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Bactericidal Activity of Mouse α-Defensin, Cryptdin-4 Predominantly Affects Non-Commensal Bacteria

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Short title: Selective Bactericidal Activity of Cryptdin-4

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Abstract

Mouse Paneth cell α-defensins, termed cryptdins, are secreted into the intestinal lumen, exert microbicidal activity and contribute to the intestinal innate immunity. Among them, cryptdin-4 (Crp4) has the most potent microbicidal activity. In the intestinal lumen, commensal bacteria colonize and elicit beneficial effects to the host. However, the effects of Crp4 against commensal bacteria are poorly understood. Thus, we investigated the bactericidal activities of Crp4 against commensal bacteria compared to non-commensal bacteria. Oxidized Crp4 showed only minimal or no bactericidal activity against 8 out of 12 commensal bacterial species, including *Bifidobacterium bifidum* and *Lactobacillus casei*. We further addressed a role of the conserved disulfide bonds of Crp4 by analyzing reduced Crp4 (r-Crp4). r-Crp4 demonstrated significantly greater bactericidal activities against 7 of 12 commensal bacteria than did oxidized Crp4. Oxidized Crp4 and r-Crp4 elicited equivalently potent bactericidal activities against 11 of 11 non-commensal bacteria tested such as *Salmonella enterica* serovar Typhimurium, and 5 of 12 commensal bacteria. Furthermore, when r-Crp4 was exposed to a processing enzyme of cryptdins, MMP-7, r-Crp4 was degraded, and bactericidal

activities disappeared. These findings suggest that Crp4 has selective bactericidal activities against intestinal microbiota and that the activities are dependent on the disulfide bonds.

Introduction

Innate immunity functions as the front line of host defense in plants, invertebrates, and mammals. Antimicrobial peptides (AMPs) are one of the major effectors of innate immunity [1-3]. In the small intestine, antimicrobial peptide α-defensins are expressed in the granules of Paneth cells and are secreted into the lumen of intestinal crypts in response to bacterial stimuli [4]. The secreted α-defensins elicit potent bactericidal activity and contribute to innate immunity in the small intestine [5, 6]. Mouse α-defensins, termed cryptdins (Crps) are activated *in vivo* in Paneth cell granules through the processing of pro-cryptdins (pro-Crps) with the proteolytic enzyme, matrix metalloproteinase-7 (matrilysin, MMP-7) [7, 8]. MMP-7 cleaves pro-Crps at three cleavage sites, and one of these sites is the N-terminus or near the N-terminus of mature Crps [9]. This processing is an essential event for the production of mature, functional

Crps [7-9]. MMP-7-deficient mice that lack active form of Crps are significantly susceptible to orally administered *Salmonella enterica* serovar Typhimurium than wild type mice [7]. These results also show the pivotal role of AMPs in innate immunity.

In the gastrointestinal tract, a highly complex microbial ecosystem is constructed by colonizing microbes. Hosts and microbiota have co-evolved in ways that have mutually beneficial effects [10]. These include host development [11], nutritional absorption [12], and functional development of the immune systems [13]. Recent studies have shown that well-balanced cross-talk between the host and commensal bacteria are important [14], as imbalances of the relationships result in inflammation [15] and cancer [16]. In the mouse small intestine, the expression of more than 20 cryptdin genes and cryptdin-related sequence genes have been reported [17-20], and these peptides are secreted into the intestinal lumen where commensal bacteria reside [21]. Among Crps, cryptdin-1 ~ -6 (Crp1~6) are characterized at peptide level. Although the amino acid sequence identities of mature regions of Crp1~3, 6 are more than 90%, those of Crp4 and Crp5 with Crp1 are 42 and 54%, respectively [18]. In particular, Crp4 has several features that distinguish it from other Crps. For example, the Crp4 polypeptide chain uniquely lacks three amino acids between fourth and fifth cysteine residue positions [18], and the unique repeated element in the region upstream of the gene transcriptional

74 start site [22]. Crp4 also has the most potent *in vitro* bactericidal activity of known
75 mouse Paneth cell α-defensins [18], suggesting that Crp4 may have pivotal role for
76 intestinal innate immunity. However, the interaction of Crp4 and small intestinal
77 microbiota is poorly understood. Thus, to clarify the effects of Crp4 on commensal
78 bacteria, we investigated the bactericidal activities of Crp4 against commensal bacteria
79 compared to non-commensal bacteria. We further tested the bactericidal activities of
80 Crp1 in addition to Crp4.

81 Crp4 is highly cationic peptide and generally believed to permeabilize bacterial
82 plasma membrane through electrostatic interaction with negatively charged bacterial
83 phospholipids followed by the insertion of hydrophobic side chains [23]. This
84 conclusion is supported by the reports which reveal that positively charged Arg residues
85 of Crp4 is critical for its bactericidal activity [24]. However, the precise mechanism(s)
86 of its bactericidal activity are not known. α-Defensins including Crp4 are characterized
87 by invariant disulfide bonds arranged between Cys^1-Cys^6, Cys^2-Cys^4, and Cys^3-Cys^5 [3].
88 The pairings of three disulfide bonds are conserved in all species which express these
89 peptides. Previously, the bactericidal activities of the Crp4 mutants in which Cys
90 residues were substituted to Ala residues were analyzed by Maemoto *et al* [25]. They
91 showed that disulfide bond-null mutants had equivalent or greater bactericidal activity

than native Crp4 [25]. However, the effects of the disulfide bonds in native Crp4 on bactericidal activity against commensal bacteria remain unknown. Therefore, we further addressed a role of the disulfide bonds on the bactericidal activity of Crp4 using reduced Crp4 (r-Crp4) which did not contain disulfide bonds.

Crp4 is processed by MMP-7 in the granules of Paneth cells. It was reported that disulfide bonds null-mutant of Crp4 was degraded by MMP-7 [25]. This result indicates that disulfide bonds of Crp4 determine proteolytic resistance to MMP-7. Therefore, to elucidate the effect of reduction on the processing of r-Crp4, we also investigated the susceptibility of r-Crp4 to MMP-7 and the effects of MMP-7 on the bactericidal activity of the processed r-Crp4.

Materials and Methods

Preparation of oxidized Crps and r-Crps

Three pairs of disulfide bonds were introduced into Crp4 (Sigma Genosys, St. Louis, MO or Medical & Biological Laboratories Co., Ltd, Nagoya, Japan) by air oxidation as described [25]. Crp4 was then purified to homogeneity using reverse-phase high

performance liquid chromatography (RP-HPLC). The Crp4 with three pairs of disulfide bonds, defined as oxidized Crp4, was purified by a C-18 column (SepaxHP-C18, 4.6x150 mm, 5 μm, Sepax Technologies, Inc., Newark, DE) in 0.1% trifluoroacetic acid with an 18-36% acetonitrile gradient developed over 30 min at 1 ml/min (online suppl. fig. S1a). Oxidized Crp4 was obtained after final lyophilization and stored at -30°C until use.

r-Crp4, in which the disulfide bonds were entirely reduced, was prepared. Oxidized Crp4 was dissolved in 500 mM dithiothreitol (DTT), and let stand at 4°C overnight. The reaction mixture was then applied to a C-18 column and r-Crp4 was purified by RP-HPLC under the same condition as used in the purification of oxidized Crp4 (online suppl. fig. S1b). Because the retention times of DTT and r-Crp4 were very different, we conclude DTT was completely removed from r-Crp4 in the purification process. Thus, r-Crp4 used in the assay did not contain DTT, and we confirmed that DTT did not affect the bactericidal assay. r-Crp4 was obtained after final lyophilization and stored at -30°C until use. Oxidized Crp1 and r-Crp1 were also prepared by the same respective methods.

Evaluation of disulfide bond formation

Disulfide bonds formation was evaluated by Acid-Urea PAGE (AU-PAGE) [26], MALDI-TOF MS, and RP-HPLC. In AU-PAGE analysis, samples (1.0 μg) of oxidized Crp4 and r-Crp4 were dissolved in 5% acetic acid and electrophoresed on 12.5% acrylamide gel containing 5% acetic acid and 5 M Urea at 150 V [26]. Thereafter, the gel was stained with Coomassie brilliant blue R-250. Molecular masses of the peptides were determined by MALDI-TOF MS (Voyager-DE PRO, Applied Biosystems, Carlsbad, CA). Oxidized Crp4 and r-Crp4 were analyzed by RP-HPLC using a C-18 column under the same condition as used in the purification of oxidized Crp4.

Bacterial strains and culture conditions

As some of the most common commensal bacteria in the small intestine [27-30], *Bifidobacterium bifidum* ATCC 11863 (*B. bifidum*), *Bifidobacterium breve* JCM 1192 (*B. breve*), *Bifidobacterium longum* ATCC 15707 (*B. longum*), *Lactobacillus acidophilus* ATCC 314 (*L. acidophilus*), *Lactobacillus casei* ATCC 393 (*L. casei*), *Lactobacillus johnsonii* JCM 2012 (*L. johnsonii*), *Bacteroides fragilis* JCM 11019 (*B. fragilis*), *Bacteroides ovatus* JCM 5824 (*B. ovatus*), *Bacteroides thetaiotaomicron* JCM 5827 (*B. thetaiotaomicron*), *Bacteroides vulgatus* JCM 5826 (*B. vulgatus*), *Enterococcus faecalis* JCM 5803 (*E. faecalis*) and *Enterococcus faecium* JCM 5804 (*E.*

146 *faecium*) were used. As examples of non-commensal bacteria in the small intestine,
147 wild-type *Salmonella enterica* serovar Typhimurium ATCC 14028 (*S. enterica* serovar
148 Typhimurium), a defensin-sensitive strain of *Salmonella enterica* serovar Typhimurium
149 *phoP*- (*S. enterica* serovar Typhimurium *phoP*-) [31], *Escherichia coli* ML35 ATCC
150 43827 (*E. coli*), *Staphylococcus aureus* ATCC 27217 (*S. aureus*), *Listeria*
151 *monocytogenes* JCM 7671 (*L. monocytogenes*), *Klebsiella oxytoca* JCM 1665 (*K.*
152 *oxytoca*), *Klebsiella pneumoniae* JCM 1662 (*K. pneumoniae*), *Proteus vulgaris* JCM
153 20013 (*P. vulgaris*), *Yersinia enterocolitica* JCM 7577 (*Y. enterocolitica*),
154 *Campylobacter coli* JCM 2529 (*C. coli*) and *Campylobacter jejuni* JCM 2013 (*C. jejuni*)
155 were used. Bacteria were cultured in the following media; *B. bifidum*: reinforced
156 clostridial medium (RCM) supplemented with 2% (w/v) of skim milk, *B. breve* and *B.*
157 *longum*: RCM, *Lactobacillus* sp.: de Man, Rogosa, and Sharpe (MRS) broth,
158 *Bacteroides* sp.: GAM broth (Nissui Seiyaku Co., Ltd., Tokyo, Japan), *Enterococcus*
159 sp.: Brain Heart Infusion (BHI), non-commensal bacteria except for *C. coli* and *C.*
160 *jejuni*: Tryptic Soy broth, *C. coli* and *C. jejuni*: GAM broth. Commensal bacteria were
161 grown in anaerobic conditions using the Anaero Pack system (Mitsubishi Gas Chemical
162 Co., Inc., Tokyo, Japan) at 37°C. Non-commensal bacteria except for *C. coli* and *C.*
163 *jejuni* were grown in a shaking incubator at 37°C with shaking at 180 rpm. *C. coli* and *C.*

164 *jejuni* were grown in microaerophilic conditions using the Anaero Pack system.

165

166 *Bactericidal peptide assay*

167 Exponential-phase bacteria cultured at 37°C were deposited by centrifugation at
168 9,300 g at 4°C for 5 min. Bacteria except for *B. vulgatus* were washed twice and
169 resuspended in Milli-Q water, *B. vulgatus* was washed twice and resuspended in PBS
170 diluted 1:4 with Milli-Q water. The OD_{620} was measured to determine bacterial cell
171 numbers. Twenty μl of samples containing 1,000 colony forming units (CFU) per
172 aliquot mixed with equal vol of oxidized Crp4, r-Crp4, oxidized Crp1 or r-Crp1 to final
173 concentrations ranging from 0.027 to 1.35 μM. The mixtures were incubated for 1 hr at
174 37°C. The incubated samples were plated on RCM Agar plates for *Bifidobacterium* sp.,
175 MRS Agar plates for *Lactobacillus* sp., GAM Agar plates for *Bacteroides* sp., BHI Agar
176 plates for *Enterococcus* sp. and Tryptic Soy Agar (TSA) plates for non-commensal
177 bacteria. The plates were then incubated in anaerobic conditions at 37°C for commensal
178 bacteria or at 37°C for non-commensal bacteria. Bacterial survival rates were
179 determined from surviving colonies relative to peptide-unexposed controls (online suppl.
180 fig. S2). Bacterial cell viability of peptide-unexposed controls was not changed during
181 bactericidal peptide assay (data not shown).

182

183 *Bactericidal peptide assay against a bacterial mixture*

184 Exponential-phase *S. enterica* serovar Typhimurium, *L. casei* and *B. thetaiotaomicron*
185 cultured at 37°C were washed with Milli-Q water and each bacteria population was
186 adjusted to 150 CFU in 20 μl. Then each bacterial solution of 20 μl was mixed and the
187 mixture was incubated with 60 μl of oxidized Crp4 or r-Crp4 with the final
188 concentration of 1.35 μM. After incubation for 1 hr at 37°C, the bacterial mixtures were
189 separated and grown on TSA plates at aerobic conditions, MRS and GAM plates at
190 anaerobic conditions using the Anaero Pack system, respectively. More than 28 colonies
191 were picked randomly from each plate as a representative of total colonies, then the
192 genomic sequences of the conserved region in 16S rRNA among three species of
193 bacteria were amplified by colony direct polymerase chain reaction (PCR) using Blend
194 Taq (TOYOBO, Tokyo, Japan). The forward primer (5'-GTTGG TGAGG TAACG
195 GCTCA CCAA-3') was paired with the reverse primer (5'-TGACG GGCGG TGTGT
196 ACAAG GC-3'). The PCR products from *S. enterica* serovar Typhimurium, *L. casei*
197 and *B. thetaiotaomicron* were digested at only one site by *Bam*H I, *Bgl* II and *Spe* I,
198 respectively. Therefore, after the digestion of the PCR products by these three enzymes,
199 each bacterium was distinguished by the resulting patterns visualized by agarose

electrophoresis. Relative bacterial distribution was shown from picked representative colonies, and the actual colony numbers of each bacterium were estimated by multiplying the relative bacterial distribution by the total colony numbers. Because each plate is appropriate for each bacterium, the numbers of *S. enterica* serovar Typhimurium, *L. casei* and *B. thetaiotaomicron* were estimated from colonies grown on TSA, MRS and GAM plates, respectively.

Antimicrobial assay with membrane potential sensitive dye

Exponential-phase bacteria were incubated in Milli-Q water at 37°C with oxidized Crp4 or r-Crp4 (1.35 μM) for 1 hr. Then the suspensions were incubated for 10 min with 1 μg/ml of the membrane potential sensitive fluorophore, bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] (Invitrogen, Carlsbad, CA) as described [32]. The suspensions were centrifuged for 5 min at 9,300 g, and the bacterial pellets were resuspended in 1 ml PBS(-). Each bacterial sample was analyzed on a desktop cell sorter JSAN (Bay Bioscience, Kobe, Japan). The median fluorescence intensity ratio (MFIR) was obtained by dividing the median fluorescence intensity of peptide treated sample by the median fluorescence intensity of non treated sample.

Cleavage of r-Crp4 with MMP-7 in vitro

Samples (1.0 μg) of oxidized Crp4 and r-Crp4 were incubated with an activated recombinant human MMP-7 (1.0 μg) catalytic domain (Calbiochem, La Jolla, CA) in 10 mM HEPES pH 7.4, 150 mM NaCl, and 5 mM $CaCl_2$ for 18-24 hr at 37°C [9, 25]. The digested samples were analyzed by Tris-Tricine SDS-PAGE, N-terminal peptide sequencing and MALDI-TOF MS. For N-terminal peptide sequencing by Edman degradation, digested r-Crp4 was resolved by RP-HPLC under the same condition as used in the purification of oxidized Crp4. Fifty pmol samples of digested r-Crp4 were subjected to 5 cycles of N-terminal peptide sequencing at The Creative Research Initiative Sousei, Hokkaido University. To determine biological activity, the digested samples were applied to bactericidal peptide assays as described above.

Results

Oxidized Crp4 shows bactericidal activities against non-commensal bacteria, but little or no bactericidal activity against some commensal bacteria

Throughout the present study, we used oxidized Crp4 containing three pairs of

disulfide bonds and r-Crp4 obtained by the reduction of oxidized Crp4. As shown in the results of AU-PAGE (fig. 1), MALDI-TOF MS (fig. 2a) and RP-HPLC (online suppl. fig. S1a), oxidized Crp4 was homogeneous. Oxidized Crp4 showed lower molecular weight than r-Crp4 by 6 Da (fig. 2), consistent with the oxidation of 6 Cys residues in the formation of disulfide pairings. Furthermore, oxidized Crp4 had bactericidal activity against two strains of *S. enterica* serovar Typhimurium, as well as *E. coli* and *S. aureus* (fig. 3). Hence, oxidized Crp4 prepared was both biologically active and contained three pairs of disulfide bonds. r-Crp4 showed reduced migration relative to oxidized Crp4 in AU-PAGE (fig. 1). This corresponds to the results of AU-PAGE for oxidized Crp4 versus Cys-to-Ala substituted Crp4, in which the disulfide-null mutant showed reduced migration compared to oxidized Crp4 [25]. MALDI-TOF MS of r-Crp4 showed a major peak (fig. 2b) and RP-HPLC of r-Crp4 showed a single peak (online suppl. fig. S1b). From these results, we judged r-Crp4 to be homogeneous and that the three pairs of disulfide bonds were reduced. The reduction state of r-Crp4 was assured by MALDI-TOF MS and AU-PAGE prior to use and after incubation in bactericidal assays. Although r-Crp4 showed secondary bands in AU-PAGE suggestive of disulfide bond formation, some Cys residues may tend to form disulfide bonds in AU-PAGE since an excess concentration of DTT is needed for the complete reduction of Crp4.

Bactericidal activities of oxidized Crp4 against small intestinal commensal and non-commensal bacteria were examined. Oxidized Crp4 killed 11 out of 11 non-commensal bacteria and 4 of 12 commensal bacteria, *B. longum*, *B. vulgatus*, *E. faecalis* and *E. faecium* in a dose-dependent manner (fig. 3, 4c, j, k, l). In contrast, oxidized Crp4 showed little or no bactericidal activities on 8 out of 12 commensal bacteria, *B. bifidum*, *B. breve*, *L. acidophilus*, *L. casei*, *L. johnsonii*, *B. fragilis*, *B. ovatus* and *B. thetaiotaomicron* at 1.35 μM (fig. 4a, b, d, e, f, g, h, i). These activities did not change at 2.7 μM peptide concentration (data not shown). Thus, oxidized Crp4 had only minimal or no effect on survival of 8 out of the 12 species of commensal bacteria tested. In contrast, oxidized Crp4 had potent bactericidal activities against 11 of the 11 non-commensal bacterial species tested, but was only active against 4 of the 12 species of commensal bacteria. The statistical analysis of bactericidal activities of oxidized Crp4 against commensal bacteria and non-commensal bacteria revealed that oxidized Crp4 showed significantly greater bactericidal activities against non-commensal bacteria than against commensals at 1.35 μM (fig. 5, commensal bacteria vs. non-commensal bacteria exposed to oxidized Crp4).

In the intestinal lumen, a wide variety of bacteria are able to colonize. To test the selective activity of oxidized Crp4 further, a mixture of commensal and non-commensal

bacteria were exposed to oxidized Crp4. Oxidized Crp4 selectively killed *S. enterica* serovar Typhimurium, while viability of the other commensal bacteria, *L. casei* and *B. thetaiotaomicron* in the mixture was retained (fig. 6, non-treated and oxidized Crp4-treated). This result supports the selective activities of Crp4.

To determine whether this selective activity is specific to oxidized Crp4, we analyzed the bactericidal activities of oxidized Crp1, the most abundant Crp, against six non-commensal bacteria, *S. enterica* serovar Typhimurium, *S. aureus*, *E. coli*, *L. monocytogenes*, *K. oxytoca* and *P. vulgaris*, and six commensal bacteria, *B. bifidum*, *B. longum*, *L. casei*, *L. johnsonii*, *B. fragilis* and *B. thetaiotaomicron*. Oxidized Crp1 showed potent bactericidal activities against 6 of 6 non-commensal bacteria and 1 of 6 commensal bacteria, *B. longum* (online suppl. table S1), however Crp1 had little or no effect on 5 of 6 commensal bacteria (online suppl. table S1). Thus, oxidized Crp1 also had predominant bactericidal activities against non-commensal bacteria as did oxidized Crp4.

r-Crp4 has bactericidal activities against both commensal and non-commensal bacteria

Since disulfide bond pairings in α-defensins are conserved in all species which

290 express these peptides, the bactericidal activities of r-Crp4 were analyzed to elucidate
291 the effects of the conserved disulfide bonds on its bactericidal activity. r-Crp4 showed
292 equivalent bactericidal activities against all non-commensal bacteria but only 5 of 12
293 commensal bacterial species, *B. longum*, *L. acidophilus*, *B. vulgatus*, *E. faecalis* and *E.*
294 *faecium* in comparisons with oxidized Crp4 (fig. 3, 4c, d, j, k, l). These data indicated
295 that the bactericidal activities of Crp4 against these bacteria were independent of the
296 existence of the disulfide bonds. On the other hand, r-Crp4 killed 7 out of 12
297 commensal bacteria, *B. bifidum*, *B. breve*, *L. casei*, *L. johnsonii*, *B. fragilis*, *B. ovatus*
298 and *B. thetaiotaomicron* in a dose-dependent manner and showed significantly greater
299 bactericidal activities compared to those of oxidized Crp4 (fig. 4a, b, e, f, g, h, i).
300 Therefore, the reduction of the disulfide bonds conferred Crp4 bactericidal activities
301 against *B. bifidum*, *B. breve*, *L. casei*, *L. johnsonii*, *B. fragilis*, *B. ovatus* and *B.*
302 *thetaiotaomicron*, but the disulfide bonds had little or no effect on bactericidal activity
303 against all non-commensal bacteria tested and *B. longum*, *L. acidophilus*, *B. vulgatus*, *E.*
304 *faecalis* and *E. faecium*. Comparison of bactericidal activities of oxidized Crp4 and
305 r-Crp4 revealed that r-Crp4 had significantly greater bactericidal activities against
306 commensal bacteria than those of oxidized Crp4 (fig. 5, commensal bacteria exposed to
307 oxidized Crp4 vs. r-Crp4). Bactericidal activities of oxidized Crp4 and r-Crp4 were not

308 significantly different against non-commensal bacteria (fig. 5, non- commensal bacteria
309 exposed to oxidized Crp4 vs. r-Crp4).

310 When the mixture of commensal and non-commensal bacteria was exposed to r-Crp4,
311 r-Crp4 had potent bactericidal activities against both commensals and non-commensals
312 (fig. 6, non-treated and r-Crp4-treated). Therefore, the selective bactericidal activities of
313 Crp4 against certain commensal bacteria were regulated by its disulfide bonds.

314 Furthermore, bactericidal activities of r-Crp1 against 6 non-commensal and 6
315 commensal bacteria were also tested for relative sensitivity to a second mouse
316 α-defensin, oxidized and reduced Crp1. r-Crp1 showed equivalent bactericidal activities
317 to those of oxidized Crp1 against 6 of 6 non-commensal bacteria and against 1 of 6
318 commensal bacteria, *B. longum* (online suppl. table S1), but it demonstrated
319 significantly greater bactericidal activity against 5 of 6 commensal bacteria (online
320 suppl. table S1) compared to that of oxidized Crp1. Thus, the predominant bactericidal
321 activities of Crp1 against non-commensal bacteria were also affected by its disulfide
322 bonds as in the case of Crp4.

323 We next tested membrane disruption activity of Crp4. It has been reported that Crp4
324 permeabilized bacterial cell membrane, and permeabilization correlated with bacterial
325 killing activity [23]. To examine the possible mechanism of bactericidal activities of

326 Crp4, a depolarization of the membrane potential was detected with membrane potential
327 sensitive fluoroprobe, $DiBAC_4(3)$ [32] after exposure to oxidized and reduced Crp4.
328 r-Crp4 exhibited the significantly greater depolarization than oxidized Crp4 in 3 of 3
329 commensal bacteria tested (fig. 7). On the other hand, the depolarization activities of
330 oxidized Crp4 and r-Crp4 were not significantly different against 2 of 3 species of
331 non-commensal bacteria (fig. 7). Both oxidized and reduced Crp4 showed a remarkable
332 depolarization against *K. oxytoca* (fig. 7).
333
334 *Digestion of r-Crp4 by MMP-7 resulted in the attenuation of its bactericidal activity*
335 Crps are processed and activated by the processing enzyme, MMP-7 *in vivo* in Paneth
336 cell granules. It was reported that disulfide bonds of Crp4 are essential for proteolytic
337 resistance to MMP-7 by using disulfide bonds null-mutant of Crp4 [25]. Therefore, to
338 elucidate the susceptibility of r-Crp4 to MMP-7, an assay for proteolytic degradation
339 was conducted. Oxidized Crp4 and r-Crp4 were incubated with or without MMP-7 and
340 then applied to Tris-Tricine SDS-PAGE. r-Crp4 was digested by MMP-7 into smaller
341 fragments, whereas oxidized Crp4 showed complete resistance to MMP-7 (fig. 8a).
342 N-terminal peptide sequencing of the peptide fragment that emerged in the Tris-Tricine
343 SDS-PAGE revealed that the N-terminus was YCRKG. MALDI-TOF MS showed that

the peptide fragment had a molecular weight of 2480 Da. These results indicated that MMP-7 cleaved r-Crp4 at $Cys^{4}\downarrow Tyr^{5}$ and $Phe^{25}\downarrow Leu^{26}$ as shown in figure 8b. Although the peptide fragment appeared to have a molecular weight of ~3.5 kDa in figure 8a, because the fragment comprises cationic amino acids at high rates (theoretical pI = 10.3), it might had a smaller migration in Tris-Tricine SDS-PAGE than that expected from molecular weight determined by MALDI-TOF MS. The observed cleavage sites correspond to the previous cleavage of reduced, alkylated pro-Crp4 by MMP-7 at the same sites [9]. Furthermore, the digested peptide fragments were assayed for bactericidal peptide activity to test for biological effects of the degradation. Previously, it was shown that MMP-7 itself had no effect on bactericidal activity [7, 25]. The bactericidal activities of r-Crp4 against commensal and non-commensal bacteria except for *L. acidophilus* significantly decreased when r-Crp4 was digested by MMP-7 (table 1). In contrast, the faint bactericidal activity of digested r-Crp4 against *L. acidophilus* remained (table 1). Thus, the degradation of r-Crp4 by MMP-7 attenuated its bactericidal activity against most bacteria tested.

Discussion

In this study, the bactericidal activities of oxidized Crp4 against commensal and non-commensal bacteria has been analyzed. Consistent with the results of previous studies [18, 25], oxidized Crp4 showed potent bactericidal activities against non-commensal bacteria. In contrast, oxidized Crp4 had only minimal or no bactericidal activity against commensal bacteria, though it showed dose-dependent activity against *B. longum*, *B. vulgatus*, *E. faecalis* and *E. faecium*. These results demonstrate that oxidized Crp4 has more selective bactericidal activity against small intestinal bacteria. Thus, it is suggested that Crp4 has a role in the regulation of intestinal microbiota by killing certain non-commensal species while retaining the viability of certain commensal bacteria. This concept was supported by the result that oxidized Crp4 selectively killed *S. enterica* serovar Typhimurium but showed no bactericidal activity against two species of commensal bacteria, *L. casei* and *B. thetaiotaomicron* when a mixture of bacterial species was exposed to oxidized Crp4. Oxidized Crp1 also showed selective bactericidal activities, suggesting that other members of Crps contribute to maintain intestinal microbiota by killing non-commensal bacteria selectively. A recent study of intestinal microbiota in MMP-7-deficient mice that lacked active form of Crps showed that a significantly higher percentage of Firmicutes and a significantly lower percentage

of Bacteroides were detected in the small bowel of MMP-7-deficient mice compared to wild-type mice [33]. Since total bacterial numbers in both mice were not changed, it appears that Crps regulated the composition of the intestinal bacteria [33], consistent with our results that demonstrate the selective bactericidal activity of Crp4.

Some commensal bacteria showed resistance to oxidized Crp4. To date, various ways of bacterial resistance mechanisms against AMPs have been reported [34]. These include proteolytic degradation of AMPs by microbial proteases [35], binding of secreted bacterial proteins to AMPs for preventing AMPs from accessing the bacterial plasma membrane [36], extruding AMPs from bacterial cell by multiple drug resistance exporter [37], and modification of bacterial cell membrane to reduce the net anionic charge, resulting in attenuation of the affinity of AMPs to surface membrane [38, 39]. A wide variety of microbicidal mechanisms, such as the permeabilization of bacterial cell membranes, and the inhibition of DNA or protein synthesis, are presented for various AMPs [40]. In the case of Crp4, it was shown that Crp4 permeabilized the phospholipid bilayer and that the activity was dependent on the membrane composition [41]. However, the precise bactericidal mechanism(s) of native Crp4 are yet to be fully elucidated and may be bacteria-dependent. Therefore, the bacterial resistance mechanisms against Crp4 may also be various and bacteria dependent. We detected

depolarization of the membrane potential in some non-commensal bacteria by Crp4. Further investigation of bacterial factors that affect bactericidal activity is needed to uncover the bacterial resistant mechanisms to Crp4.

In this study, *Bifidobacterium* sp., *Lactobacillus* sp., *Bacteroides* sp. and *Enterococcus* sp. were used as types of small intestinal commensal bacteria. These bacteria were reported to colonize the mouse small intestine where Crp4 is present [42, 43]. Oxidized Crp4 did not show bactericidal activities against *B. Bifidum*, *Lactobacillus* sp., *B. thetaiotaomicron* and *B. fragilis*, while it killed *B. Longum*, *B. vulgatus* and *Enterococcus* sp. *B. bifidum* comprises the major portion of the intestinal microbiota in breast-fed infants [27]. *B. longum* is mainly found in the adult intestine [28], and *L. acidophilus*, *L. casei*, *L. johnsonii*, *B. thetaiotaomicron*, *B. fragilis* and *B. vulgatus* colonize the intestinal tract in both infants and adults [29, 30]. *Enterococcus* sp. are commensal bacteria that can cause opportunistic infection. It is speculated that the bacteria-dependent bactericidal activities of oxidized Crp4 against commensal bacteria reflect *in vivo* regulatory role of Crp4 to tune microbial homeostasis.

The bactericidal activities of r-Crp4 which contained no disulfide bond against small intestinal bacteria were analyzed. r-Crp4 had significantly greater bactericidal activities against *B. bifidum*, *B. breve*, *L. casei*, *L. johnsonii*, *B. fragilis*, *B. ovatus* and *B.*

416 *thetaiotaomicron* than did oxidized Crp4. This result shows that the bactericidal
417 activities of Crp4 against certain commensal bacteria were regulated by a function of its
418 disulfide bonds, indicating a novel role of the conserved disulfide bonds of Crp4 in
419 controlling bactericidal activities. r-Crp4 showed significantly greater depolarization
420 activity than oxidized Crp4 against the three commensal bacterial species tested. These
421 results suggest that disruption of membrane integration may account for part of the
422 potent bactericidal activities of r-Crp4 relative to oxidized Crp4, especially with respect
423 to certain commensals. Meanwhile, r-Crp4 and oxidized Crp4 showed equivalent
424 bactericidal activities against 11 of 11 non-commensal bacteria, 5 of 12 commensal
425 bacteria. These results demonstrate that bactericidal activities of Crp4 against these
426 bacteria have no relationship with the presence of disulfide bonds. Previously, Maemoto
427 *et al* showed that the bactericidal activity of Crp4 against non-commensal bacteria was
428 independent of its disulfide array by using a disulfide-null mutant of Crp4 [25]. Our
429 results show that the reduction of disulfide bonds has the compatible effect with the
430 previous study against non-commensal bacteria. Thus, the regulatory effect of disulfide
431 bonds depends on bacterial species. r-Crp1 also showed the bacteria-dependent
432 regulatory effects of disulfide bonds. It is speculated that this property of disulfide
433 bonds may applicable to other Crps. Native Crp4 consists of a triple-stranded

antiparallel β-sheet [44], whereas the NMR spectroscopy of Cys-to-Ala-substituted Crp4 indicates the mutant peptide is disordered [25]. According to these structural data, r-Crp4 used in this study may also have a random coil structure. In that case, it is suggested that the flexibility of r-Crp4 makes it bactericidal against *B. bifidum*, *L. casei*, *L. johnsonii*, *B. thetaiotaomicron and B. fragilis* those oxidized Crp4 did not kill completely.

In vitro degradation assays revealed that r-Crp4 was digested by MMP-7. Further, the bactericidal activities of r-Crp4 against small intestinal microbiota, except for *L. acidophilus* were attenuated by this digestion. If r-Crp4 was secreted into the lumen of the small intestine, r-Crp4 would kill both non-commensal and commensal bacteria, resulting in the perturbation of small intestinal microbial homeostasis. Thus, it is considered that degradation and inactivation of r-Crp4 before secretion would prevent this perturbation. This suggests that the host has a management mechanism to avoid the release of aberrant Crp4 that is disadvantageous to the host.

Previously, it was reported that the proform of human Paneth cell α-defensin, HD5 was reduced in some patients with Crohn's disease [45]. The reduced pro-HD5 was degraded by trypsin, a processing enzyme of HD5 *in vivo*. This resulted in diminished production of mature HD5 [45]. If Crp4 were reduced *in vivo*, degradation of Crp4 by

MMP-7 would lead to the dysfunction of innate immunity. Therefore, these results indicated that the protease resistance of α-defensins due to the disulfide bonds may contribute to the maintenance of intestinal innate immunity as well as pathology of diseases such as inflammatory bowel disease.

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Figure Legends

Fig. 1. AU-PAGE analysis of oxidized Crp4 and r-Crp4. Samples (1.0 μg) of oxidized Crp4 and r-Crp4 were resolved by AU-PAGE (12.5% acrylamide gel containing 5% acetic acid and 5 M Urea) and then stained with Coomassie brilliant blue R-250.

Fig. 2. MALDI-TOF MS analysis of oxidized Crp4 and r-Crp4. Molecular weights of oxidized Crp4 and r-Crp4 analyzed by MALDI-TOF MS are shown in (**a**) and (**b**), respectively.

Fig. 3. Bactericidal activities of oxidized Crp4 and r-Crp4 against non-commensal bacteria. Survival rates of *S. enterica* serovar Typhimurium (**a**), *S. enterica* serovar Typhimurium *phoP*- (**b**), *E. coli* ML35 (**c**), *S. aureus* (**d**), *L. monocytogenes* (**e**), *K.*

614 *oxytoca* (**f**), *K. pneumoniae* (**g**), *P. vulgaris* (**h**), *Y. enterocolitica* (**i**), *C. coli* (**j**) and *C.*
615 *jejuni* (**k**) exposed to oxidized Crp4 (●) or r-Crp4 (□) at 0.027, 0.054, 0.135, 0.27 and
616 1.35 μM are shown. Data were expressed as the means ± S.E., n = 6 for **a**, **b**, **c** and **d**, n
617 = 3 for **e**, **f**, **g**, **h**, **i**, **j** and **k** performed in triplicate.
618
619 **Fig. 4.** Bactericidal activities of oxidized Crp4 and r-Crp4 against commensal bacteria.
620 Survival rates of *B. bifidum* (**a**), *B. breve* (**b**), *B. longum* (**c**), *L. acidophilus* (**d**), *L. casei*
621 (**e**), *L. johnsonii* (**f**), *B. fragilis* (**g**), *B. ovatus* (**h**), *B. thetaiotaomicron* (**i**), *B. vulgatus* (**j**),
622 *E. faecalis* (**k**) and *E. faecium* (**l**) exposed to oxidized Crp4 (●) or r-Crp4 (□) at 0.027,
623 0.054, 0.135, 0.27 and 1.35 μM are shown. Data were expressed as the means ± S.E., n
624 = 6 for **a**, **c**, **d, e**, **i** and **j**, n = 3 for **b**, **f**, **g**, **h**, **k** and **l** performed in triplicate, **P* < 0.01 by
625 Student's *t* test.
626
627 **Fig. 5.** Statistical analysis of bactericidal activities**.** Survival rates of 12 commensal
628 bacteria and 11 non-commensal bacteria (Material and Methods) exposed to oxidized
629 Crp4 or r-Crp4 at 1.35 μM are shown. Each point represents a survival rate of each
630 bacterium. The medians were expressed as horizontal lines; the data were evaluated for
631 statistical significance by Mann-Whitney U test, where differences at values of **P* <

632 0.05 were considered to be significant. NS: Not significant.

633

634 **Fig. 6.** Bactericidal activities of oxidized Crp4 and r-Crp4 against the mixture of
635 commensal and non-commensal bacteria. The bacterial mixture of exponentially
636 growing *S. enterica* serovar Typhimurium as non-commensal bacteria, *L. casei* and *B.*
637 *thetaiotaomicron* as commensal bacteria were exposed to oxidized Crp4 or r-Crp4 at
638 1.35 μM. Surviving bacteria were counted as colony forming units.

639

640 **Fig. 7.** Antimicrobial assay with membrane sensitive dye. The median fluorescence
641 intensity ratio (MFIR) of commensal bacteria (**a**) and non-commensal bacteria (**b**)
642 exposed to oxidized Crp4 (■) or r-Crp4 (□) are shown. Data were expressed as the
643 means ± S.E. performed in triplicate, and evaluated for statistical significance by
644 Student's *t* test, where differences at values of $*P < 0.05$ were considered to be
645 significant. NS: Not significant.

646

647 **Fig. 8.** Proteolytic degradation assay of oxidized Crp4 and r-Crp4 by MMP-7. **a**
648 Tris-Tricine SDS-PAGE analysis of oxidized Crp4 and r-Crp4 incubated with or without
649 MMP-7. Samples (1.0 μg) of oxidized Crp4 and r-Crp4 were incubated with or without

MMP-7 (1.0 μg) catalytic domain. The digested samples were analyzed by Tris-Tricine SDS-PAGE. The positions of bands corresponding to MMP-7 and Crp4 are noted at the *right*. The *bold arrow* denotes the position of the MMP-7-digested fragment of r-Crp4. **b** MMP-7 cleavage sites in r-Crp4. The digested r-Crp4 was analyzed by 5 cycles of N-terminal peptide sequencing and MALDI-TOF MS. Cleavage sites are noted by *downwards arrows* (↓). *Numerals* below the r-Crp4 sequence refer to residue positions in reference to the N-terminal Gly of r-Crp4 as residue position number 1.

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670

671 **Table**

672

Table1. Bactericidal activities of r-Crp4 and MMP-7-digested r-Crp4

Bacteria	**Survival rates (%)[a] of bacteria exposed to:**	
	r-Crp4	**MMP-7-digested r-Crp4**
S. enterica serovar Typhimurium	1.4 ± 1.2	98.3 ± 9.9*
S. enterica serovar Typhimurium *phoP-*	3.2 ± 2.7	96.3 ± 12.0*
E. coli ML35	0.0 ± 0.0	118.1 ± 10.9*
S. aureus	0.0 ± 0.0	49.5 ± 13.4*
B. bifidum	13.1 ± 7.3	96.4 ± 10.4*
B. longum	0.2 ± 0.2	87.3 ± 17.0*
L. casei	28.8 ± 7.3	72.4 ± 10.3*
L. acidophilus	53.9 ± 6.3	64.8 ± 0.9‡
B. thetaiotaomicron	33.9 ± 5.7	86.5 ± 9.5*
B. vulgatus	13.6 ± 6.6	101.6 ± 8.9*

673 [a] Values are means ± S.E., for survival rates of bacteria exposed to r-Crp4 (1.35 μM)
674 and MMP-7-digested r-Crp4 (1.35 μM). n = 6 for r-Crp4, n = 3 for MMP-7-digested
675 r-Crp4.
676 *Value is significantly different ($P < 0.01$) compared to that exposed to r-Crp4 as
677 calculated by Student's *t* test.
678 ‡Not significant.

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Online Supplementary

Online supplementary Table S1. Bactericidal activities of oxidized Crp1 and r-Crp1

Bacteria	Survival rates (%)[a] of bacteria exposed to:	
	Oxidized Crp1	**r-Crp1**
Commensal bacteria		
B. bifidum	97.7 ± 5.2	0 ± 0*
B. longum	0 ± 0	0 ± 0‡
L. casei	71.1 ± 7.8	0 ± 0*
L. johnsonii	73.5 ± 13.0	0 ± 0*
B. fragilis	66.8 ± 6.2	15.1 ± 7.0*
B. thetaiotaomicron	89.8 ± 3.9	4.0 ± 8.1*
Non-commensal bacteria		
S. enterica serovar Typhimurium	1.5 ± 0.5	1.9 ± 1.3‡
S. aureus	0 ± 0	0 ± 0‡
E. coli	11.0 ± 2.1	0 ± 0‡
L. monocytogenes	24.5 ± 8.0	10.0 ± 3.1‡
K. oxytoca	0 ± 0	0 ± 0‡
P. vulgaris	7.9 ± 2.0	1.0 ± 0.4‡

[a] Values are means ± S.E., for survival rates of bacteria exposed to oxidized Crp1 or r-Crp1 (1.35 μM) performed in triplicate.

*Value is significantly different ($P < 0.01$) compared to that exposed to oxidized Crp1 as calculated by Student's *t* test.

‡Not significant.

Online Supplementary Fig. S1. Purification of oxidized Crp4 and r-Crp4 by RP-HPLC. Oxidized Crp4 (**a**) and r-Crp4 (**b**) were purified by a C-18 column (SepaxHP-C18, 4.6x150 mm, 5 μm, Sepax Technologies, Inc., Newark, DE) in 0.1% trifluoroacetic acid with an 18-36% acetonitrile gradient developed over 30 min at 1 ml/min.

Online Supplementary Fig. S2. Bactericidal peptide assay. The colonies of *Salmonella enterica* serovar Typhimurium incubated with oxidized Crp4 are shown as a represented example. Exponential-phase bacteria cultured at 37°C were washed and the bacteria populations were adjusted to 1,000 CFU in 20 μl. Then the bacteria solution was mixed with equivalent volumes of oxidized Crp4 with the final concentrations of 0.027, 0.054, 0.135, 0.27, and 1.35 μM. The mixtures were plated after incubation for 1 hr at 37°C. The assay measured bactericidal activity because the peptides affect bacteria only at the incubation time. Bacteria survival rates were determined from the numbers of surviving colonies relative to peptide-unexposed controls.

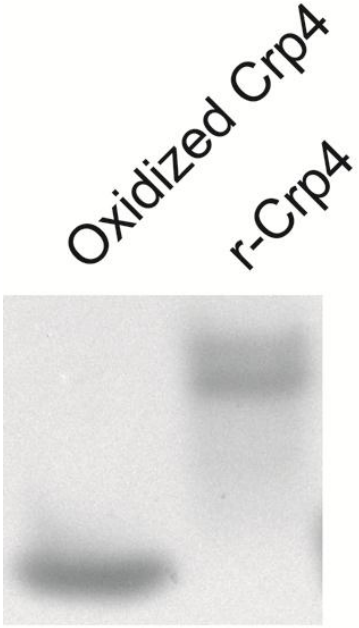
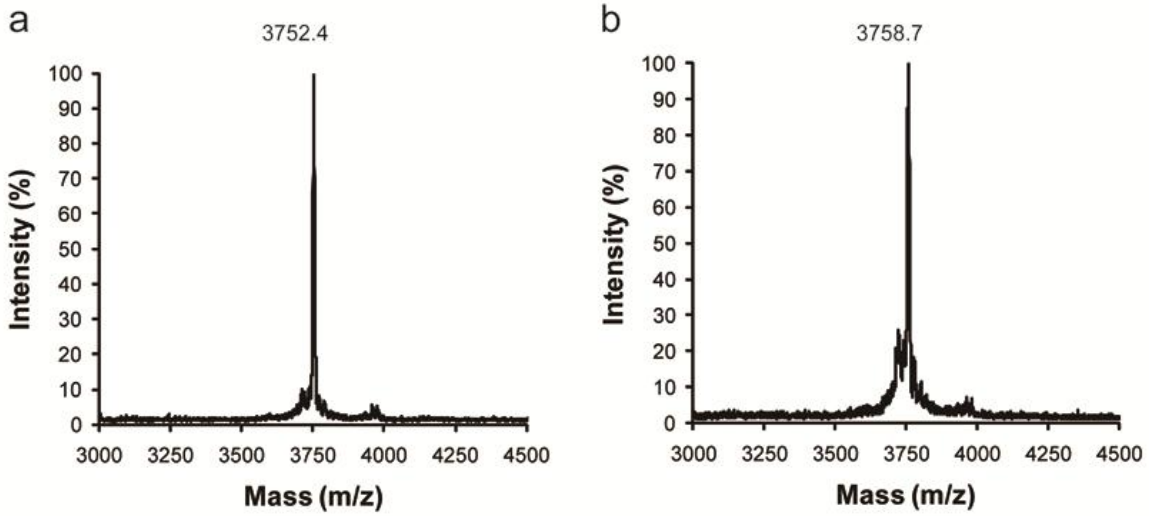


Fig. 1. Koji Masuda



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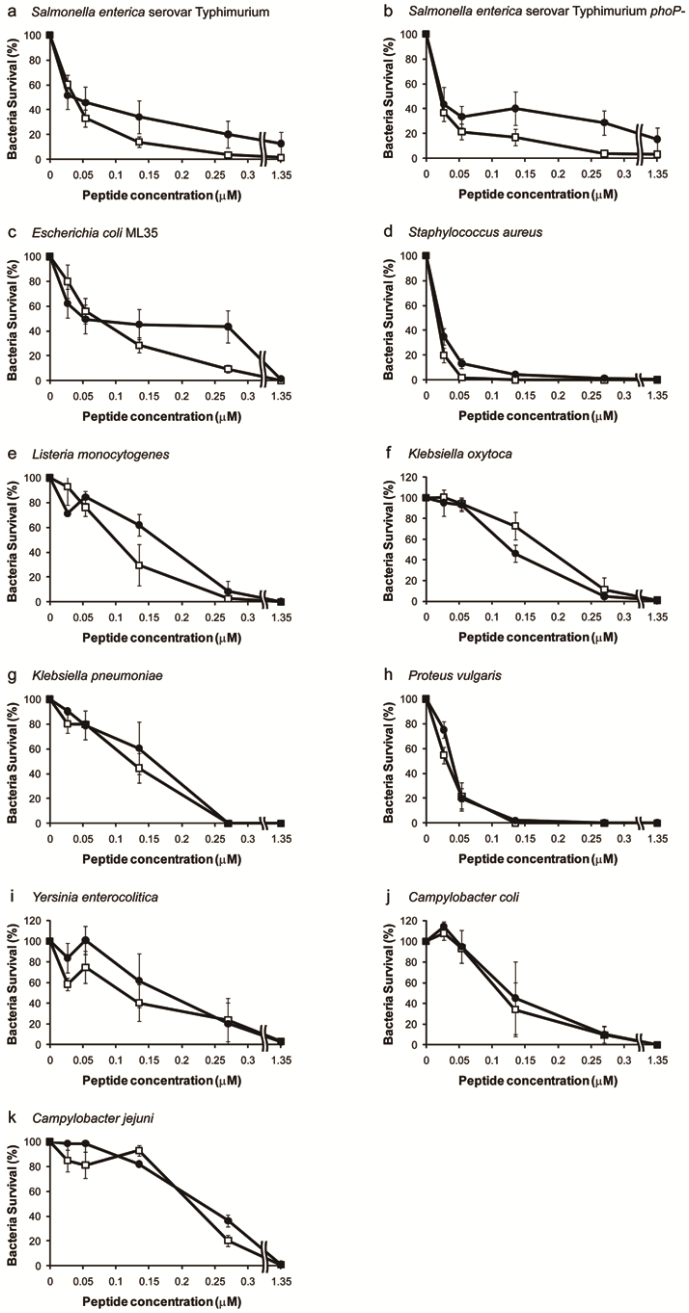

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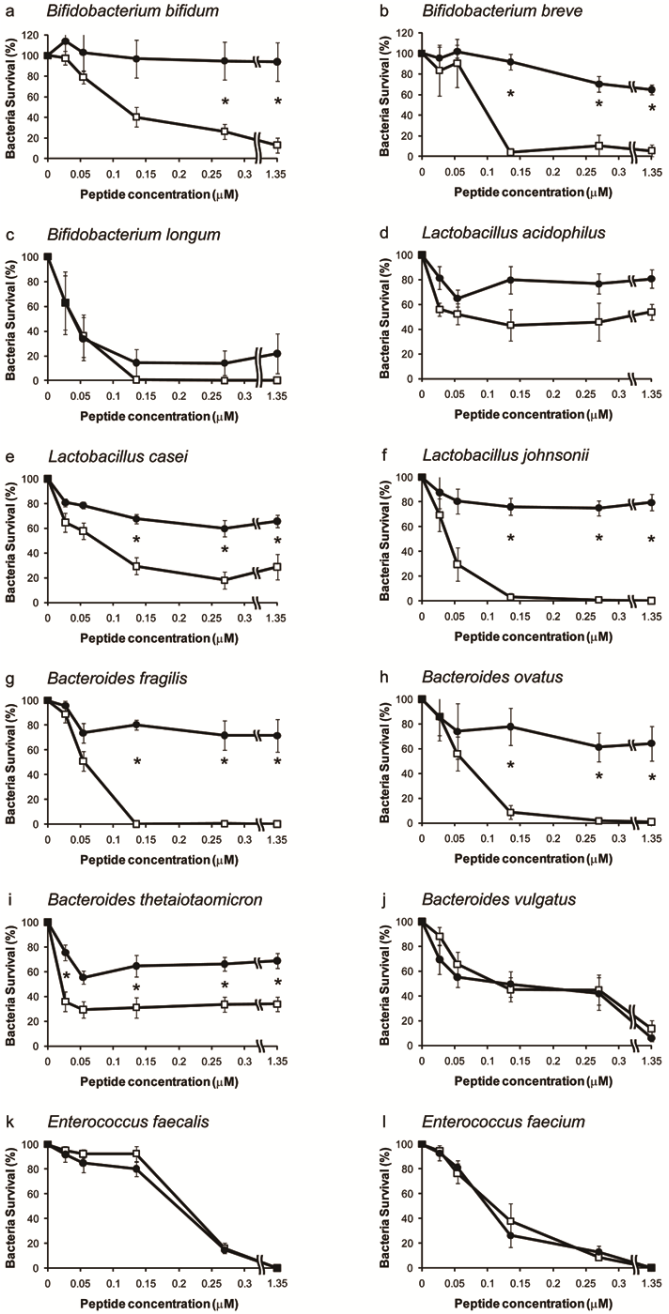

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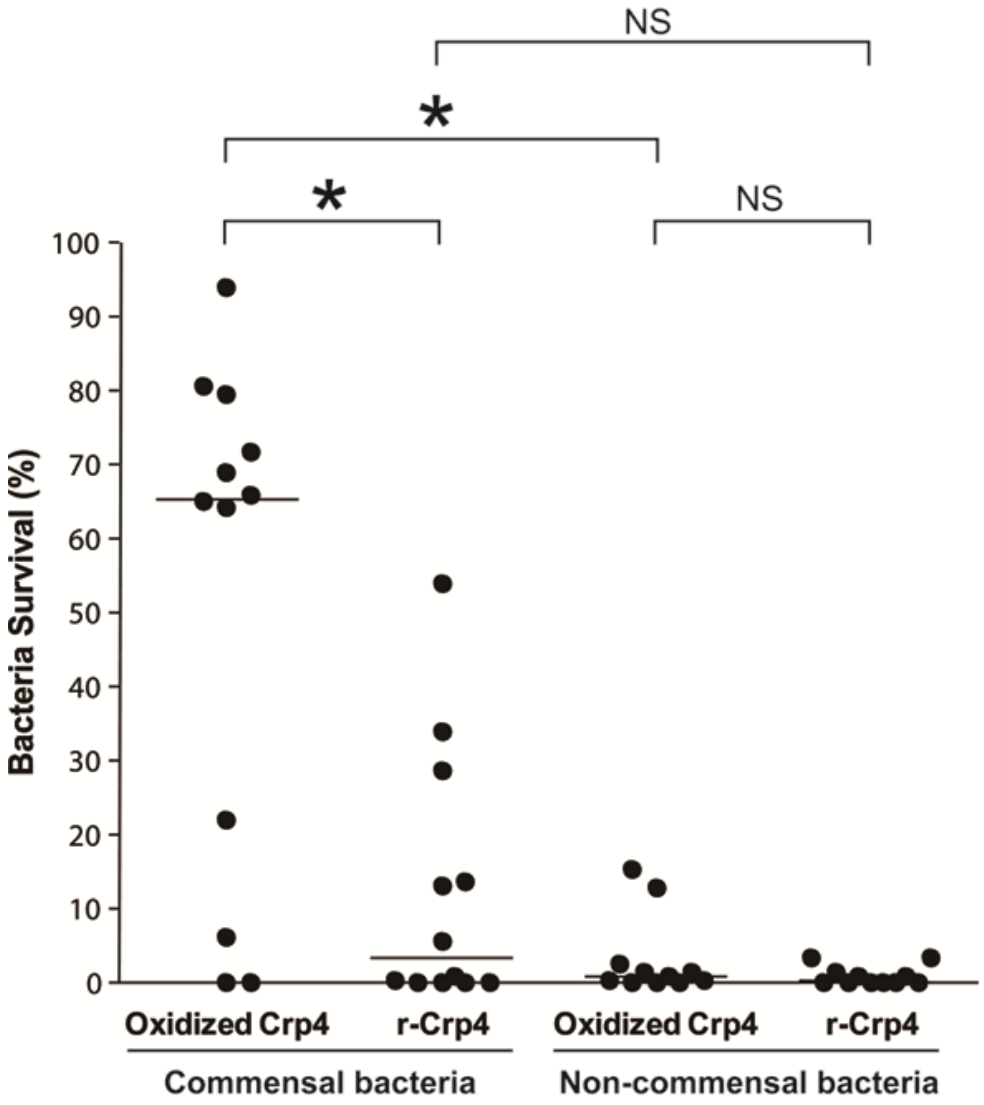

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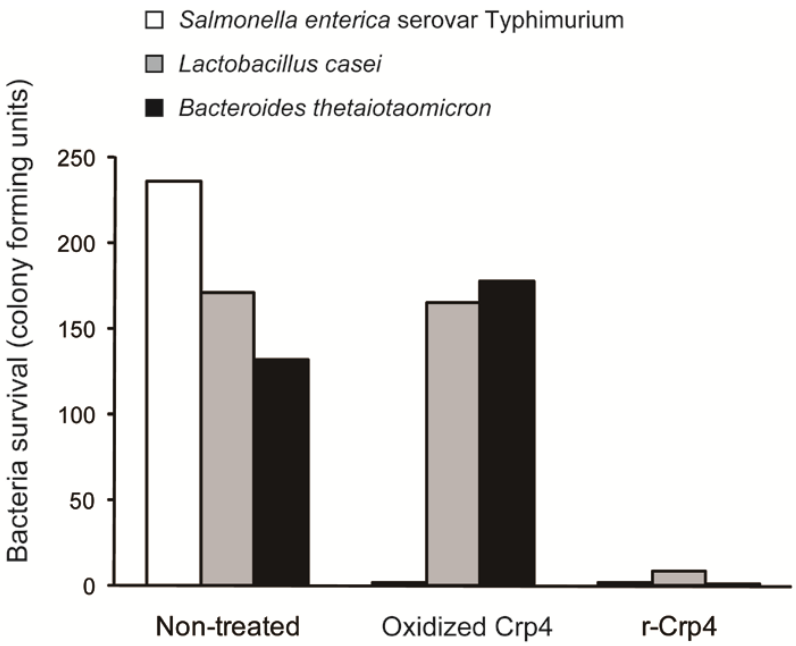

Fig. 6. Koji Masuda

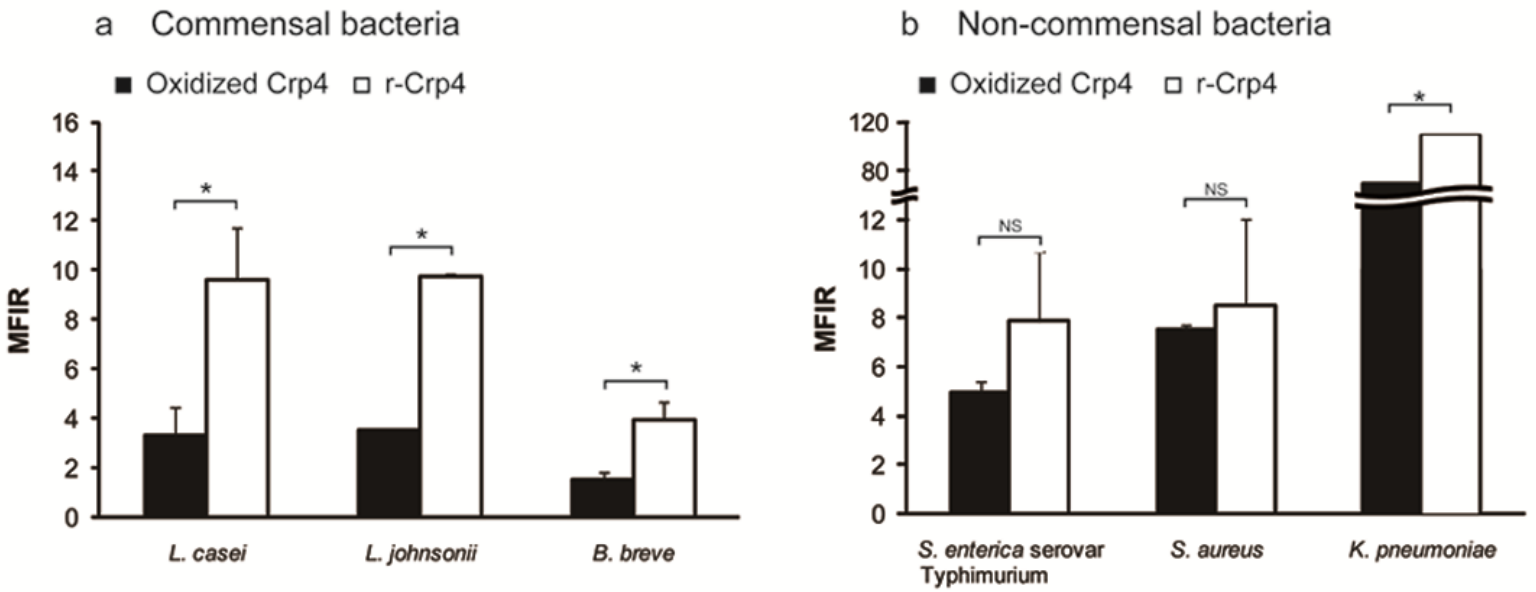

Fig. 7. Koji Masuda

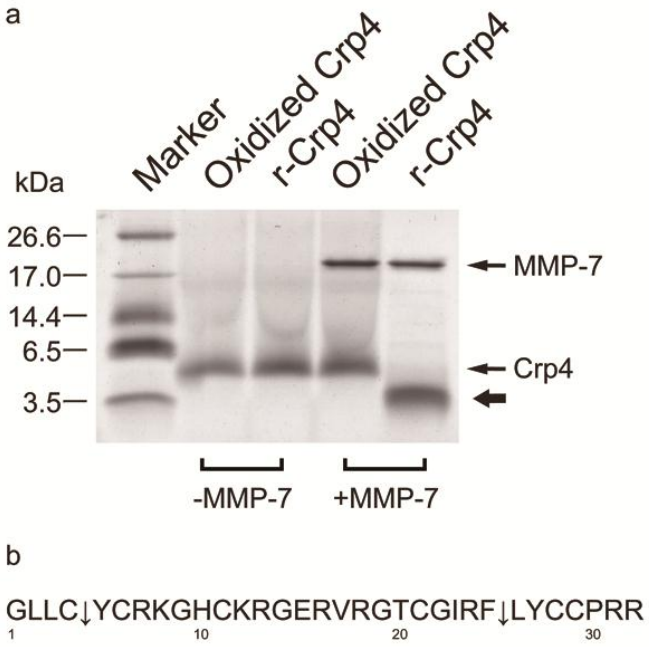

Fig. 8. Koji Masuda