsin, pH 3.0). The bacterial suspensions were incubated at 37°C anaerobically with agitation for 180 min. Then, the cells were collected, suspended in 10 mL of artificial intestinal fluids (0.1% pancreatin, 0.15% oxgall in distilled water, pH 8.0), incubated as previously described, and followed by immunohistochemical staining.

Quantification of *rpsS* mRNA levels in *Lactobacillus* strains using real-time RT-PCR

Total RNA was extracted from FSMM15, FSMM22, and LGG at the mid-exponential growth phase using RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed to synthesize single strand cDNA, according to the provided protocol. In this study, two housekeeping genes, *gapdh* and the 16S

rRNA gene, were used as internal controls to predict the relative expression level of *rpsS* genes. Standard curves were constructed in duplicate using the PCR products of *rpsS*, *gapdh*, and 16S rRNA gene using a single colony of LGG as a template. To obtain a 10-fold serial dilution in the range of 10^8 to 10^1 for real-time PCR, cDNA concentration was adjusted to 500 ng/μL of EB buffer (Qiagen), diluted 10-fold, and subjected to real-time PCR reaction using Power CYBR Green PCR master mix (Thermo Fisher Scientific). Real-time PCR was performed by using the STEP ONE plus real-time PCR System (Thermo Fisher Scientific). The cycle conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 9 sec, 57.5°C or 60.5°C for 1 min, and followed by a dissociation step of 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec to determine the arbitrarily-place threshold (C_T) values of the amplicons. The gene copy numbers of the samples were analysed using the absolute quantification method by extrapolating the C_T values of the samples and the standard curves. The analysis was performed using StepOne software for StepOne and StepOnePlus real-time PCR system Version 2.2.2. Primers used in this study are listed in Table 1.

Western blotting

Proteins in an SDS-PAGE gel were transferred to a polyvinylidene difluoride membrane using mini-trans-blot electrophoretic transfer cell (Bio-Rad Laboratories). Blocking was performed with 5% (w/v) blocking agent (GE Healthcare) in PBS-T at RT for 2 h. After rinsing with PBS-T, the membrane was incubated with an anti-RpsS antibody (diluted 1:5,000) in PBS-T at 4°C overnight. After washing with PBS-T, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (diluted 1:50,000) at RT for 1 h. Signals were developed with ECL prime Western blotting detection reagent (GE Healthcare) and analysed using Ez-Capture MG (Atto, Tokyo, Japan). An anti-RNA polymerase antibody (diluted 1:1,000; Neoclone, Madison, WI, USA) was used to detect RNA polymerase as a cytosolic protein marker.

Table 1

Statistical analysis

Experiments were performed in triplicate from three independent cultures and expressed as the mean ± standard deviation. Cell viability and protein concentration were analysed by Student's t-test. Analysis of variance with post-hoc Dunnet's test was used for ELISA experiments.

Results

Profiles of CSPs extracted from FSMM15 and FSMM22 with 1 M LiCl

CSPs yielded 143 ± 12 and 580 ± 60 μg mL^{-1} in FSMM15 and FSMM22, respectively. Cell viabilities before and after extraction were 1.6 x 10^8 and 1.5 x 10^8 colony forming units (CFU) mL^{-1} for FSMM15, respectively, and 1.3 x 10^8 and 1.2 x 10^8 CFU mL^{-1} for FSMM22, respectively, indicating that cell damage was negligible (Table S1). Removal of CSPs led to an approximately 2-fold decrease

Table S1

in the laminin-binding ability of FSMM22 but not of FSMM15, indicating that CSPs act as laminin adhesins on the cell surface (Fig. 1). As shown in Fig. 2, the band patterns of CSPs in FSMM15 and FSMM22 1M LiCl extracts were highly similar. In contrast, proteins that bound to laminin were present in trace amounts in FSMM15, whereas several bands were observed in FSMM22. The FSMM22 CSPs extracted with 1M LiCl solution significantly decreased the ELISA titre compared to that of the control (Fig. 3), supporting the result obtained through SDS-PAGE analysis. Mass spectrometry analysis revealed that DNA-binding protein HU (HUP), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH), and 30S ribosomal protein S19 (RpsS) were a part of the LBPs in the FSMM22 CSPs extracted with 1M LiCl solution (Fig. S1).

Fig. 1 Fig. 2 Fig. 3 Fig. S1

Detection of RpsS present on the cell surface of FSMM22 by immunohistochemical staining

The binding specificity of the primary antibody was confirmed by Western blotting (Fig. S2). RpsS was clearly detected in the cell surface region of FSMM22 but not in FSMM15 (Fig. 4). The number of RpsS on the surface of living FSMM22 cells decreased after the cells were damaged by treatment with artificial gastric and intestinal fluids, because the thickness of the DAB-stained dark brown layer surrounding the bacterial cells apparently decreased. Binding of the primary antibody against RpsS was inhibited under the presence of the antigen peptide (Fig. S3).

Fig. S2 Fig. 4 Fig. S3

Gene and protein expression levels of RpsS in *L. rhamnosus* FSMM15 and FSMM22

To better understand the different RpsS numbers on the cell surface of FSMM15 and FSMM22 cells, the mRNA and protein expression levels of RpsS in the two strains were investigated. Consequently, there was no significant difference in the expression level of *rpsS* during the mid-exponential growth phase of FSMM15 and FSMM22; *gapdh* and 16S rRNA genes were used as controls (Fig. 5A). In contrast, RpsS was detectable in the 1 M LiCl and cell-free FSMM22 extracts but not in FSMM15 extracts (Fig. 5B).

Fig. 5

Discussion

Nishiyama et al. (2015) reported that a FSMM22 mucus-binding protein deletion mutant lost one-half of its laminin-binding ability; therefore, our results suggest that the other half should be attributed to anchorless CSPs that are extractable with 1 M LiCl. FSMM15 adhered to laminin to some extent (Shi et al. 2012), despite the lower amount of extracted LBPs in FSMM15 compared to that in FSMM22; thus, FSMM15 may express different types of CSPs, such as lmb, a LBP found in *Streptococcus agalactiae* (Spellerberg et al. 1999) and laminin-binding microbial surface components recognizing adhesive matrix molecules (Sillanpää et al. 2004). The distribution of CSPs responsible for adhesion to the host seems to be bacterial strain dependent as was previously reported (Mackenzie et al. 2010). Whether the FSMM strains are piliated is unknown. Laminin-binding ability has already

been described for GAPDH, which associates with the cell wall of *Candida albicans* (Gozalbo et al. 1998). It is also likely for HUP because an HUP homolog in *Mycobacterium tuberculosis* showed 78% identity of amino acids towards a 21-kDa LBP found in *Mycobacterium leprae* (Shimoji et al. 1999). LDH is also known to function as a moonlighting protein, e.g. an eye lens protein in geckos (van Rheede et al. 2003); however, there is currently no report in relation to laminin binding. Further experiments are needed to confirm the laminin-binding ability of LDH given the possibility of complex formation between LDH and GAPDH, as was found in a multicomponent Oct-1 coactivator that is essential for S phase-dependent histone H2B transcription (Zheng et al. 2003), cannot be excluded. Previously reported laminin binding moonlighting proteins, such as enolase (Antikainen et al. 2007a), glutamine synthetase (Kainulainen et al. 2012), and malate synthase (Kinhikar et al. 2006), were not found in this study.

RpsS is a small protein with an approximate molecular mass of 10,000 that exists in a complex with 30S ribosomal protein S13, which binds to 16S rRNA in the prokaryotic small ribosomal subunit (Schwarzbauer and Craven 1985). Among lactic acid bacteria, RpsS has been found on the cell surface of *Lactococcus lactis* NZ900 grown in M17 medium supplemented with 0.5% glucose (Berlec et al. 2011) and *L. rhamnosus* grown under heavy metal stress (Sreevani et al. 2014), while 30S ribosomal protein S5 was abundantly present in the surface-exposed proteome of LGG after bile stress (Koskenniemi et al. 2011). Extraribosomal functions of ribosomal proteins have been well studied; these functions expand beyond protein synthesis to encompass many biological processes, including replication, transcription, and RNA processing (Wool 1996). Thus far, laminin-binding ability has been attributed to the 40S ribosomal protein SA, which is a 67-kDa laminin receptor in vertebrates (Auth and Brawerman 1992; Ardini et al. 1998). Although our data strongly suggest that the RpsS moonlights on the cell surface as an LBP, further experiments are needed, e.g. inhibition of bacterial cell adhesion to laminin using an appropriate anti-RpsS antibody. However, our preliminary experiment attempting to inhibit FSMM22 adherence to immobilized laminin through the addition of anti-RpsS antibodies was unsuccessful (Fig S4). Immunohistochemical staining was successful only when the bacterial cell wall peptidoglycan was partially degraded by lysozyme; therefore, the binding epitope seems to not be exposed to the solvent, and this might be the reason why the preliminary experiment did not succeed.

Fig. S4

There was no significant difference between the mRNA expression levels of the *rpsS* gene in FSMM15 and FSMM22. On the other hand, no positive band could be detected even in the cell-free extract of FSMM15 by Western blotting analysis, implying the occurrence of an unknown variation in the N-terminal region of FSMM15 RpsS. These observations led us to assume the existence of a specific RpsS transport pathway from the cytosol to the cell surface of FSMM22 cells, although further experiments are needed. In fact, the molecular mechanism of transporting anchorless CSPs is controversial. There is experimental evidence to support the presence of an unknown export pathway of moonlighting proteins (Boël et al. 2005), secretion from dead or traumatized cells (Stephenson et

al. 1999), and increased membrane permeability (Saad et al. 2009). These alternatives are not entirely mutually exclusive as mentioned by Kainulainen and Korhonen (2014). RpsS is a highly basic protein with a theoretical *p*I value of around 10, which may support its presence on the bacterial cell surface *via* electrostatic interaction; however, the pH-dependent attachment/detachment observed in acidic enolase (*p*I = 4.8) and GAPDH (*p*I = 5.2) from *Lactobacillus crispatus* (Antikainen et al. 2007b) is not likely the case for RpsS.

To summarize, HUP, GAPDH, LDH, and RpsS were extractable with 1 M LiCl in *L. rhamnosus* FSMM22, but not FSMM15, as a part of CSPs that enhanced the bacterial adhesion to laminin. The cell surface localisation of RpsS in lactobacilli was immunohistochemically confirmed for the first time. Our findings suggest that the host-bacterial interaction is influenced by the abundance of anchorless CSPs in addition to contributions by pilus adhesins and anchored CSPs.

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Figure legends

Fig. 1. Effects of CSP removal on the adhesive properties of *L. rhamnosus* FSMM15 and FSMM22 on laminin. Filled bars, relative bacterial cell numbers that bound to laminin prior to 1 M LiCl extraction; diagonal bars, relative bacterial cell numbers that bound to laminin after 1M LiCl extraction. The asterisk represents a statistically significant difference with $p < 0.05$ ($n = 3$).

Fig. 2. SDS-PAGE profiles of the CSPs and LBPs extracted with 1 M LiCl from *L. rhamnosus* FSMM15 and FSMM22. CSPs, cell surface proteins; LBPs, laminin-binding proteins; control, 0.1% BSA in PBS was used in the isolation procedure of LBPs instead of CSP solution; size marker, a precision plus protein dual color standard from 10-250 kDa (Bio-Rad Laboratories). Protein bands were visualized by silver staining. The bands indicated by numbers were subjected to protein identification by mass spectrometry analysis. GAPDH and LDH were found in band 5, whereas RpsS and HUP were detected in band 8.

Fig. 3. Inhibition ELISA using the CSPs extracted with 1 M LiCl from *L. rhamnosus* FSMM15 and FSMM22. As a control, 0.1% BSA in PBS (filled bar) was used for ELISA, whereas the CSP solutions were extracted with either PBS (diagonal bars) or 1 M LiCl (dotted bars) as described in the Materials and Methods. When proteins in the CSP solutions bound to the immobilized laminin, binding of the primary antibody to the immobilized laminin was hampered, and thereby, the titre showed a significant decrease compared to that in the control. The asterisk represents a statistically significant difference to the control with $p < 0.001$ ($n = 3$).

Fig. 4. Anti-RpsS immunohistochemical staining of *L. rhamnosus* FSMM15 and FSMM22. 'Before' and 'After' indicate before and after treatment with artificial gastric and intestinal fluids, respectively. DAPI, PI, and Anti-RpsS indicate microscopic images of the bacterial cells stained with 4′,6-diamidino-2-phenylindole, propidium iodide, and 3,3′-diaminobenzidine tetrahydrochloride, respectively. Identical microscopic fields are shown for each staining method. Areas in which viable cells were observed (stained not with PI but with DAPI) were squared in the DAB staining images and are shown at higher magnification (High mag). White bars in the DAPI staining images represent a length of 1 μm.

Fig. 5. Expression levels of RpsS (A) mRNA and (B) protein in *L. rhamnosus* FSMM15 and FSMM22. In panel (A), the *rpsS* mRNA expression levels of the two FSMM strains were evaluated by real-time RT-PCR using the 16S rRNA (filled bars) and *gapdh* (diagonal bars) genes as controls. Error bars in the graphs represent the standard deviation ($n = 3$). In panel (B), the presence of RpsS in the 1 M LiCl extracts and in the cell-free extracts is shown. The cell-free extracts were prepared from

bacterial cells obtained from 100 mL of the 20-h-culture broth. Cells were harvested, suspended in 20 mL of PBS, and then sonicated (20% amplitude for 3 min with 1 min interval, 7 times, on ice) using the Vibra-Cell VC505 (Sonics & Materials, Newtown, CT, USA).

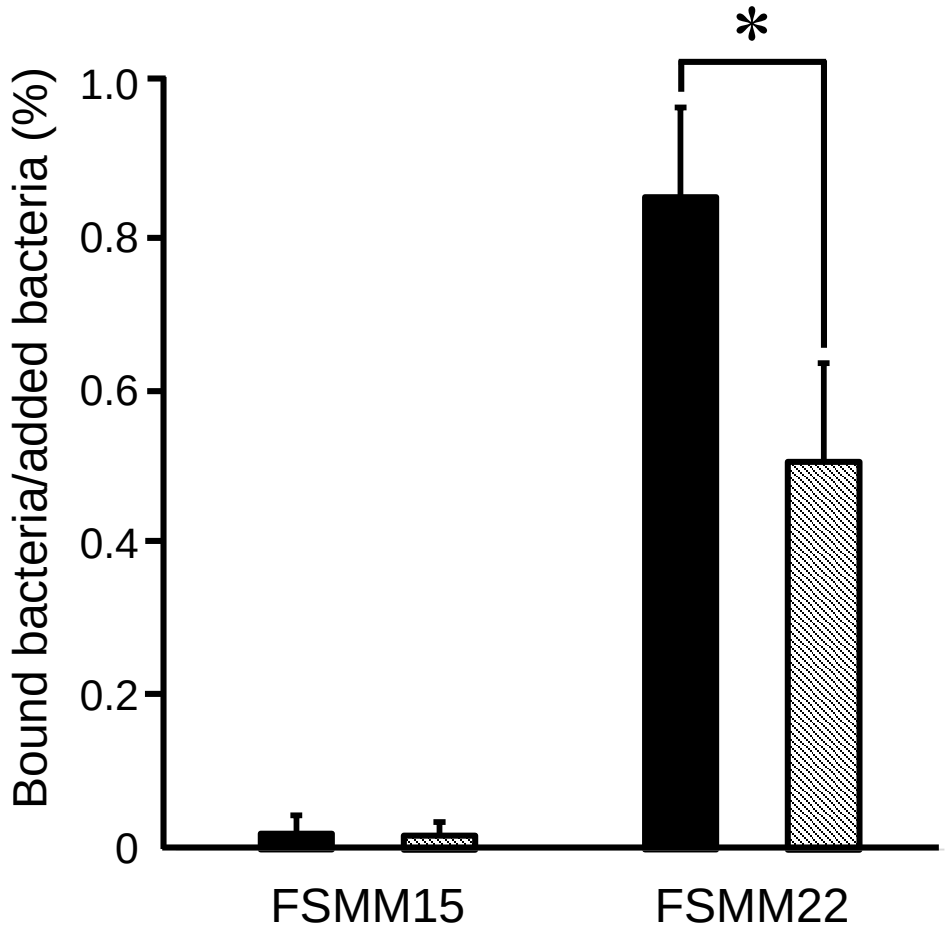

Fig. 1. Aryantini et al.

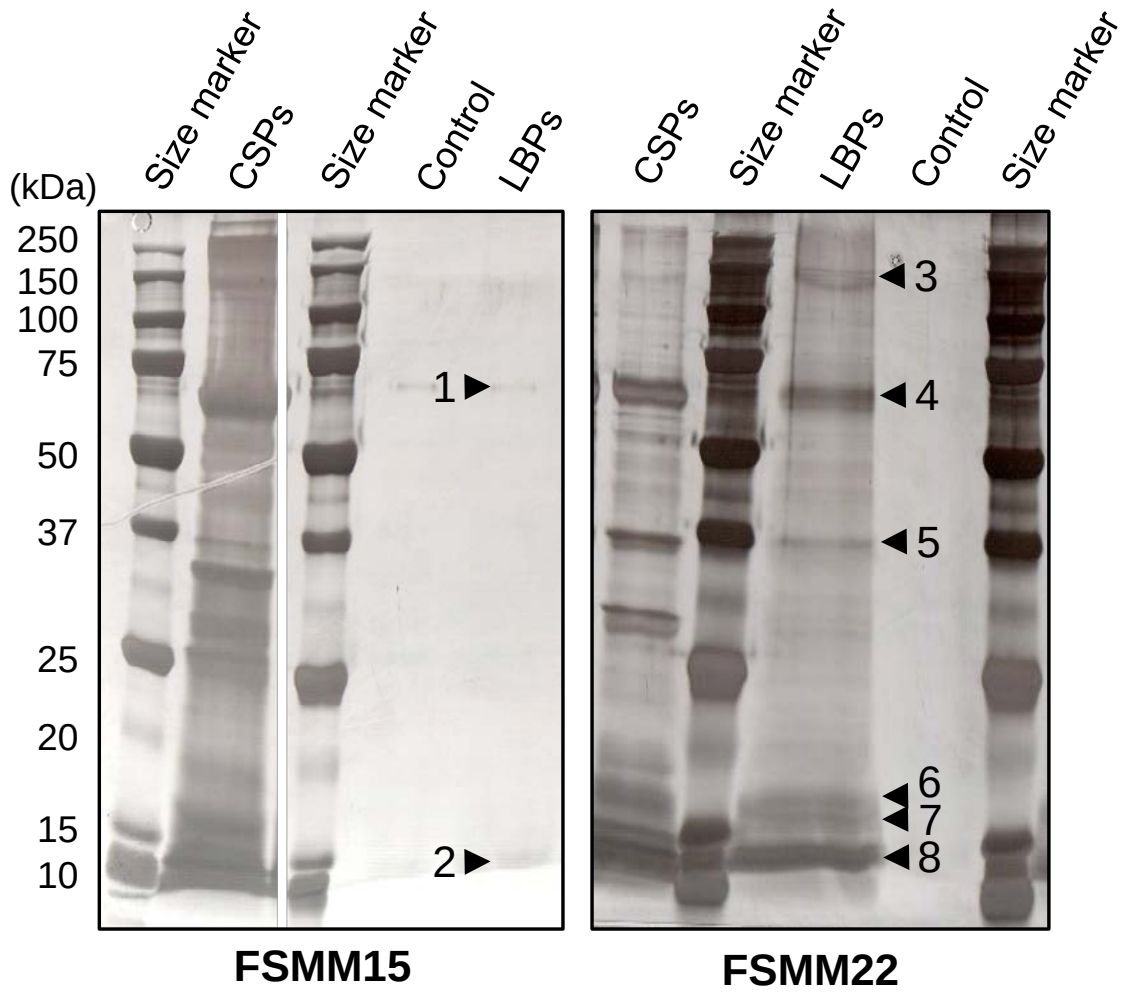

Fig. 2. Aryantini et al.

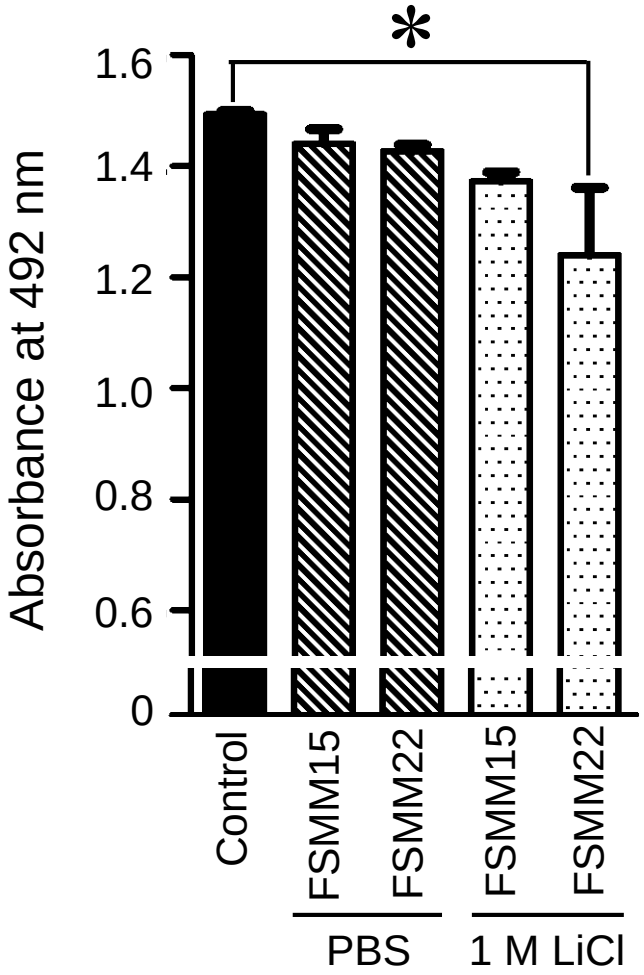

Fig. 3. Aryantini et al.

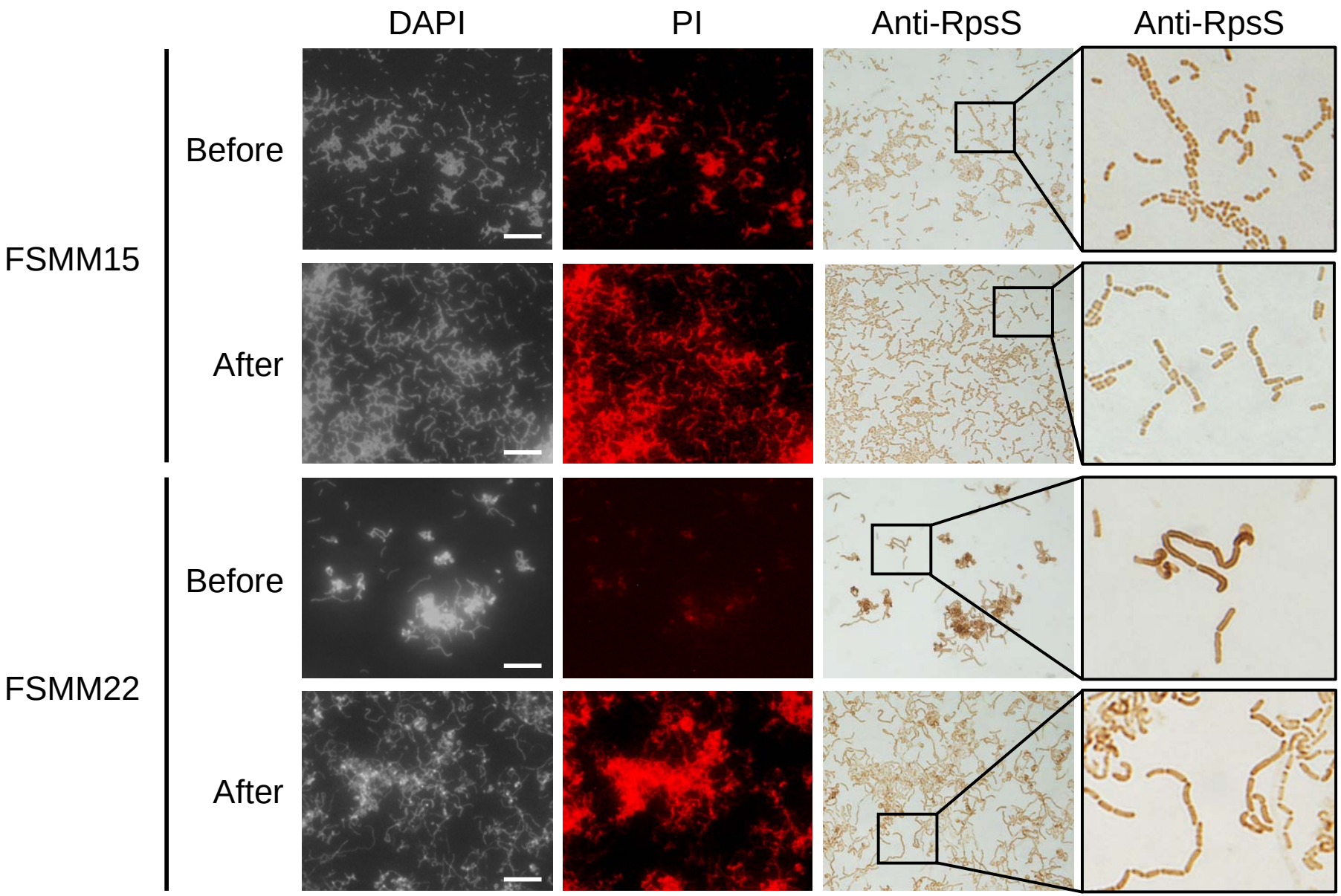

Fig. 4. Aryantini et al.

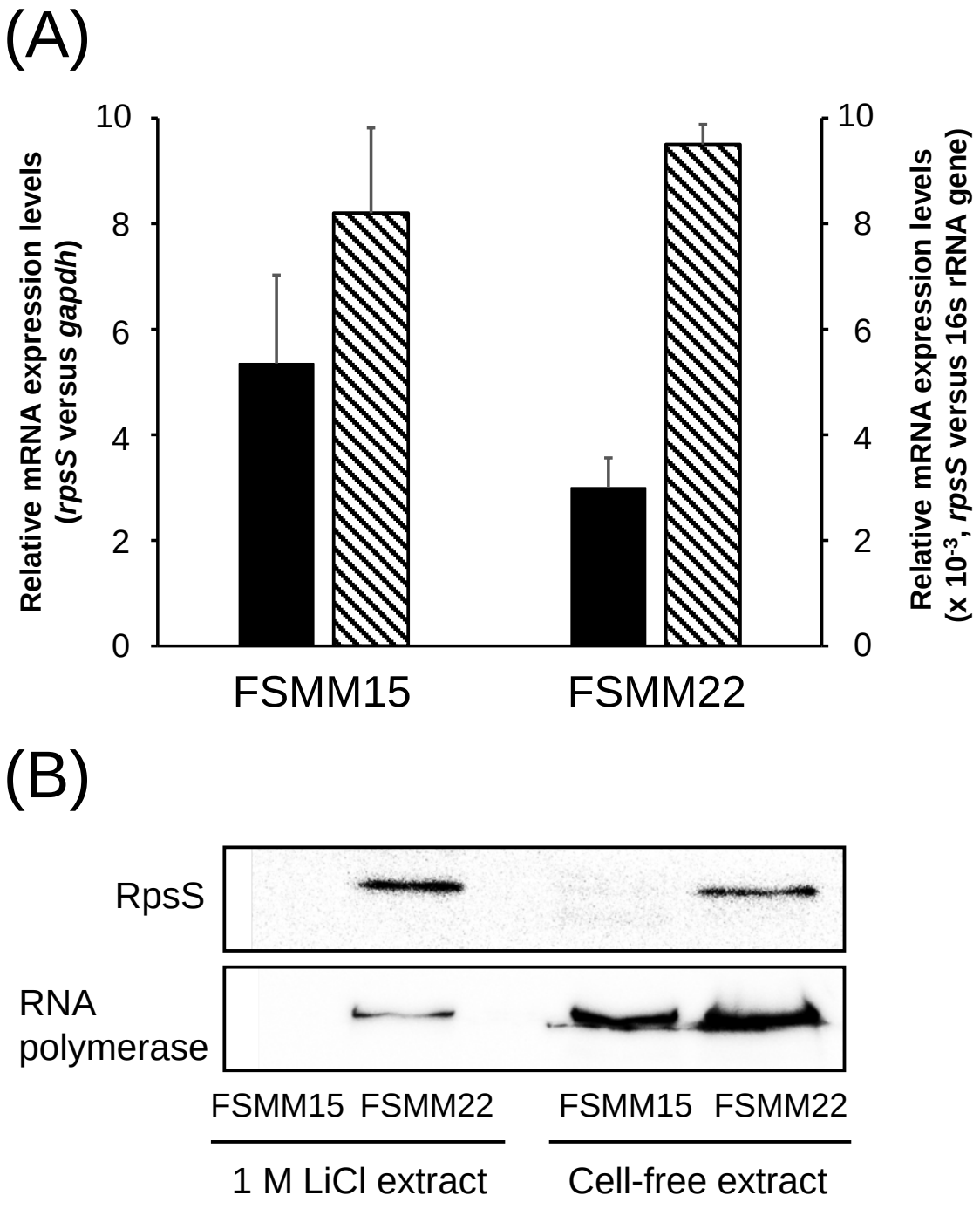

Fig. 5. Aryantini et al.

Table 1. Primers used in this study for real-time RT-PCR.

Genes[1)]	Primers	Sequence (5'→3')	Length (bp)	T_m (°C)	GC (%)	Amplicon size (bp)
rpsS (gi|258506995)	rpsS-F	ATGGGTCGCAGTCTTAAAAAAG	22	54	40.9	282
	rpsS-R	CTAGCGTGCTGTTGTCTTCTTGTC	24	60	50.0	
	rpsS qPCR-F	TACACCATCGCCGTTTAC	18	54	50.0	82
	rpsS qPCR-R	TTCGCCTAACTTGTGACC	18	54	50.0	
16s rRNA (gi|507147971)	27F[2)]	AGAGTTTGATCCTGGCTCAG	20	56	50.0	1528
	1492R[2)]	TACCTTGTTACGACTT	16	45	37.5	
	16s rRNA qPCR-F	GTAGGGAATCTTCCACAATGGACG	24	60	50.0	321
	16s rRNA qPCR-R	GTTCCACTGTCCTCTTCTGCAC	22	61	54.5	
gapdh (gi|258506995)	gapdh-F	TACTTTCCCTGGTGAAGTTAGT	22	54	40.9	533
	gapdh-R	CCTGTAACTTGCCGTTCAATTC	22	57	45.5	
	gapdh qPCR-F	CAAAGCGTGTTCTGATTTCTGC	22	57	45.5	158
	gapdh qPCR-R	CCTGGTTCAGGAAGTAAGCC	20	58	55.0	

[1)]GenBank accession numbers are in parentheses.
[2)]Frank et al.