Ecl1 is a zinc-binding protein involved in the zinc-limitation-dependent extension of chronological lifespan in fission yeast

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Running title: Regulation of lifespan by zinc and Ecl1 in *S. pombe*

Abstract

Overexpression of Ecl1-family genes (*ecl1*$^{+}$, *ecl2*$^{+}$, and *ecl3*$^{+}$) results in the extension of the chronological lifespan in *Schizosaccharomyces pombe*. However, the mechanism for this extension has not been defined clearly. Ecl1-family proteins consist of approximately 80 amino acids, and four cysteine residues are conserved in their N-terminal domains. This study focused on the Ecl1 protein, mutating its cysteine residues sequentially to confirm their importance. As a result, all mutated Ecl1 proteins nearly lost the function to extend the chronological lifespan, suggesting that these four cysteine residues are essential for the Ecl1 protein. Utilizing ICP-AES (inductively coupled plasma atomic emission spectroscopy) analysis, we found that wild type Ecl1 proteins contain zinc while cysteine-mutated Ecl1 proteins do not. We also analyzed the effect of environmental zinc on the chronological lifespan. We found that zinc limitation extends the chronological lifespan, and this extension depends on the Ecl1-family proteins.

Key words: zinc / Ecl1 / fission yeast / *Schizosaccharomyces pombe* / lifespan

Introduction

Fission yeast, *Schizosaccharomyces pombe*, as well as budding yeast, *Saccharomyces cerevisiae*, have been used in aging research as good model organisms (Roux *et al*., 2010). In such research, chronological lifespan is defined as the survival period after entry into a non-dividing state (Fabrizio and Longo, 2003). Previously, we identified the Ecl1 family-genes (*ecl1*$^{+}$, *ecl2*$^{+}$, and *ecl3*$^{+}$) whose overexpression extends chronological lifespan in *S. pombe* (Ohtsuka *et al*., 2009), and one orthologue of the Ecl1-family genes was identified in *Saccharomyces cerevisiae* (Azuma *et al*., 2009). However, it has not been clear how these proteins extend the chronological lifespan.

Previously, we reported that Ecl1-family proteins are essential for sexual development caused by zinc depletion in *S. pombe* (Ohtsuka *et al*., 2015). Ecl1-family proteins are also important for cellular response to zinc limitation; therefore, we expected that Ecl1-family proteins would function as a zinc sensor. Zinc is one of the essential trace elements required for many important biological processes (Andreini and Bertini, 2012). Intracellular zinc balance is regulated strictly, and collapse of this balance leads to harmful effects for the cell (Choi and Bird, 2014). In this study, we show that four cysteine residues conserved among Ecl1-family proteins are essential for protein functions, such as extension of chronological lifespan and inducing of sexual development. When conserved cysteine residue was mutated singularly, zinc binding was lost, indicating the importance of the cysteine residue for zinc binding. We also show that when zinc is limited in the growth medium, the chronological lifespan is extended, and this extension depends on the Ecl1-family proteins.

Material and Methods

Strains and media. The *S. pombe* strains used this study are listed in Table 1. These strains were grown in SD or EMM medium added necessary nutrients (Moreno *et al*, 1991). The synthetic SD medium that was used in the zinc-depletion experiments had the following composition: NH_4Cl (5.0 g/L), Na_2SO_4 (5.0 g/L), biotin (2.0 μg/L), calcium pantothenate (0.4 mg/L), folic acid (2.0 μg/L), inositol (2.0 mg/L), nicotinic acid (0.4 mg/L), p-aminobenzoic acid (0.2 mg/L), pyridoxine hydrochloride (0.4 mg/L), riboflavin (0.2 mg/L), thiamine hydrochloride (0.4 mg/L), H_3BO_3 (0.5 mg/L), $CuSO_4$ (0.04 mg/L), KI (0.1 mg/L), $FeCl_3$ (0.2 mg/L), $MnSO_4$ (0.4 mg/L), MoO_4 (0.2 mg/L), $ZnSO_4$ (0.4 mg/L), KH_2PO_4 (1.0 g/L), $MgCl_2$ (0.4 g/L), NaCl (0.1 g/L), $CaCl_2$ (0.02 g/L), and glucose (20 g/L). For the zinc-depleted

media, $ZnSO_4$ was omitted.

Plasmid construction. The plasmid pEcl1 was used for overexpression of *ecl1*$^+$ as described previously (Ohtsuka *et al*., 2012). To construct Cys-to-Ser mutation in Ecl1 protein, two-step PCR was performed. In the first step, two different DNA fragments were generated by using the plasmid pEcl1 as template. T7 and Reverse primer were used for one, M4 and each mutation specific primer were used for the other. In the second step, these generated DNA fragments were purified and mixed, then target DNA fragments were amplified by using Reverse and M4 primer. The sequences of used primers are as follows. T7 : GTAATACGACTCACTATAGGGC, Reverse : GGAAACAGCTATGACCATG, M4 : GTTTTCCCAGTCACGAC, Ecl1^{C6S} : CCACACACTGTGGAAAAATCC, Ecl1^{C9S} : TGGTGGCTCCAGACACTGTGCAA, Ecl1^{C20S} : ATTCAGAAGAGGAGTACAAACTGCC, Ecl1^{C24S} : CCAAAAGATGGGATTCAGAAGAG. Each mutated *ecl1*$^+$ gene was cloned onto vector plasmid, pLB-Dblet, and resultant plasmids were named as pC6S-Ecl1, pC9S-Ecl1, pC20S-Ecl1, and pC24S-Ecl1. They express Ecl1^{C6S}, Ecl1^{C9S}, Ecl1^{C20S}, and Ecl1^{C24S} proteins, respectively.

Construction and purification of GST tagged Ecl1 proteins. To overproduce the GST-tagged Ecl1 protein in *E. coli*, pGEX-6P-1 (GE Healthcare Life Sciences) was used as vector plasmid. DNA fragments encoding wild type Ecl1 or cysteine mutated Ecl1 were amplified by PCR using following primers and cloned on the plasmid. F : ACTGAATTCATGGATTTGGATTTTTGCACAGTG, R : TTCTAAGTCGACTACGCATGTAGTTTTGTGTAAGC.

The GST-tagged Ecl1 protein was purified as follows. *E. coli* cells (BL21) carrying the expression plasmid were cultured in LB medium containing ampicillin (50 μg/ml) and IPTG (1 mM) at 30°C for 6 h, then cells were harvested. Cells were suspended in lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% NP40, 2.5 mM PMSF) and were sonicated by sonicator UD100 (TOMY), then total lysate was centrifuged to obtain its supernatant. It was applied to column containing COSMOGEL GST-Accept (nacalai tesque). After the wash of column with lysis buffer, elution buffer (10 mM glutathione, 50 mM Tris-HCl pH8.0) was added to the column to elute the GST-tagged Ecl1 protein.

Construction of Δ*zrt1* mutant. To construct *zrt1*-deletion (Δ*zrt1*) mutant, the ORF region of *zrt1*$^+$ gene

was replaced with *kan*^{r} cassette using plasmid pFA6a-kanMX6 and method described previously (Ohtsuka *et al*., 2013). Primers used were follows. F1 : TTGCTGATCACTCCTGCTCC, F2 : TTAATTAACCCGGGGATCCGCAAGTCACAGTAGCAGAGCT, R1 : GCGCTTTGCGATTTACCTGA, R2 : GTTTAAACGAGCTCGAATTCACCTTTTGGCATGCTCGATG.

Analyses of chronological lifespan and mating rate. Chronological lifespan and mating rate was measured as described previously (Oshiro *et al*., 2003, Ito *et al*., 2010, Ohtsuka *et al*., 2013; 2015). To determine cell survival, cells were grown in SD liquid medium, sampled in each growth phase, and then plated in yeast extract peptone dextrose agar plates after suitable dilution. We counted the number of colonies appeared derived from 1 mL of culture on the plate and then divided the number by the cell turbidity at the sampling time. Cell growth was monitored by turbidity using a Bactomonitor (BACT-550) equipped with a 600-nm filter (Nissho Denki Co.)

ICP-AES analysis. ICP-AES analysis was performed to quantify zinc. Concentrations of the purified proteins were measured by Protein assay kit (BIO-RAD). Equimolar amounts of proteins (137.8 μg) were used for following analysis. The ICP-AES analysis was carried out essentially as described previously (Reuscher *et al*., 2014). Equal volume of nitric acid was added to each protein solution, and boiled at 95°C for 30 min. Then, total sample volume was normalized with ultrapure water. The sample was analyzed by ICP-AES (iCAP6500, Thermo Fisher Scientific). The multielement standard solution 5 for ICP (SIGMA-ALDRICH) was used for standard.

Results

Ecl1 family proteins have four conserved cysteine residues that are essential for protein function. We analyzed the amino acid sequences of Ecl1-family proteins and found four conserved cysteine residues in their N-terminal domains (Fig. 1A). These four cysteine residues are also conserved in the Ecl1 protein of *S. cerevisiae* (Fig. 1B). Conservation of these cysteine residues indicates that they are important for Ecl1-protein function; therefore, we replaced each of the four cysteine residues with serine, one at a time, and analyzed its effects as follows.

First, we measured the viability of strains overexpressing each cysteine-mutated Ecl1 protein (Fig.

2A). Wild-type Ecl1 protein extended the chronological lifespan significantly, while cysteine-mutated Ecl1 proteins (Ecl1^{C6S}, Ecl1^{C9S}, Ecl1^{C20S}, and Ecl1^{C24S}) were not as successful. We also found, correspondingly, that the cysteine residues in the Ecl1 protein of *S. cerevisiae* were important for the extension of chronological lifespan in that species (supplemental Fig. 1). Previously, we reported that the Δ*ecl1.2.3* mutant has a mating defect after entry into the stationary phase (Ohtsuka *et al*., 2015). So, we next analyzed whether cysteine-mutated Ecl1 is able to rescue this phenotype. As shown in Fig. 2B, the Δ*ecl1.2.3* mutant carrying vector plasmid displayed a low mating rate after 2 days of cultivation in EMM medium (Ohtsuka *et al*., 2015). The mutant carrying pEcl1 that encodes wild-type Ecl1 protein complemented the mating defect; however, the mutants overexpressing each mutated Ecl1 protein (Ecl1^{C6S}, Ecl1^{C9S}, Ecl1^{C20S}, and Ecl1^{C24S}) failed to complement the mating defect. These results suggested that the four conserved cysteine residues are essential for the function of the Ecl1 protein.

Ecl1 is a zinc-binding protein. What is the role of conserved cysteines in the Ecl1 protein? Based on amino acid sequence analysis, we were able to identify a domain ($CX_2CX_{10}CX_3C$) resembling a zinc binding domain (Kyba and Brock, 1998; Nagano *et al*., 2001). We hypothesized that the Ecl1 protein might have the ability to bind zinc. Our hypothesis was tested as follows.

First, we prepared plasmids that express wild-type Ecl1 or Ecl1^{C9S} protein with which an N-terminus GST tag was fused. The plasmid-carrying proteins were purified from *E. coli*. By using ICP-AES analysis, we found that GST-tagged wild-type Ecl1 protein contains zinc, but not at the correct molar ratio (Fig. 3, data not shown). Whereas the GST-tagged Ecl1^{C9S} protein displayed diminished zinc binding. It should be noted that, because the Ecl1 protein degraded in the purification step for unknown reasons, it was difficult to determine the molar ratio of zinc to Ecl1 protein exactly (data not shown). Nevertheless, we can conclude that the Ecl1 is a zinc-binding protein and the zinc binding is dependent on Cys-9.

Zinc limitation extends chronological lifespan in fission yeast. Previously, we proposed that Ecl1-family proteins are involve in zinc sensing (Ohtsuka *et al*., 2015). In this study, we found that the Ecl1 protein binds zinc. Because the Ecl1 protein was originally identified as the extender of chronological lifespan, we analyzed whether the limitation of zinc in the medium affects chronological

lifespan in fission yeast.

First, we measured viability of wild-type strain grown in SD medium. As shown in Fig. 4A, when EDTA was added to the medium, the chronological lifespan of wild-type strain was extended. Because EDTA is a chelator of metal ions, we prepared SD medium containing a limited amount of zinc, and then analyzed cell viability in the medium. As shown in Fig. 4B, chronological lifespan was extended in wild-type cells with zinc limitation. Limitation of zinc to one-tenth of the control medium showed the same effect as found in medium with no zinc. Finally, we measured the viability of the Δ*zrt1* mutant. Because the Zrt1 protein is a zinc transporter that imports zinc from medium into cells, intracellular zinc concentrations are constitutively low in the Δ*zrt1* mutant (Boch *et al*., 2008; Dainty *et al*., 2008). As a result, the Δ*zrt1* mutant exhibited a chronologically long-lived phenotype compared with the wild-type strain (Fig. 4C). These data together suggest that the decrease in intracellular zinc extends the chronological lifespan in fission yeast.

Lifespan extension caused by zinc limitation depends on Ecl1-family proteins. After determining that, zinc limitation extended the chronological lifespan of fission yeast, we analyzed whether this lifespan extension was dependent on Ecl1-family proteins. Previously, we found that when zinc was limited in the medium, zinc-responsive genes, *zrt1* and *SPBC1348.06c*, were induced in the wild-type but not in the Δ*ecl1.2.3* mutant. Based on this result, we proposed that Ecl1/2/3 proteins might function as zinc sensors (Ohtsuka *et al*., 2015). We also found that overexpression of Ecl1 did not affect the expression of *SPBC1348.06c*-mRNA, suggesting the intracellular zinc level was not affected by the overexpression of Ecl1 (data not shown). Based on our previous results, we proposed that lifespan extension due to zinc limitation would diminish in the Δ*ecl1.2.3* mutant cells. We confirmed this hypothesis by growing wild-type and Δ*ecl1.2.3* mutant cells in zinc-limited SD medium, and then measuring the chronological lifespans of each (Fig. 5). Wild-type cells exhibited an extended lifespan in zinc-limited SD medium; however, lifespan extension was diminished almost entirely in the Δ*ecl1.2.3* mutant. These results indicate that the lifespan extension caused by zinc limitation primarily depends on Ecl1-family proteins, and are consistent with our previous research..

Again, we analyzed whether cysteine-mutated Ecl1 could rescue the phenotype caused by the Δ*ecl1.2.3* mutation. Under zinc-limited conditions, the Δ*ecl1.2.3* mutant cells overexpressing only Ecl1

(not Ecl1^{C9S}) extended chronological lifespan at the same level as that of the wild-type cells carrying vector plasmid (supplemental Fig. 2). This result is consistent with the idea that Cys-9 is necessary for the function of the Ecl1 protein.

Discussion

In this study, we found that four cysteine residues conserved among Ecl1-family proteins are essential for Ecl1 protein function. Our analysis showed that Cys-9, one of the conserved four cysteine residues, is necessary for zinc binding to the Ecl1 protein. Most zinc-binding proteins, such as the zinc finger protein, require zinc to maintain its protein structure (Laity *et al*., 2001). Commonly, loss of zinc-binding ability leads to a loss of function in these proteins; therefore, the structure and function of the Ecl1 protein might be maintained by binding with zinc. At present, we have not analyzed Ecl2 and Ecl3 proteins; they also may bind zinc as well as the Ecl1 protein. Our data indicate that a limitation of zinc in the medium, resulting in the decrease of intracellular zinc, causes an extension of chronological lifespan in fission yeast, and this phenomenon mainly depends on the Ecl1-family proteins.

Recently, it was reported that zinc limitation extended lifespan in *Caenorhabditis elegans* (Kumar *et al*., 2016), indicating that zinc levels regulate lifespan, not only fission yeast, but also in other species. Furthermore, we analyzed the effect of other mineral limitations on chronological lifespan, and found that limited-supplies of both iron and manganese extended chronological lifespan in fission yeast (data not shown). These results suggest that trace elements are highly involved in the regulation of lifespan.

Thus far, it is known that the Ecl1-family proteins are required for the regulation of sexual development and gene expression due to zinc limitation (Ohtsuka *et al*., 2015). In this process, we believed that the Ecl1-family proteins would function as a zinc sensor. Because the Δ*ecl1.2.3* mutant cannot respond to zinc limitation appropriately, showing a defect in lifespan extension, we currently do not know the relevance between the zinc-binding function and zinc-sensing function, as carried out by Ecl1. This area of study will be our future focus.

It was also suggested that the lifespan extension due to zinc limitation is partially independent of Ecl1-family proteins. That is, both Δ*ecl1.2.3* mutant cells (Fig. 5) and the Δ*ecl1.2.3* mutant cells carrying vector plasmid (supplemental Fig. 2) extended their chronological lifespan under zinc limitation to some extent. Because zinc is involved in numerous protein functions, zinc depletion may affect their activity,

directly, resulting in lifespan extension. The presence of plasmid and/or leucine in the medium may affect the response.

Recently we found that Ecl1-family proteins are necessary for cellular response to sulfur limitation in *S. pombe*, and that sulfur limitation also extends chronological lifespan. Under sulfur-limited condition, many ribosomal genes were down-regulated in the Ecl1 family proteins dependent manner (unpublished result). Zinc, as well as sulfur, is an essential nutrient that is required for a wide range of biological processes.

Finally, microarray analysis revealed that zinc limitation causes down-regulation of several ribosomal genes (Dainty *et al*., 2008), so it is possible that lifespan extension by zinc limitation also arose from the down-regulation of ribosomal biogenesis in the Ecl1-family proteins.

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Figure Legends

Fig. 1. Four cysteine residues conserved among Ecl1 family proteins. The amino acid sequences of the Ecl1-family proteins of *S. pombe* (Ecl1, Ecl2, and Ecl3) and *S. cerevisiae* (Sc-Ecl1). The identical amino acid residues are marked with asterisks below the sequences. (A) Alignment of amino acid sequences of Ecl1-family proteins. (B) Alignment of amino acid sequences between Ecl1 proteins of *S. pombe* (Sp-Ecl1) and *S. cerevisiae* (Sc-Ecl1). The four conserved cysteine residues are marked as crosses above the sequences.

Fig. 2. Conserved cysteine residues are important for the Ecl1 function. (A) Wild type cells (JY333) carrying a plasmid (the empty vector, pEcl1, pC6S-Ecl1, pC9S-Ecl1, pC20S-Ecl1, and pC24S-Ecl1) were cultured in SD medium, and their chronological lifespans were measured. (B) Mating rates of wild-type (JY808) and Δ*ecl1.2.3* mutant (JY808 Δ*ecl1.2.3*) each carrying a plasmid (the empty vector, pEcl1, pC6S-Ecl1, pC9S-Ecl1, pC20S-Ecl1 and pC24S-Ecl1) were analyzed after cultivation for two days in EMM medium. The data were shown as the means of three independent experiments with standard deviations.

Fig. 3. Cysteine residue, Cys-9, is necessary for zinc binding in the Ecl1 protein. The GST-tagged Ecl1 and the GST-tagged Ecl1^{C9S} proteins were purified from *E. coli*, and zinc content in each protein sample was measured by ICP-AES analysis. GST protein was also purified and used as control. The data were shown as the means of three independent experiments with standard deviations.

Fig. 4. Zinc limitation extends chronological lifespan of *S. pombe*. (A) Chronological lifespan of wild-type cells (JY333) cultured in SD medium with or without EDTA (5mM) was analyzed. (B) Chronological lifespan of wild-type cells (JY333) cultured in SD medium containing indicated amounts of zinc (1/10 Zn and –Zn indicate that one tenth zinc concentration, and no zinc are in the medium, respectively). (C) Chronological lifespan of Δ*zrt1* mutant cultured in SD medium. Left panels show growth, while right panels show lifespan. Data were shown as the means of three independent experiments with standard deviations.

Fig. 5. Lifespan extension by zinc limitation depends on Ecl1 family proteins. Wild-type and Δ*ecl1.2.3* mutant cells were cultured in SD medium containing indicated amounts of zinc. Growth (left panel) and chronological lifespan (right panel) were measured. The data were shown as the means of three independent experiments with standard deviations.

Supplemental Fig. 1. Conserved cysteine residues are important for the ScEcl1 function. *Saccharomyces cerevisiae* strain YPH250 was transformed with plasmid carrying ScEcl1 or each of four cysteine-mutated ScEcl1 (C9S, C12S, C47S, and C51S). Vector plasmid pG1 was used for transformation. Each strain was cultured in SD medium, and viability was measured at each time point after entry into stationary phase. The experiment was performed more than three times independently; representative data with similar results are shown.

Supplemental Fig. 2. Wild type (JY333) strain and Δ*ecl1.2.3* mutant carrying each plasmid were cultured in SD medium (Control) or zinc-limited SD medium (1/10 Zn). The viability was measured at each time point after entry into stationary phase. The experiment was performed more than three times independently; representative data with similar results are shown.

Table 1. List of *S. pombe* strains used in this work

Strains	Genotype
JY333	*h⁻ leu1-32 ade6-M216*
JY808	*h^{90} leu1-32 ade6-M216*
JY333 Δ*zrt1*	*h⁻ leu1-32 ade6-M216 zrt1:: kan^{r}*
JY333 Δ*ecl1.2.3*	*h⁻ leu1-32 ade6-M216 ecl1:: kan^{r} ecl2:: kan^{r} ecl3:: kan^{r}*
JY808 Δ*ecl1.2.3*	*h^{90} leu1-32 ade6-M216 ecl1:: kan^{r} ecl2:: kan^{r} ecl3:: kan^{r}*

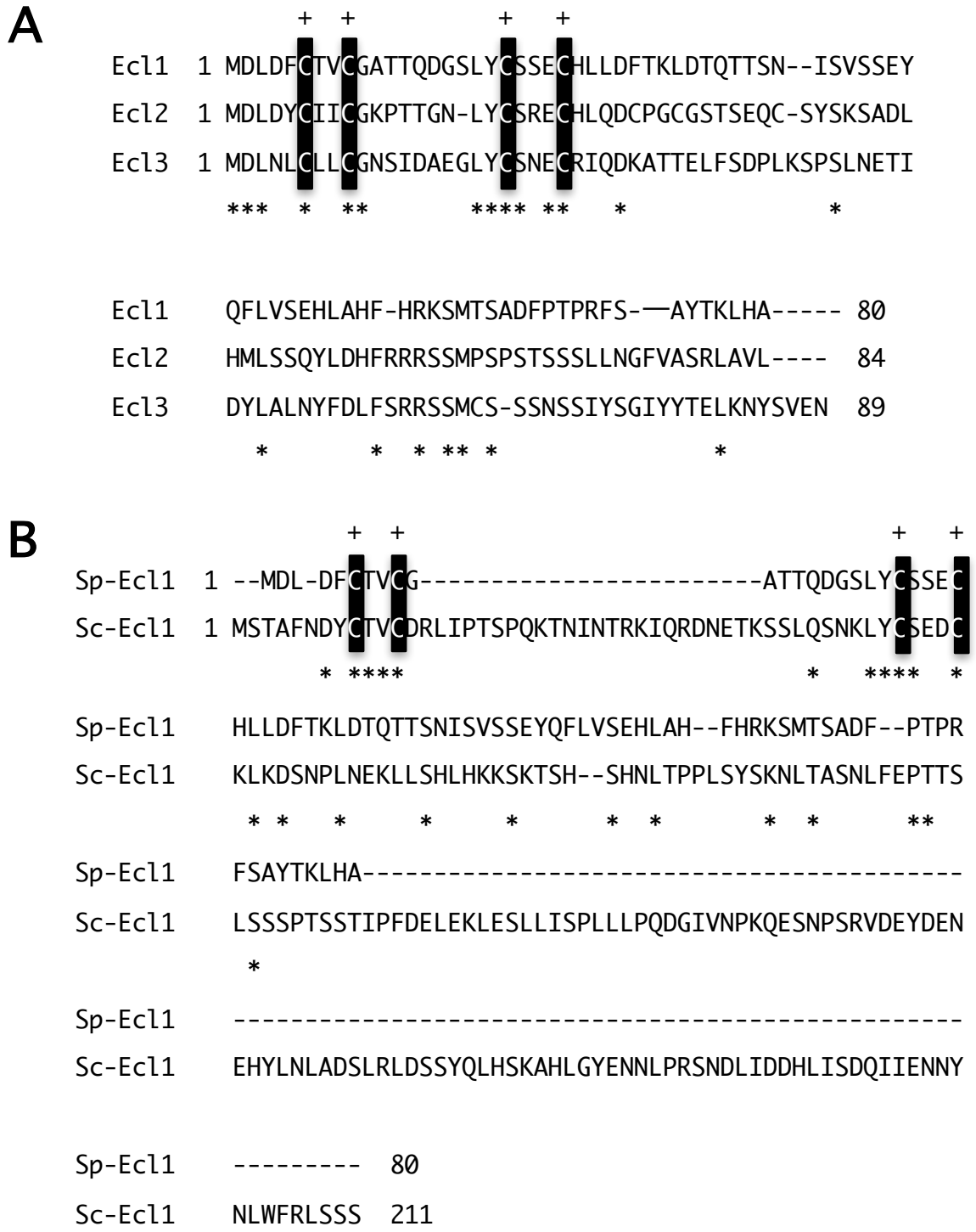


Fig. 1 Shimasaki *et al*.

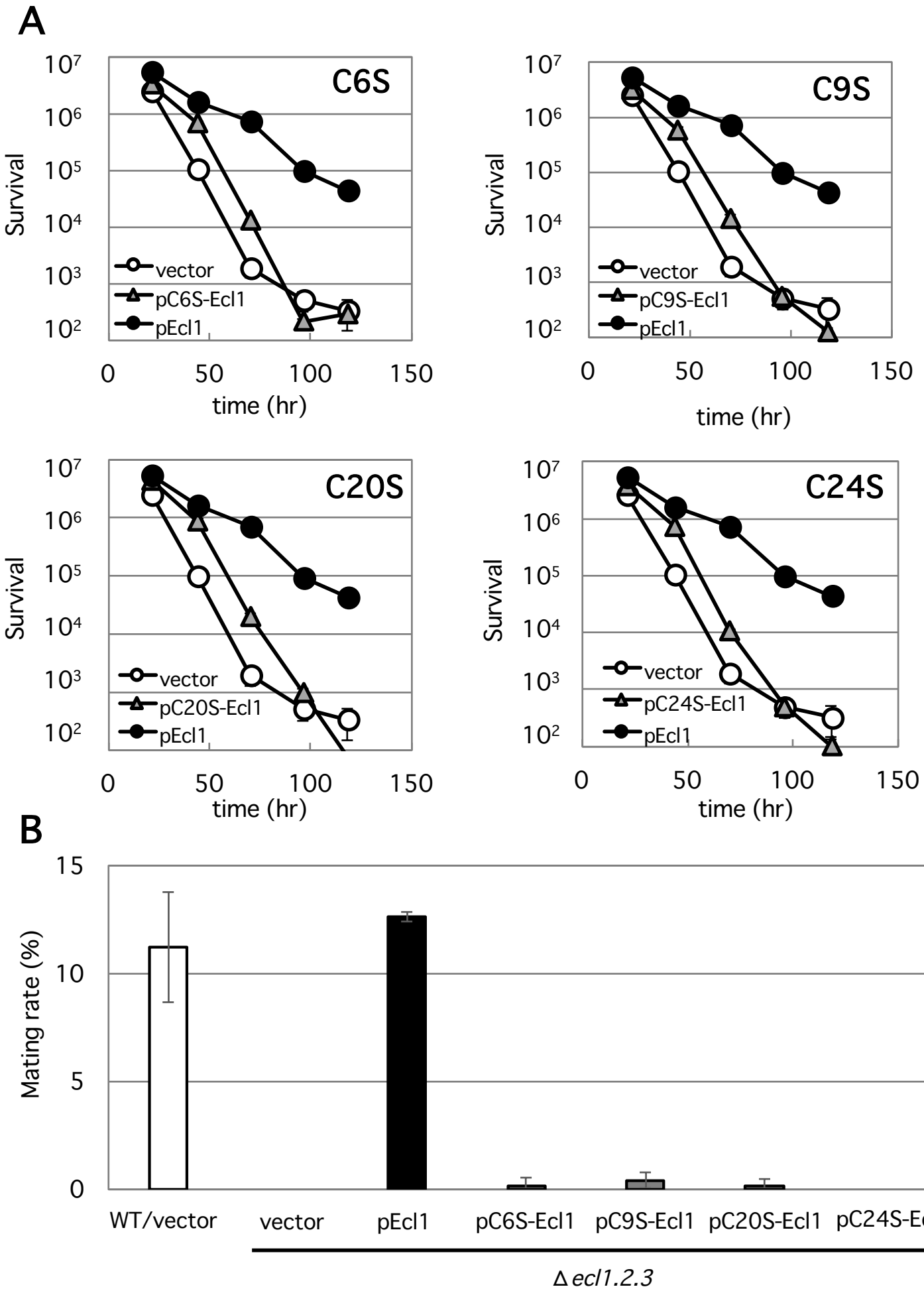
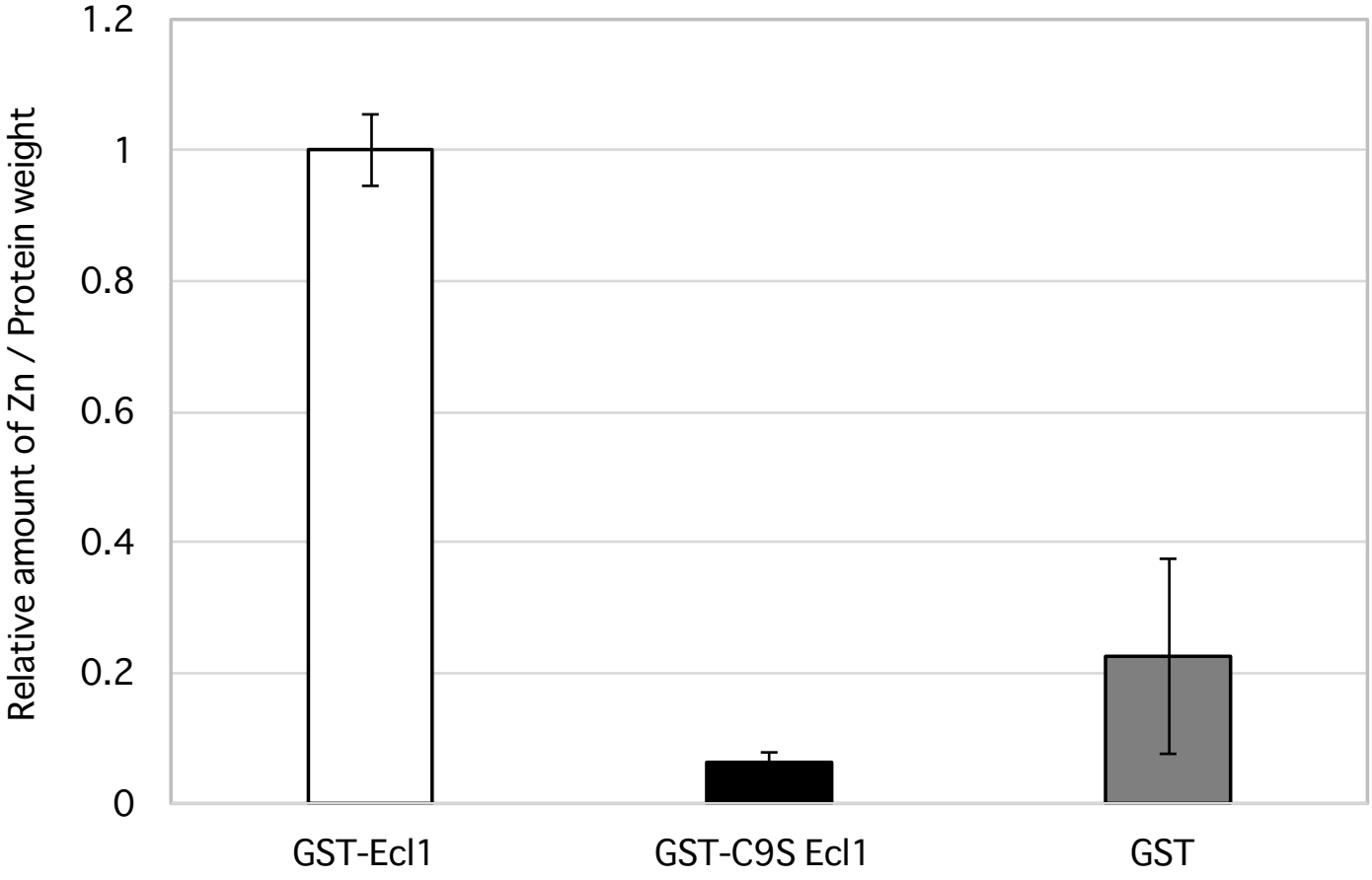


Fig. 2 Shimasaki *et al.*



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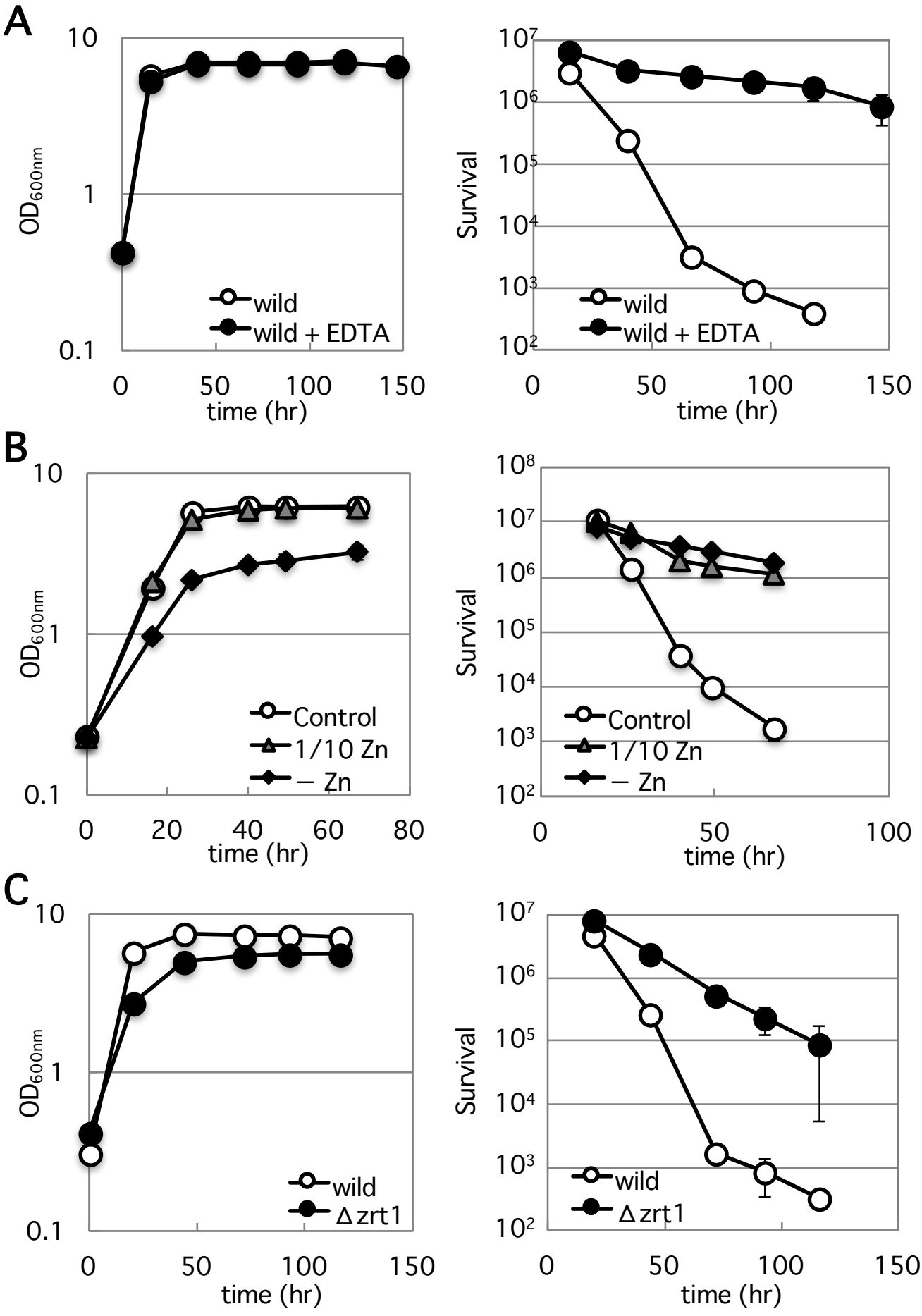

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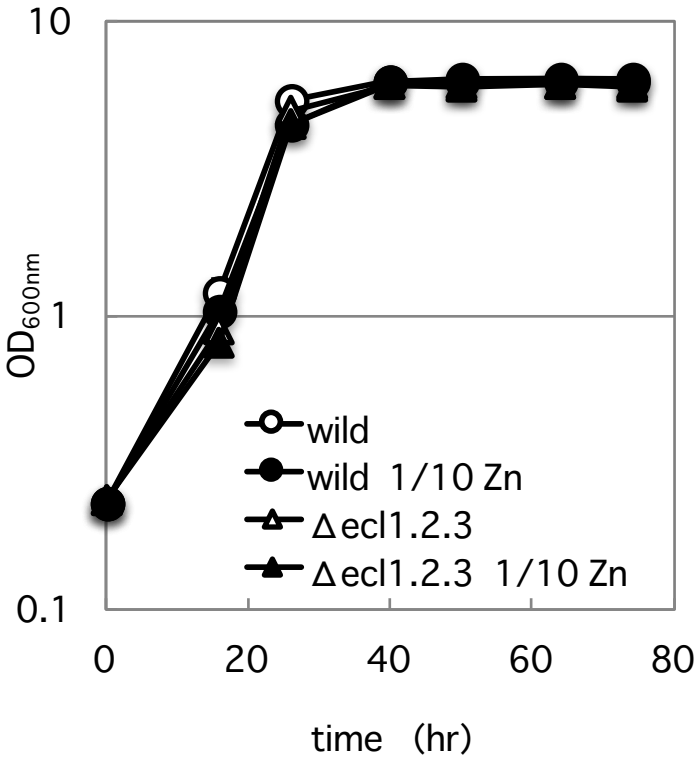


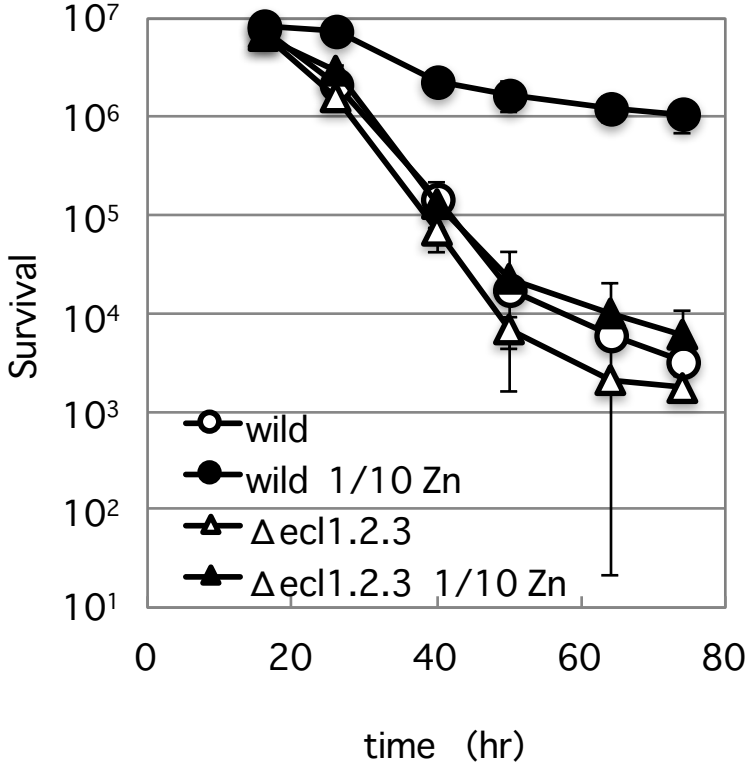
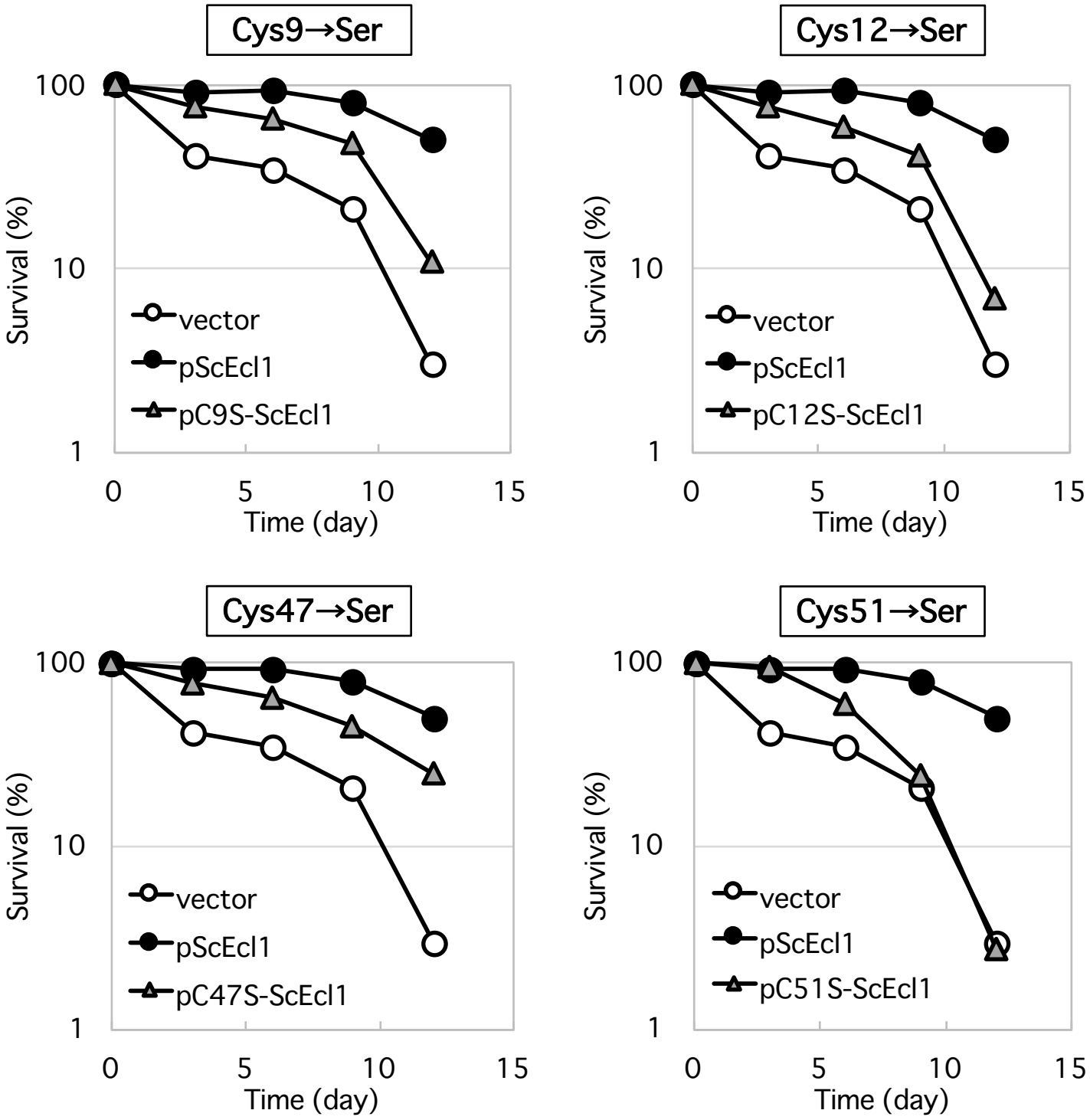
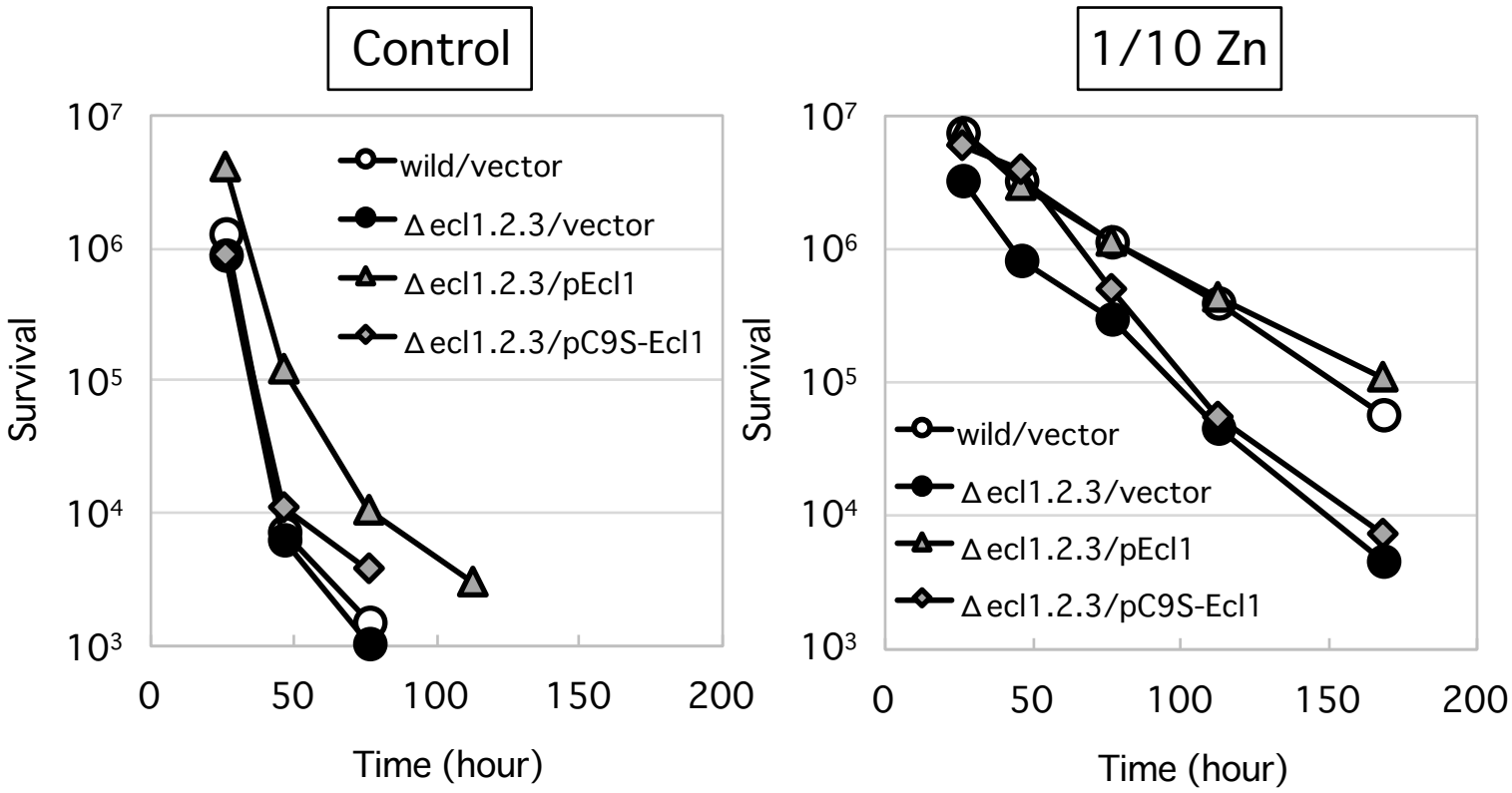


Fig. 5 Shimasaki *et al*.



Supplemental Fig. 1 Shimasaki *et al*.



Supplemental Fig. 2 Shimasaki *et al*.