Expression of a biologically active GFP-α_{S1}-casein fusion protein in *Lactococcus lactis*

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Running Head: α_{S1}-casein Expression in *Lc. Lactis*

Abstract

In this study, we successfully developed a recombinant strain of *Lactococcus lactis* NZ9000 (NZ9000) that produced green fluorescent protein fused to α_{S1}-casein (GFP-α_{S1}Cas). A modified lactic acid bacterial vector (pNZ8148#2) was constructed by inserting genes for GFP and α_{S1}-casein, a major cow's milk allergen, and the resulting vector, pNZ8148#2-GFP-α_{S1}Cas, was applied to the expression of recombinant GFP-α_{S1}Cas protein (rGFP-α_{S1}Cas) in NZ9000. After inducing expression with nisin, the production of rGFP-α_{S1}Cas was confirmed by confocal laser microscopic analysis, and the expression conditions were optimized based on fluorescent analysis and Western blotting results. Moreover, the *in-vitro* treatment of splenocytes isolated from α-casein (≥70% α_S-casein)-immunized mice with rGFP-α_{S1}Cas resulted in increased IL-13 mRNA expression. The observed allergic activity is indicative of the Th2-cell mediated immune response and is similar to the effects induced by exposure to α-casein. Our results suggest that the expression of rGFP-α_{S1}Cas in NZ9000 may facilitate *in-vivo* applications of this system aimed at improving the specificity of immunological responses to specific milk allergen.

Keywords; milk allergen, α_{S1}-casein, GFP, *Lactococcus lactis*, nisin

Introduction

Milk is one of the first components introduced into human diet. It also represents one of the first allergen sources, which induces immunoglobulin E (IgE)-mediated allergies in childhood ranging from gastrointestinal, skin, and respiratory manifestations to severe life-threatening manifestations, such as anaphylaxis [18]. Cow's milk allergy is the most common food allergy in young children, affecting 1–2% of all infants [18]. Casein represents a major allergen in cow's milk [3] and consists of four proteins: α_{S1}-casein, α_{S2}-casein, β-casein and κ-casein, representing 32%, 10%, 28%, and 10%, respectively, of the total milk protein [17]. Of these four proteins, α_{S1}-casein thought to be the most potent for inducing a specific IgE response [1]. α_{S1}-casein is considered as a true food allergen, also classified as class I food allergen, which is comparable to the major fish allergen parvalbumin [21], the major peanut allergens [13], and the major shrimp allergen [15], and it can induce severe and life-threatening anaphylactic reactions. α_{S1}-casein, which is a single-chain linear phosphoprotein of 199 amino acid residues, has only a small amount of secondary structure and lacks disulfide bonds, which results in a reduction of tertiary interactions [8]. However, as the potential of an α_{S1}-casein delivery system for mucosal immunization is not known, we speculated that such a system may aid in the study and modulation of the specific IgE immune response induced by α_{S1}-casein.

Current evidence suggests that probiotic bacteria can play a beneficial role in the prevention or treatment of the Th2-biased allergic response [6]. *Lactococcus (Lc.) lactis* is a highly efficient probiotic microorganism with a wide range of benefits for human health. Experimental data and genomic analyses indicate that *Lc. lactis* only naturally secretes a few proteins [14], and a plasmid-free *Lc. lactis* strain does not produce the extra cytoplasmic protease PrtP [5]. These features have drawn the attention of researchers to the potential use of *Lc. lactis* for the secretion of biotechnologically important proteins. The nisin-controlled gene expression (NICE) system has become a widely used tool for regulated gene expression in Gram-positive bacteria [2], including lactic acid bacteria such as *Lc. lactis*. The use of such a system in lactic acid bacteria may represent a suitable approach for generating a novel type of topical antigen delivery vehicle for mucosal immunization [9].

Here, we engineered a *Lc. lactis* NZ9000 (NZ9000) recombinant strain that produces green fluorescent protein (GFP) fused to α_{S1}-casein (rGFP-α_{S1}Cas). The fluorescence signal of GFP allows the rapid, simple, and accurate measurement of target protein expression by microscopic analysis [4]. The aim of the present study was to investigate the biological activity of splenocytes isolated from mice immunized with α-casein and subsequently treated with rGFP-α_{S1}Cas.

Materials and methods

Bacterial strain and growth conditions

NZ9000 is a standard host strain for the NICE system (MoBiTec, Goettingen, Germany). NZ9000 is derived from *Lc. lactis* subsp. *cremoris* MG1363 (MG1363) and contains the regulatory genes *nisR* and *nisK* integrated into the *pepN* gene. NZ9000 was grown in M17 medium supplemented with 0.5% glucose at 30 °C, and gene expression was induced with nisin, as described previously [7]. Plasmid maintenance was ensured by growing recombinant NZ9000 strains in medium supplemented with 25 µg/ml chloramphenicol.

Construction of a recombinant NZ9000 strain

pNZ8148#2 vector is a modified plasmid of the *Lc. lactis* expression vector pNZ8148 (MoBiTec; Goettingen, Germany) and contains the PnisA promoter and terminator upstream and downstream, respectively, of a multicloning site (MCS). A 6xHis-tag and factor Xa recognition site are positioned between the PnisA promoter and MCS (Fig. 1A). The GFP and GFP-α_{S1}Cas coding sequences were optimized for MG1363 codon usage to increase its expression level in NZ9000. The codon-optimized GFP and GFP-α_{S1} Cas genes were synthesized by Operon Biotechnologies (Tokyo, Japan) and subcloned into pCR2.1 (Invitrogen, Carlsbad, CA, USA). The resulting plasmid was digested by *BamH*I and *Hind*III to excise the GFP or GFP-α_{S1}Cas gene cassette, which was then cloned into the pNZ8148#2 expression vector. The resulting plasmid (pNZ8148#2-GFP or GFP-α_{S1}Cas) contained the GFP or GFP-α_{S1}Cas gene cassette under control of the nisin-inducible PnisA promoter (Fig. 1B, C) and was introduced in NZ9000 by electroporation using a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Inc., CA, USA) following the manufacturer's instructions. The resulting NZ9000 recombinant strain produced rGFP-α_{S1}Cas and was named NZ9000-GFP-α_{S1}Cas. NZ9000 was also electroporated with empty plasmid pNZ8148#2 to generate a NZ9000 vector control strain (NZ9000-Vc).

Recombinant GFP-α_{S1}-casein expression and purification

Optimal parameters for rGFP-α_{s1}Cas expression were assessed by growth curves (OD_{600}), external pH, GFP fluorescence signal, and Western blotting analysis for various nisin concentrations and induction times (h). After induction with nisin, a cell pellet obtained by centrifugation was broken by grinding with aluminum oxide

powder (3 g per 1 g of cells; Wako, Osaka, Japan) for 15 min, after which the broken cells were suspended in a standard buffer containing protease inhibitor (Sigma). Protein content was measured using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Fluorescence, reported as relative fluorescence units (RFU), was measured using a Fluoroskan Ascent FL Microplate Fluorometer (Thermo Scientific, Tokyo, Japan) by excitation at 485 nm and detection of emission at 538 nm.

rGFP and rGFP-α_{S1}Cas were purified using a HisTrap HP column (1 mL, precharged with Ni^{2+}; GE Healthcare Japan, Tokyo, Japan) under native conditions, according to the manufacturer's instructions. The flow-through fractions were collected by washing the column with 20 mM imidazole phosphate buffer (pH 8.0). The absorbed factions were eluted using 31.25, 62.5, 125, 250, and 500 mM imidazole phosphate buffer (pH 8.0). After dialysis, the eluted fractions were freeze-dried and analyzed by SDS-PAGE and Western blotting. For SDS-PAGE, the cell lysates and purified protein samples were boiled for 5 min in SDS sample buffer. The boiled samples were run on 15% SDS-PAGE gels and then transferred onto PVDF membranes. Western blotting was performed with primary antibodies (Abs) against the 6xHis tag, GFP and casein (BioLegend, Inc., San Diego, CA, USA), followed by HRP-conjugated secondary Ab (Sigma). Signals were visualized by treating the membranes with TMB peroxidase substrate.

α-casein-immunized mice

Pathogen-free female BALB/c mice (4 weeks of age) were purchased from Japan SLC (Shizuoka, Japan) and housed under temperature- and light-controlled conditions. Mice were fed a standard diet of Labo MR Breeder (Nihon Nosan Co., Kanagawa, Japan) and sterile water *ad libitum*. After a preliminary breeding period of 2 weeks, BALB/c mice (6 weeks of age, n=3) were intraperitoneally (i.p.) sensitized once weekly for 3 weeks with 100 μg of bovine milk α-casein (Sigma, MO, USA), consisting of ≥70% α_S-casein and alum (allergen/adjuvant ratio of 1/50). All experimental procedures were conducted in accordance with the Regulations for Animal Experimentation of Shinshu University.

Biologic activity of recombinant GFP and GFP-α_{S1}-casein

The biologic activities of rGFP and rGFP-α_{S1}Cas were assessed by real-time quantitative PCR, which was used to detect IL-13 mRNA expression induced by rGFP-α_{S1}Cas in splenocytes isolated from α-casein- immunized mice. Splenocytes were prepared using standard methods [19] and were then cultured in

medium at a final concentration of 2 x 10^6 cells per well (total volume, 1 mL per well). Splenocytes were stimulated with 10 and 50 μg/mL purified rGFP, rGFP-α_{S1}Cas or α-casein as a control for 72 h, harvested, and then monitored for IL-13 mRNA expression by real-time quantitative PCR analysis, as previously described [20]. Fluorescent real-time quantitative PCR reactions were performed on SYBR Premix Ex Taq (Takara Bio Inc.) using specific primers, with each reaction containing 5 ng of cDNA in 25 mL. The β-actin and IL-13 primers were purchased from Takara Bio Inc. The PCR cycling conditions were 10 s at 95°C, followed by 45 cycles of 5 s at 95°C, and 30 s at 60°C. As a control, poly (A)+RNA samples were used as templates to check for the presence of contaminating genomic DNA. The sensitivity of the reaction and amplification of contaminant products such as the extension of self-annealed primers were evaluated by amplifying serial dilutions of the cDNA. For cross-sample comparison of results obtained following various treatments, cytokine mRNA levels were first normalized to mRNA levels obtained for β-actin. The results represent the means ± SD of three or four independent experiments.

Statistical analysis

All results represent the average of three separate experiments. Statistical significance was evaluated using the Student's *t*-test. A p value of <0.05 was considered statistically significant.

Results

Optimal conditions of rGFP-α_{S1}-casein expression in NZ9000

We constructed the vector pNZ8148#2-GFP-α_{S1}Cas, which was modified from vector pNZ8148 and contains α_{S1}-casein fused with GFP at the N-terminus (Fig. 1A, B). A nisin concentration of 50 ng/mL was found to be optimal for inducing the expression of recombinant protein by NZ9000-GFP-α_{S1}Cas, as indicated by the highest levels of fluorescence observed at this concentration (Fig. 2A). The maximum detectable GFP activity occurred 2-3 h after induction (Fig. 2B). In cell cultures treated with 50 ng/mL nisin, the external pH of the medium dropped from 6.5 to 5.0 (Fig. 2C) and stationary phase was reached 4 h after induction (Fig. 2D).

The expression of rGFP-α_{S1}Cas (55.2 kDa) was detected by Western blotting analysis with anti-His tag (Fig. 2E) and anti-casein Abs (Fig. 2F) from induced cultures of NZ9000-GFP-α_{S1} Cas. The control NZ9000-Vc strain did not express detectable amounts of α-casein protein (Fig. 2E, F). To confirm the expression of rGFP-α_{S1}Cas by NZ9000-GFP-α_{S1} Cas, NZ9000-Vc and NZ9000-GFP-α_{S1} Cas cells were microscopically

examined under visible light (Fig. 2G, H) and by confocal laser microscopy (Fig. 2H, K) after a 2 h induction with nisin. As shown in the merged images (Fig. 2I and 2L), NZ9000-GFP-α_{S1} Cas cells clearly expressed recombinant protein. From the results of the Western blotting and confocal laser microscopy analyses, we successfully isolated and purified His-tagged and GFP-fused α_{S1}Cas recombinant protein using NZ9000 transformed with the pNZ8148#2 vector containing the GFP-α_{S1}Cas gene cassette (Fig. 2).

Expression of IL-13 mRNA induced by purified rGFP-α_{S1}-casein

rGFP and rGFP-α_{S1}Cas isolated from NZ9000-GFP and GFP-α_{S1} Cas cells were purified using a HisTrap HP column. The purification of rGFP and rGFP-α_{S1}Cas were monitored by staining SDS-PAGE gels with CBB (Fig. 3A, Supplementary 1). Approximately 7.0-10 mg of rGFP and rGFP-α_{S1}Cas were obtained from 3 L of culture broth by column purification and elution with 62.5 mM imidazole. Western blotting analysis using anti-His-tag (Fig. 3B), anti-GFP (Supplementary 2), and anti-casein Abs (Fig. 3C) showed a clear band corresponding to rGFP and rGFP-α_{S1}Cas in the fractions eluted with 31.25, 62.5, and 125 mM imidazole (Fig. 3A, B, C, Supplementary 2). Endotoxin was not detected in the eluted fractions containing rGFP and rGFP-α_{S1}Cas. We next examined the ability of purified rGFP-α_{S1}Cas to induce IL-13 mRNA expression in splenocytes isolated from α-casein-immunized mice. Commercial α-casein was capable of inducing IL-13 mRNA expression in splenocytes at a concentration of 10 μg/mL casein (Fig. 3D). Splenocytes stimulated with 10 μg/mL rGFP-α_{S1}Cas for 72 h also significantly expressed IL-13 mRNA compared with the 10 μg/mL rGFP (Fig. 3E).

Discussion

Food-grade Lactic Acid Bacteria (LAB) has been safely consumed for centuries by humans in fermented foods. Thus, they are good candidates to develop novel oral vectors, constituting attractive alternatives to attenuated pathogens, for mucosal delivery strategies. Today, sufficient data is available supporting the fact that LAB, notably lactococci and lactobacilli, are excellent candidates as delivery vectors of therapeutic proteins, in the development of novel preventive and therapeutic strategies for humans. The immunogenicity of soluble proteins administered orally or intranasally is generally low and can be significantly enhanced by either coupling the protein to a bacterial carrier or by the genetic engineering of bacteria to produce the target antigen. As previously mentioned, food-grade or commensal Gram-positive bacteria constitute an attractive alternative to

attenuated pathogenic bacteria for inducing immunity [23]. In particular, lactic acid bacteria such as *Lc. lactis* and certain species of lactobacilli possess a number of attractive properties that make them suitable candidates for the development of mucosal vaccines [10].

In this study, we successfully generated a recombinant NZ9000 strain expressing a GFP-α_{S1} Cas fusion protein. Maximal expression of the GFP-α_{S1}Cas fusion in NZ9000-GFP-α_{S1} Cas cells grown at 30 °C was achieved with the following culture parameters: OD_{600} of 0.4, 50 ng/ml nisin, and 2-4 h of induction. As fluorescence is one of the most convenient methods to evaluate protein expression and purification [12, 22], we constructed a GFP-fusion expression vector in NZ9000, which is generally applicable for GFP-α_{S1}Cas fusion expression based on the results of His-tagged GFP fusion expression in this strain. We were able to optimize expression levels by simply measuring GFP RFU, because of the good correlation between RFU and the amount of rGFP-α_{S1}Cas (data not shown).

To clarify the biological activity of rGFP-α_{S1}Cas, we examined whether purified rGFP-α_{S1}Cas could induce Th2-mediated allergic responses in splenocytes isolated from α-casein-immunized mice. Th2-cell mediated immune responses against “innocuous” antigens play a role in triggering allergic diseases [16] and are characterized by the prevalent production of IL-4, IL-5, IL-9, and IL-13 [11]. Here, we showed that splenocytes isolated from α-casein-immunized mice and subsequently treated with purified rGFP-α_{S1}Cas for 72 h strongly expressed IL-13 mRNA, demonstrating that rGFP-α_{S1}Cas is biologically active.

In conclusion, we have described a convenient method for rGFP-α_{S1}Cas fusion protein production with good protein yield and high allergic activity in an advantageous probiotic host. Purified rGFP-α_{S1}Cas from NZ9000 may find significant applications in basic research into food allergic disease. Finally, strain NZ9000-GFP-α_{S1}Cas may be a useful candidate organism for the development of allergic vaccines, particularly oral vaccines. However, the future application of prophylactic and therapeutic strategies based on NZ9000-GFP-α_{S1} Cas requires clear demonstration of their efficacy in *in-vivo* trials.

Acknowledgments

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Figure legends

Figure 1. Maps of plasmids used for the transformation of NZ9000. Map of the modified vector pNZ8148#2 (A) and construction of the rGFP (B) or rGFP-α_{S1}Cas expression vector (C). 714 bp (238 aa) and 639 bp (213 aa) gene fragments encoding GFP and α_{S1}-casein were excised with restriction enzymes from pCR2.1 containing the complete GFP and α_{S1}-casein genes and then inserted into the MCS of pNZ8148#2. NisinA promoter-region (PnisA), ribosome binding site (RBS), Factor Xa recognition site (Fxa), multiple cloning site (MCS), Terminator (T), Chloramphenicol acetyltransferase gene (CM), and replication gene (rep) are indicated in the plasmid maps.

Figure 2. Optimal conditions for GFP-α_{S1}Cas expression in the NZ9000 recombinant strain was evaluated by measuring fluorescence (A, B), pH (C), and growth curves (D) after induction with various nisin concentrations and times (h). Relative fluorescence index: relative fluorescence units (RFU) are displayed as relative values *vs.* blank (water). Expression of rGFP-α_{S1}Cas (55.2 kDa) was detected by Western blotting (E, F) and confocal laser microscopic analyses (G-L). Induced NZ9000-Vc (G, H, I) and NZ9000-GFP-α_{S1}Cas (J, K, L) cells were analyzed under visible light (G, J) and fluorescence (H, K), and then merged images were generated (I, L). The arrow indicates rGFP-α_{S1}Cas (55.2kDa). **P<0.01, ***P<0.001 *vs*. vector control (A, B). Bar=5μm.

Figure 3. Purification and biological activity analysis of rGFP-α_{S1}Cas. CBB-stained SDS-PAGE gel (A) and Western blot analysis using anti-His tag (B) or anti-casein Abs. (C). Real-time quantitative PCR analysis of IL-13 mRNA levels in splenocytes isolated from α-casein-immunized mice and subsequently treated with commercial α-casein or rGFP-α_{S1}Cas (D, E). Commercial α-casein was capable of inducing IL-13 mRNA expression at a concentration of 10 μg/mL (D). **P<0.01 vs. cells cultured in the presence of 0.01 μg/mL casein (D) or 10 μg/mL rGFP (E). Similar results were obtained from at least three different mice. Lane 1, molecular mass standard; Lane 2, crude lysate before purification; Lane 3, flow-through fractions; Lanes 4, 5, 6, 7, and 8, fractions eluted using 31.25, 62.5, 125, 250, and 500 mM imidazole, respectively. The arrow indicates rGFP-α_{S1}Cas (55.2kDa).

Supplementary 1. Expression analysis of rGFP. CBB-stained SDS-PAGE gel (A) and Western blot analysis anti-GFP Abs. (B). Lane 1, molecular mass standard; Lane 2, Vector control; Lane 3, NZ9000-pNZ8148#2-GFP. The arrow indicates rGFP recombinant protein (30.8kDa).

Supplementary 2. Purification analysis of rGFP. CBB-stained SDS-PAGE gel (A) and Western blot analysis using anti-His tag (B) or anti-GFP Abs. (C). Lane 1, molecular mass standard; Lane 2, crude lysate before purification; Lane 3, flow-through fractions; Lanes 4, 5, 6, 7, and 8, fractions eluted using 31.25, 62.5, 125, 250, and 500 mM imidazole, respectively. The arrow indicates rGFP recombinant protein (30.8kDa).

Figure 1

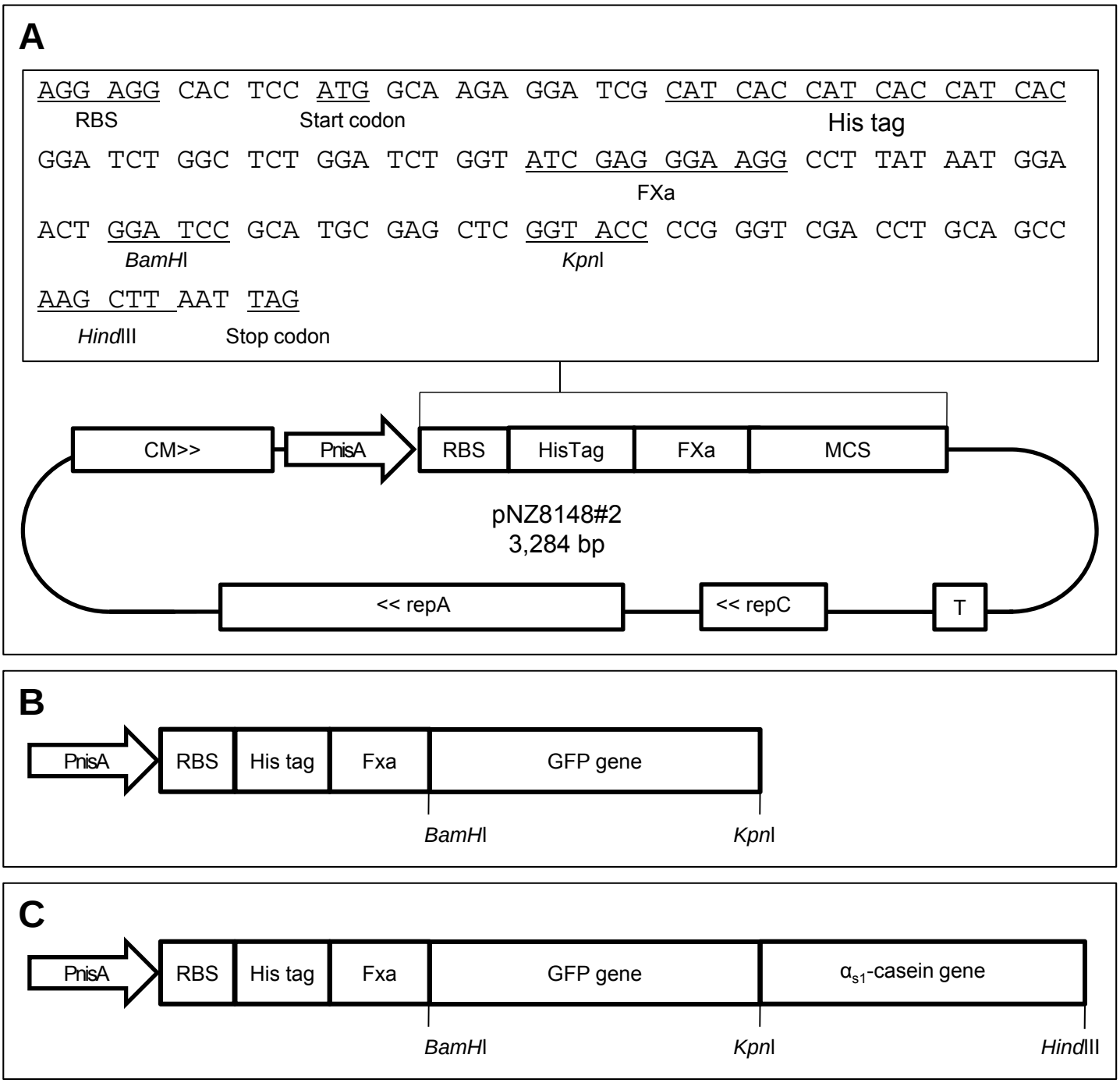
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Figure 2

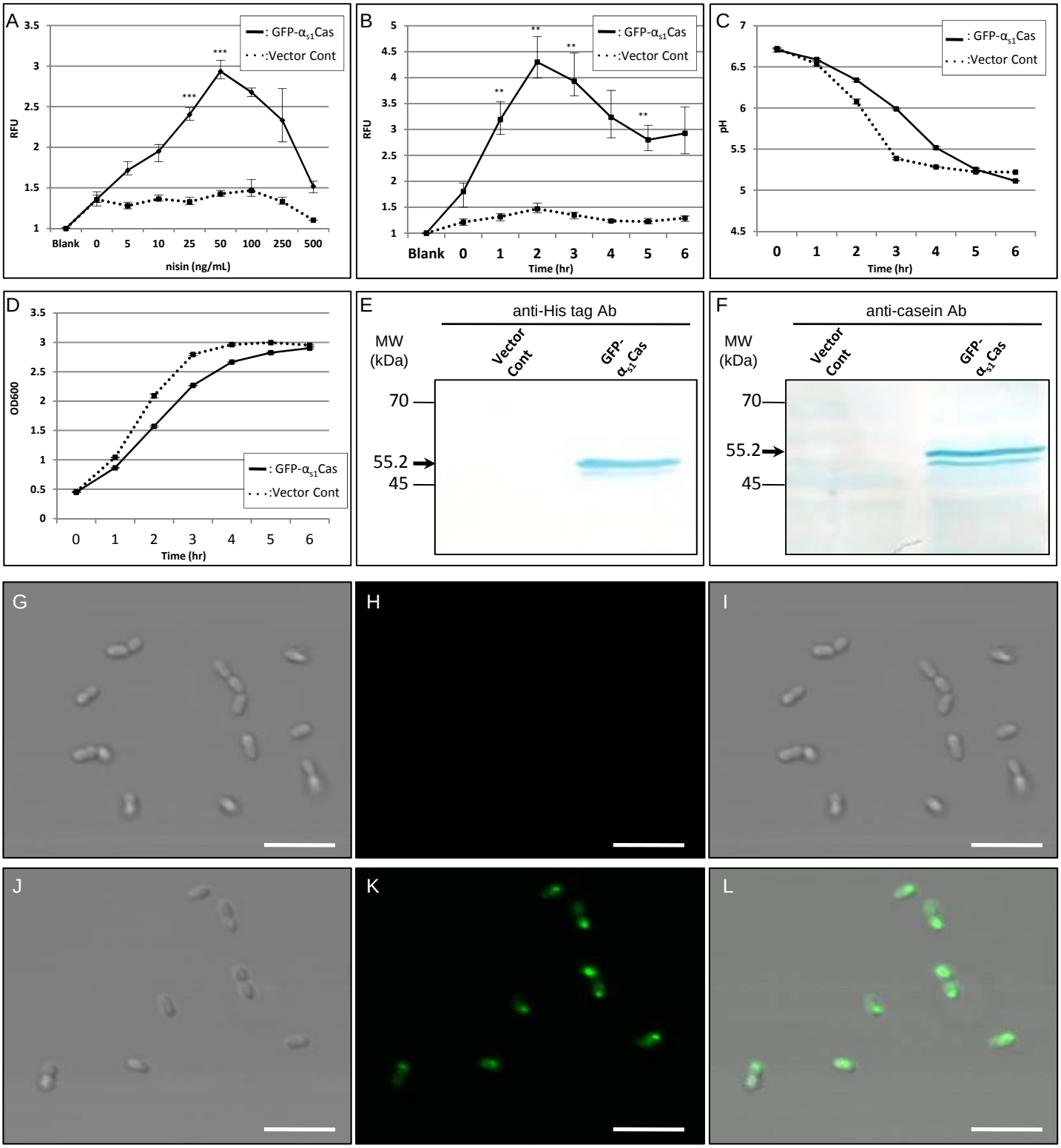
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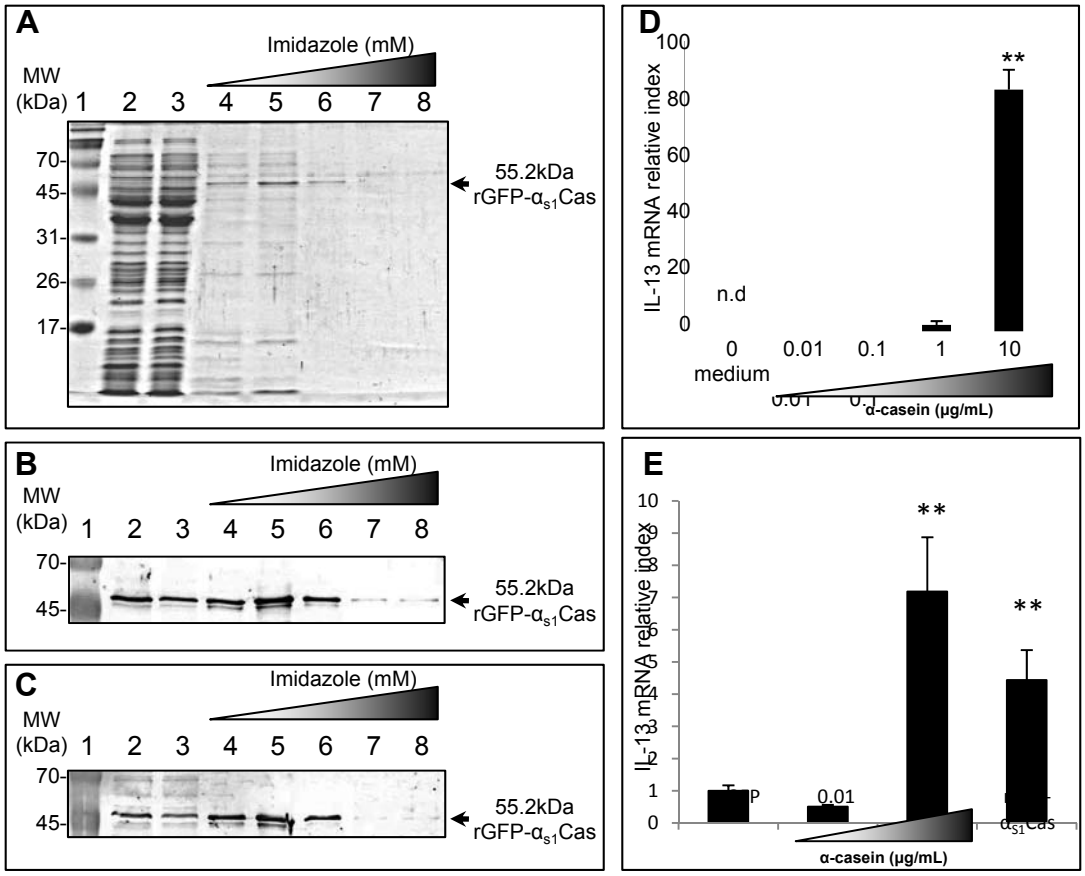
Figure 3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MW (kDa)
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70-
45-
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Imidazole (mM)
MW (kDa)
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40
20
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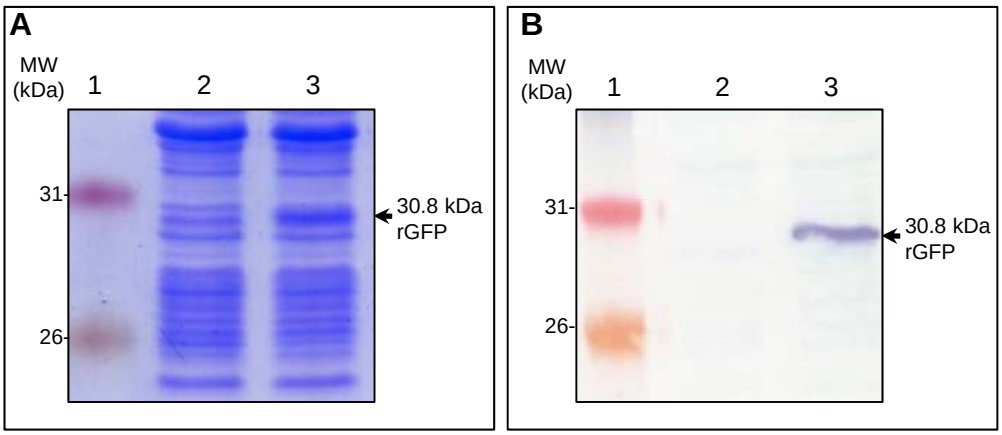
Supplementary 1



Supplementary 2