



Title	Bactericidal Activity of Mouse $\alpha$ -Defensin, Cryptdin-4 Predominantly Affects Noncommensal Bacteria
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1 Bactericidal Activity of Mouse  $\alpha$ -Defensin, Cryptdin-4  
2 Predominantly Affects Non-Commensal Bacteria  
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4

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13 Short title: Selective Bactericidal Activity of Cryptdin-4

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19

20 **Abstract**

21

22 **Mouse Paneth cell  $\alpha$ -defensins, termed cryptdins, are secreted into the intestinal**  
23 **lumen, exert microbicidal activity and contribute to the intestinal innate immunity.**

24 **Among them, cryptdin-4 (Crp4) has the most potent microbicidal activity. In the**

25 **intestinal lumen, commensal bacteria colonize and elicit beneficial effects to the**

26 **host. However, the effects of Crp4 against commensal bacteria are poorly**

27 **understood. Thus, we investigated the bactericidal activities of Crp4 against**

28 **commensal bacteria compared to non-commensal bacteria. Oxidized Crp4 showed**

29 **only minimal or no bactericidal activity against 8 out of 12 commensal bacterial**

30 **species, including *Bifidobacterium bifidum* and *Lactobacillus casei*. We further**

31 **addressed a role of the conserved disulfide bonds of Crp4 by analyzing reduced**

32 **Crp4 (r-Crp4). r-Crp4 demonstrated significantly greater bactericidal activities**

33 **against 7 of 12 commensal bacteria than did oxidized Crp4. Oxidized Crp4 and**

34 **r-Crp4 elicited equivalently potent bactericidal activities against 11 of 11**

35 **non-commensal bacteria tested such as *Salmonella enterica* serovar Typhimurium,**

36 **and 5 of 12 commensal bacteria. Furthermore, when r-Crp4 was exposed to a**

37 **processing enzyme of cryptdins, MMP-7, r-Crp4 was degraded, and bactericidal**

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

41

42

### 43 **Introduction**

44

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

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

59 In the gastrointestinal tract, a highly complex microbial ecosystem is constructed by  
60 colonizing microbes. Hosts and microbiota have co-evolved in ways that have mutually  
61 beneficial effects [10]. These include host development [11], nutritional absorption [12],  
62 and functional development of the immune systems [13]. Recent studies have shown  
63 that well-balanced cross-talk between the host and commensal bacteria are important  
64 [14], as imbalances of the relationships result in inflammation [15] and cancer [16]. In  
65 the mouse small intestine, the expression of more than 20 cryptdin genes and  
66 cryptdin-related sequence genes have been reported [17-20], and these peptides are  
67 secreted into the intestinal lumen where commensal bacteria reside [21]. Among Crps,  
68 cryptdin-1 ~ -6 (Crp1~6) are characterized at peptide level. Although the amino acid  
69 sequence identities of mature regions of Crp1~3, 6 are more than 90%, those of Crp4  
70 and Crp5 with Crp1 are 42 and 54%, respectively [18]. In particular, Crp4 has several  
71 features that distinguish it from other Crps. For example, the Crp4 polypeptide chain  
72 uniquely lacks three amino acids between fourth and fifth cysteine residue positions  
73 [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  $\alpha$ -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.  $\alpha$ -Defensins including Crp4 are characterized  
87 by invariant disulfide bonds arranged between Cys<sup>1</sup>-Cys<sup>6</sup>, Cys<sup>2</sup>-Cys<sup>4</sup>, and Cys<sup>3</sup>-Cys<sup>5</sup> [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

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

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

102

103

## 104 **Materials and Methods**

105

### 106 *Preparation of oxidized Crps and r-Crps*

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

110 performance liquid chromatography (RP-HPLC). The Crp4 with three pairs of disulfide  
111 bonds, defined as oxidized Crp4, was purified by a C-18 column (SepaxHP-C18,  
112 4.6x150 mm, 5  $\mu$ m, Sepax Technologies, Inc., Newark, DE) in 0.1% trifluoroacetic acid  
113 with an 18-36% acetonitrile gradient developed over 30 min at 1 ml/min (online suppl.  
114 fig. S1a). Oxidized Crp4 was obtained after final lyophilization and stored at -30°C until  
115 use.

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

126

127 *Evaluation of disulfide bond formation*



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

136

#### 137 *Bacterial strains and culture conditions*

138 As some of the most common commensal bacteria in the small intestine [27-30],  
139 *Bifidobacterium bifidum* ATCC 11863 (*B. bifidum*), *Bifidobacterium breve* JCM 1192  
140 (*B. breve*), *Bifidobacterium longum* ATCC 15707 (*B. longum*), *Lactobacillus*  
141 *acidophilus* ATCC 314 (*L. acidophilus*), *Lactobacillus casei* ATCC 393 (*L. casei*),  
142 *Lactobacillus johnsonii* JCM 2012 (*L. johnsonii*), *Bacteroides fragilis* JCM 11019 (*B.*  
143 *fragilis*), *Bacteroides ovatus* JCM 5824 (*B. ovatus*), *Bacteroides thetaiotaomicron* JCM  
144 5827 (*B. thetaiotaomicron*), *Bacteroides vulgatus* JCM 5826 (*B. vulgatus*),  
145 *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<sub>620</sub> 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

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

206

#### 207 *Antimicrobial assay with membrane potential sensitive dye*

208 Exponential-phase bacteria were incubated in Milli-Q water at 37°C with oxidized  
209 Crp4 or r-Crp4 (1.35 µM) for 1 hr. Then the suspensions were incubated for 10 min with  
210 1 µg/ml of the membrane potential sensitive fluorophore, bis-(1,3-dibutylbarbituric  
211 acid) trimethine oxonol [DiBAC<sub>4</sub>(3)] (Invitrogen, Carlsbad, CA) as described [32]. The  
212 suspensions were centrifuged for 5 min at 9,300 g, and the bacterial pellets were  
213 resuspended in 1 ml PBS(-). Each bacterial sample was analyzed on a desktop cell  
214 sorter JSAN (Bay Bioscience, Kobe, Japan). The median fluorescence intensity ratio  
215 (MFIR) was obtained by dividing the median fluorescence intensity of peptide treated  
216 sample by the median fluorescence intensity of non treated sample.

217

218 *Cleavage of r-Crp4 with MMP-7 in vitro*

219 Samples (1.0 µg) of oxidized Crp4 and r-Crp4 were incubated with an activated  
220 recombinant human MMP-7 (1.0 µg) catalytic domain (Calbiochem, La Jolla, CA) in 10  
221 mM HEPES pH 7.4, 150 mM NaCl, and 5 mM CaCl<sub>2</sub> for 18-24 hr at 37°C [9, 25]. The  
222 digested samples were analyzed by Tris-Tricine SDS-PAGE, N-terminal peptide  
223 sequencing and MALDI-TOF MS. For N-terminal peptide sequencing by Edman  
224 degradation, digested r-Crp4 was resolved by RP-HPLC under the same condition as  
225 used in the purification of oxidized Crp4. Fifty pmol samples of digested r-Crp4 were  
226 subjected to 5 cycles of N-terminal peptide sequencing at The Creative Research  
227 Initiative Sousei, Hokkaido University. To determine biological activity, the digested  
228 samples were applied to bactericidal peptide assays as described above.

229

230

## 231 **Results**

232

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

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

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

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

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



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

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

286

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

289 Since disulfide bond pairings in  $\alpha$ -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 *thetaitotaomicron*, 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  $\alpha$ -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<sub>4</sub>(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

344 the peptide fragment had a molecular weight of 2480 Da. These results indicated that  
345 MMP-7 cleaved r-Crp4 at Cys<sup>4</sup>↓Tyr<sup>5</sup> and Phe<sup>25</sup>↓Leu<sup>26</sup> as shown in figure 8b. Although  
346 the peptide fragment appeared to have a molecular weight of ~3.5 kDa in figure 8a,  
347 because the fragment comprises cationic amino acids at high rates (theoretical pI =  
348 10.3), it might had a smaller migration in Tris-Tricine SDS-PAGE than that expected  
349 from molecular weight determined by MALDI-TOF MS. The observed cleavage sites  
350 correspond to the previous cleavage of reduced, alkylated pro-Crp4 by MMP-7 at the  
351 same sites [9]. Furthermore, the digested peptide fragments were assayed for  
352 bactericidal peptide activity to test for biological effects of the degradation. Previously,  
353 it was shown that MMP-7 itself had no effect on bactericidal activity [7, 25]. The  
354 bactericidal activities of r-Crp4 against commensal and non-commensal bacteria except  
355 for *L. acidophilus* significantly decreased when r-Crp4 was digested by MMP-7 (table  
356 1). In contrast, the faint bactericidal activity of digested r-Crp4 against *L. acidophilus*  
357 remained (table 1). Thus, the degradation of r-Crp4 by MMP-7 attenuated its  
358 bactericidal activity against most bacteria tested.

359

360

361 **Discussion**

362

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

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

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

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

401 In this study, *Bifidobacterium* sp., *Lactobacillus* sp., *Bacteroides* sp. and  
402 *Enterococcus* sp. were used as types of small intestinal commensal bacteria. These  
403 bacteria were reported to colonize the mouse small intestine where Crp4 is present [42,  
404 43]. Oxidized Crp4 did not show bactericidal activities against *B. Bifidum*,  
405 *Lactobacillus* sp., *B. thetaiotaomicron* and *B. fragilis*, while it killed *B. Longum*, *B.*  
406 *vulgatus* and *Enterococcus* sp. *B. bifidum* comprises the major portion of the intestinal  
407 microbiota in breast-fed infants [27]. *B. longum* is mainly found in the adult intestine  
408 [28], and *L. acidophilus*, *L. casei*, *L. johnsonii*, *B. thetaiotaomicron*, *B. fragilis* and *B.*  
409 *vulgatus* colonize the intestinal tract in both infants and adults [29, 30]. *Enterococcus* sp.  
410 are commensal bacteria that can cause opportunistic infection. It is speculated that the  
411 bacteria-dependent bactericidal activities of oxidized Crp4 against commensal bacteria  
412 reflect *in vivo* regulatory role of Crp4 to tune microbial homeostasis.

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



416 *thetaitotaomicron* 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 be applicable to other Crps. Native Crp4 consists of a triple-stranded

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

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

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

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

456

457

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459

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470

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### 601 **Figure Legends**

602

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

606

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

610

611 **Fig. 3.** Bactericidal activities of oxidized Crp4 and r-Crp4 against non-commensal  
612 bacteria. Survival rates of *S. enterica* serovar Typhimurium (a), *S. enterica* serovar  
613 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 *thetaitotaomicron* as commensal bacteria were exposed to oxidized Crp4 or r-Crp4 at  
638 1.35  $\mu$ M. 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  $\pm$  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  $\mu$ g) of oxidized Crp4 and r-Crp4 were incubated with or without

650 MMP-7 (1.0  $\mu\text{g}$ ) catalytic domain. The digested samples were analyzed by Tris-Tricine  
651 SDS-PAGE. The positions of bands corresponding to MMP-7 and Crp4 are noted at the  
652 *right*. The *bold arrow* denotes the position of the MMP-7-digested fragment of r-Crp4.  
653 **b** MMP-7 cleavage sites in r-Crp4. The digested r-Crp4 was analyzed by 5 cycles of  
654 N-terminal peptide sequencing and MALDI-TOF MS. Cleavage sites are noted by  
655 *downwards arrows* ( $\downarrow$ ). *Numerals* below the r-Crp4 sequence refer to residue positions  
656 in reference to the N-terminal Gly of r-Crp4 as residue position number 1.

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671 **Table**

672

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

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

673 <sup>a</sup> 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 <sup>‡</sup>Not significant.

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**Online Supplementary**

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

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

690 <sup>a</sup> Values are means ± S.E., for survival rates of bacteria exposed to oxidized Crp1 or  
691 r-Crp1 (1.35 μM) performed in triplicate.

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

694 ‡Not significant.

695  
696



697

698

699 **Online Supplementary Fig. S1.** Purification of oxidized Crp4 and r-Crp4 by RP-HPLC.

700 Oxidized Crp4 (**a**) and r-Crp4 (**b**) were purified by a C-18 column (SepaxHP-C18,

701 4.6x150 mm, 5  $\mu$ m, Sepax Technologies, Inc., Newark, DE) in 0.1% trifluoroacetic acid

702 with an 18-36% acetonitrile gradient developed over 30 min at 1 ml/min.

703

704 **Online Supplementary Fig. S2.** Bactericidal peptide assay. The colonies of *Salmonella*

705 *enterica* serovar Typhimurium incubated with oxidized Crp4 are shown as a represented

706 example. Exponential-phase bacteria cultured at 37°C were washed and the bacteria

707 populations were adjusted to 1,000 CFU in 20  $\mu$ l. Then the bacteria solution was mixed

708 with equivalent volumes of oxidized Crp4 with the final concentrations of 0.027, 0.054,

709 0.135, 0.27, and 1.35  $\mu$ M. The mixtures were plated after incubation for 1 hr at 37°C.

710 The assay measured bactericidal activity because the peptides affect bacteria only at the

711 incubation time. Bacteria survival rates were determined from the numbers of surviving

712 colonies relative to peptide-unexposed controls.

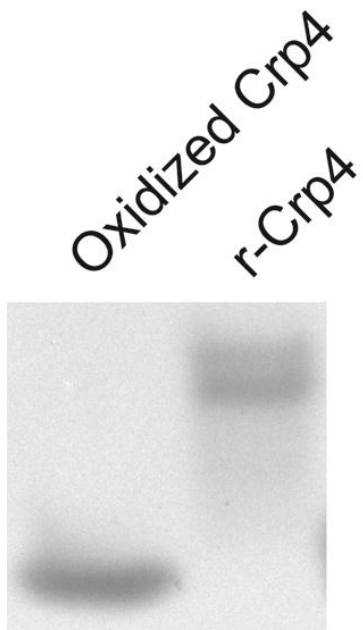


Fig. 1. Koji Masuda

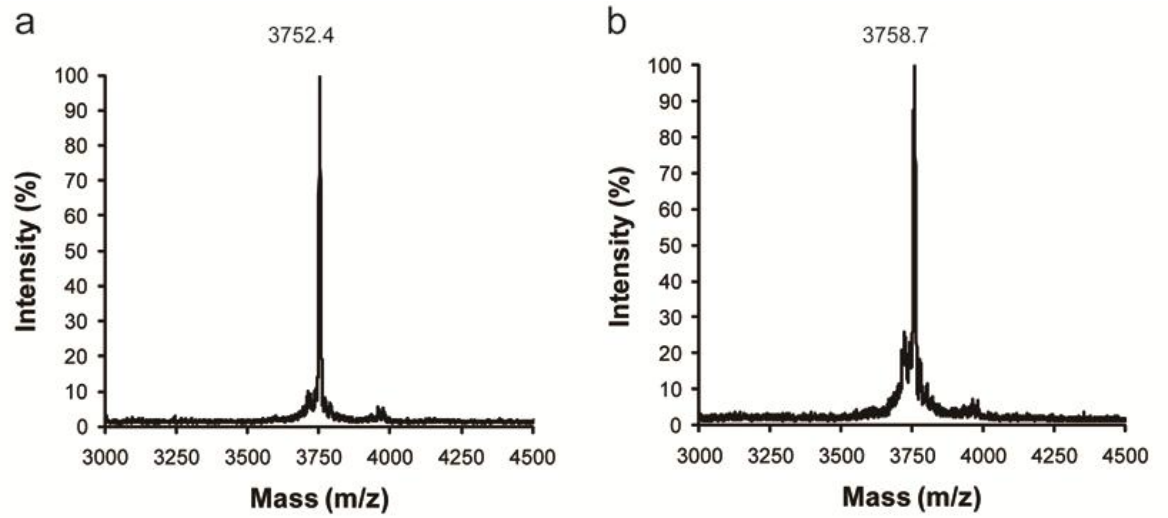


Fig. 2. Koji Masuda

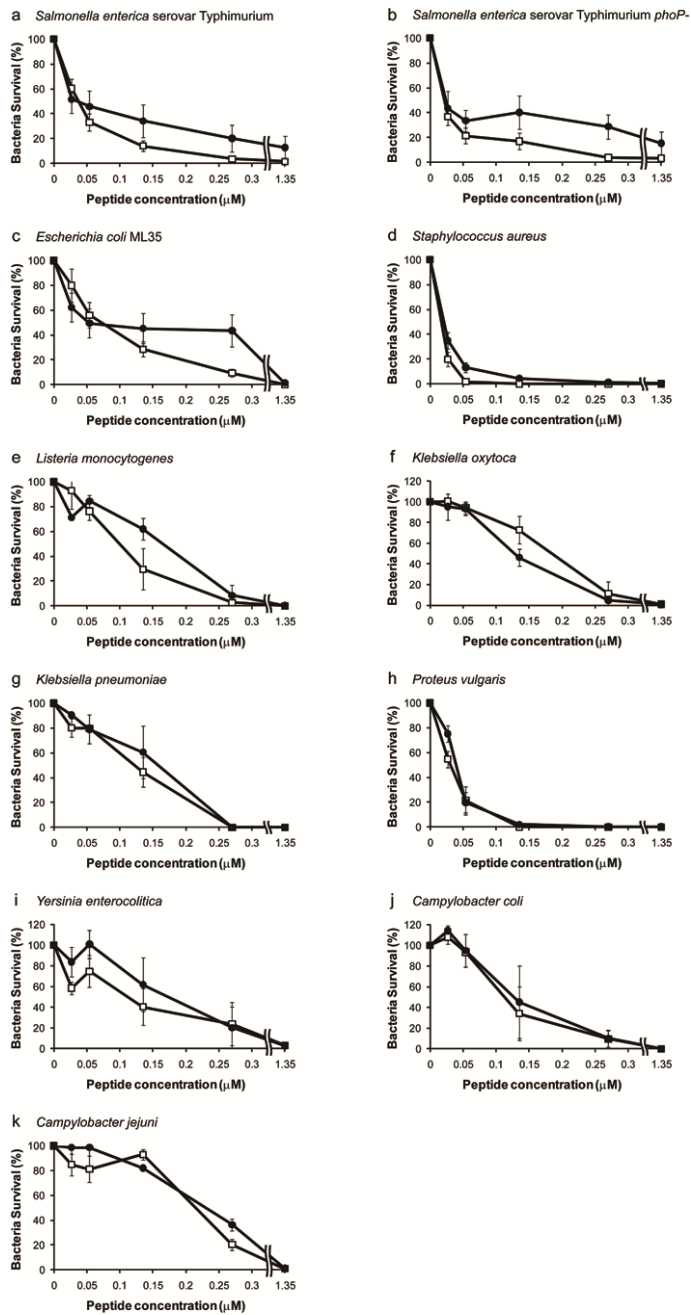


Fig. 3. Koji Masuda

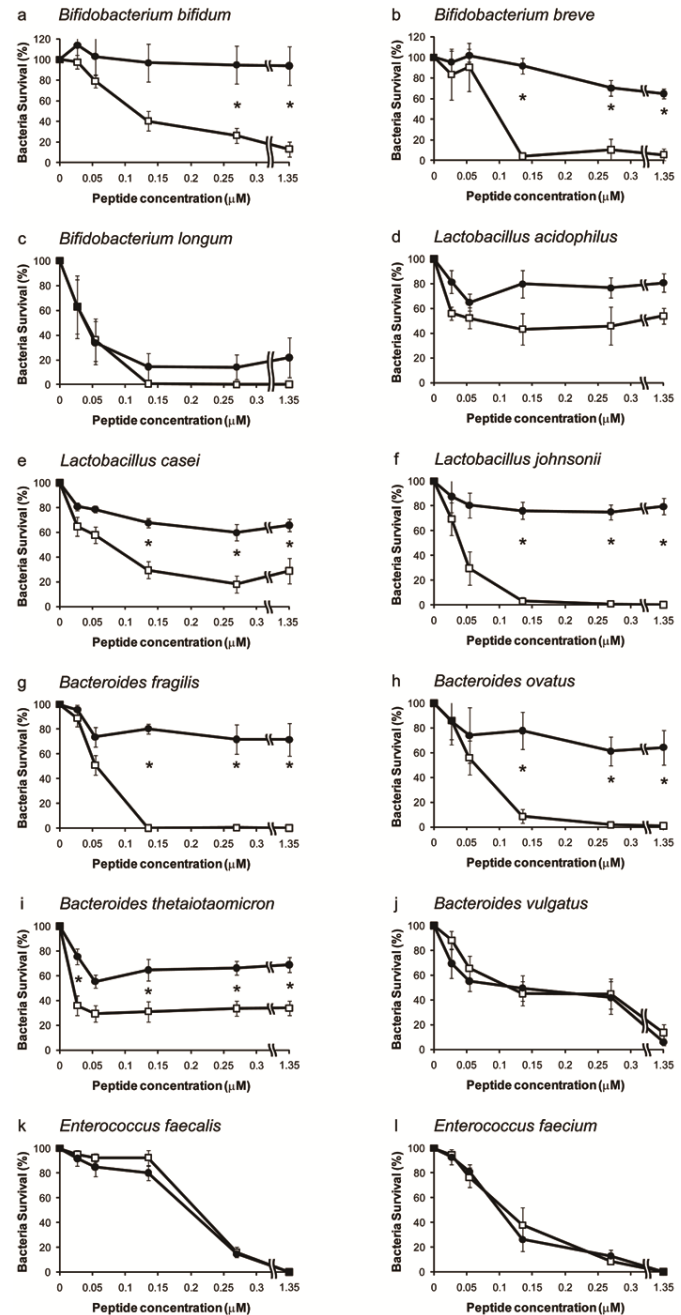


Fig. 4. Koji Masuda

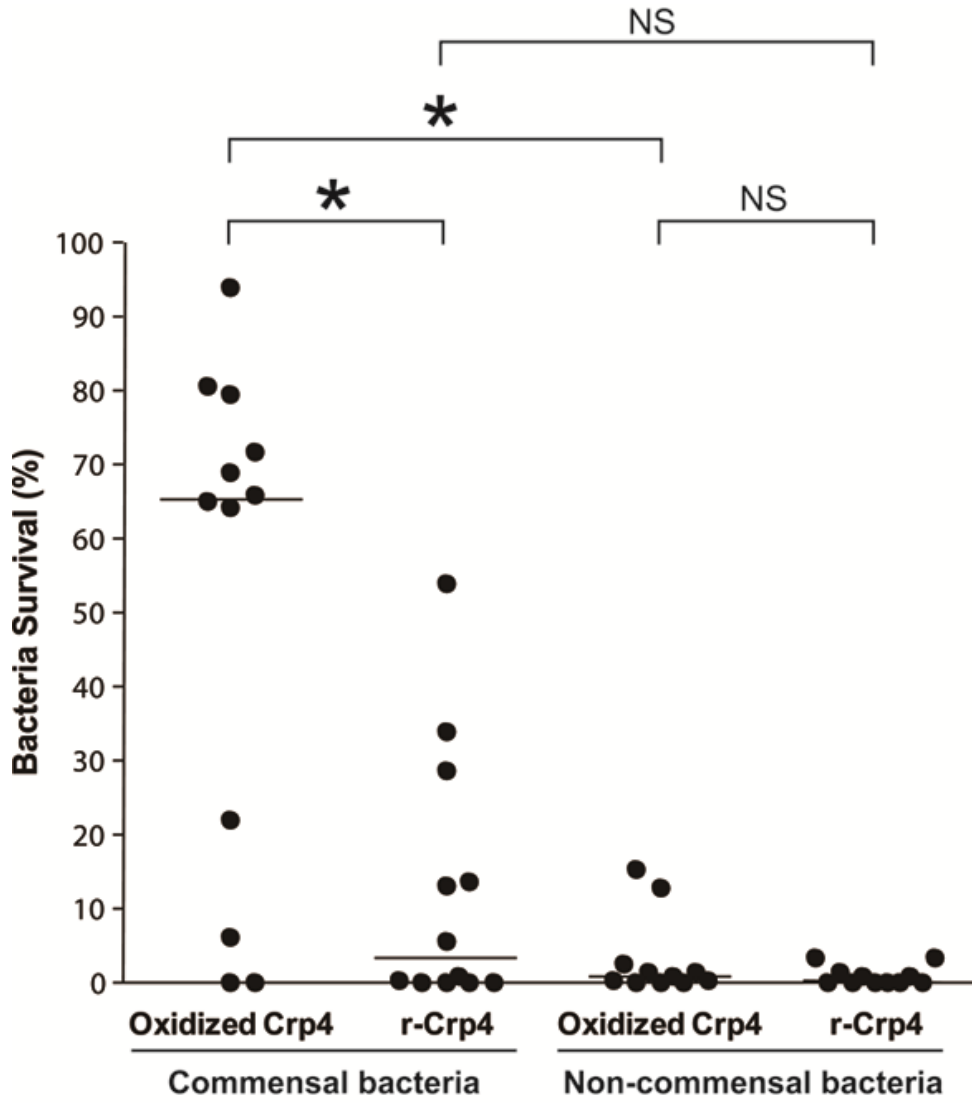


Fig. 5. Koji Masuda

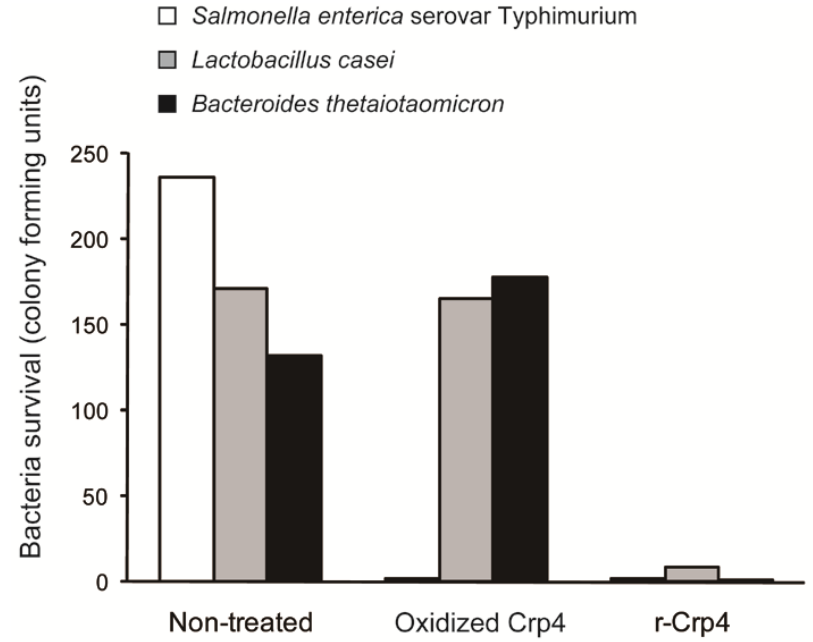


Fig. 6. Koji Masuda

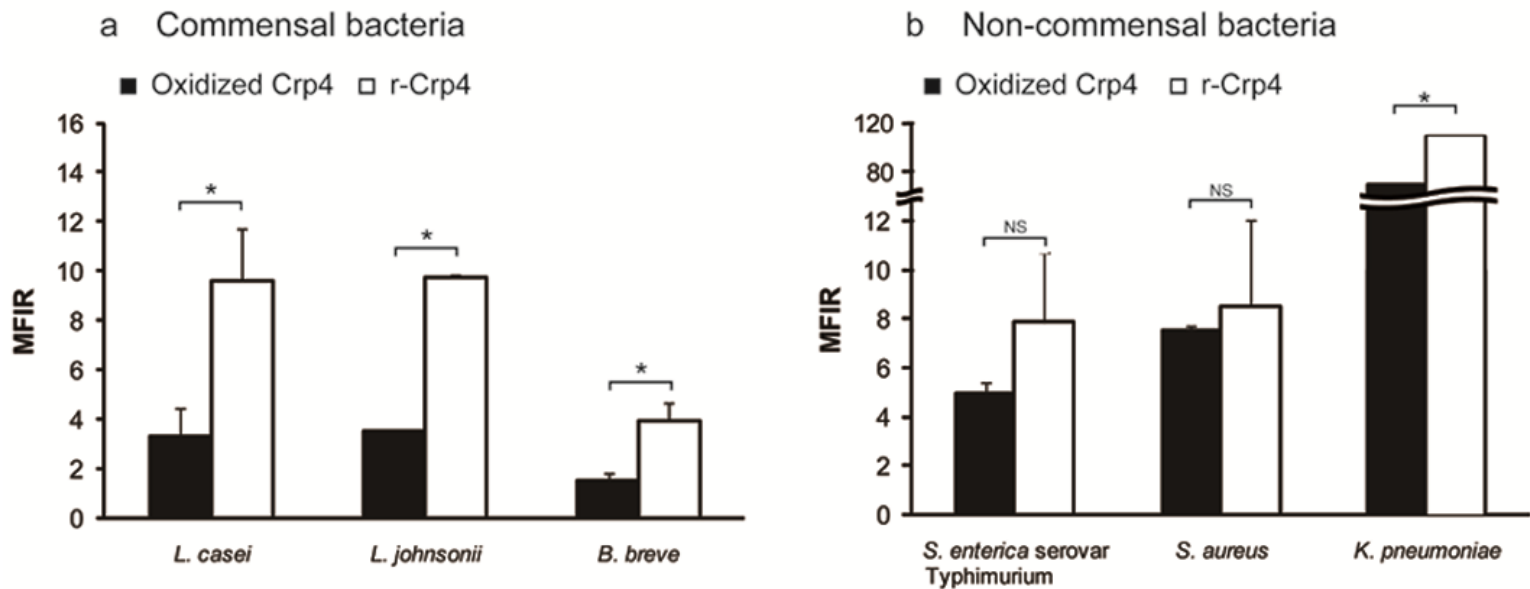


Fig. 7. Koji Masuda

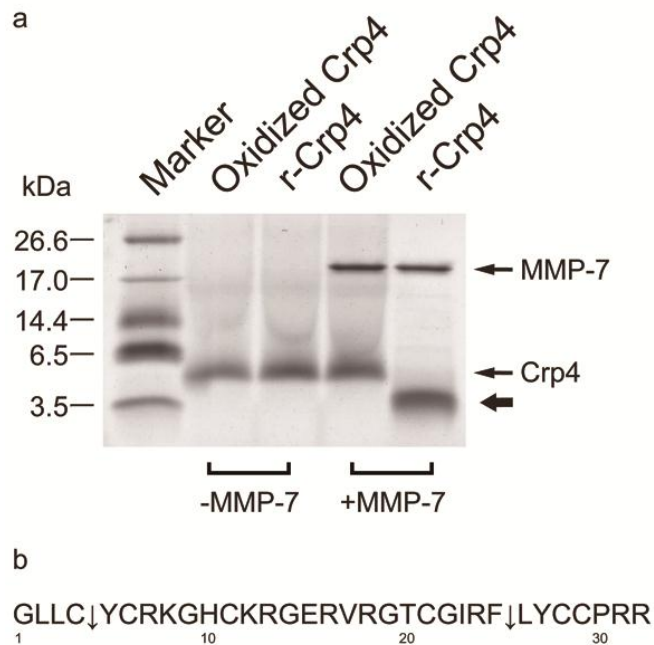


Fig. 8. Koji Masuda