Title 論文題目	Novel antimicrobial activities of a peptide derived from a Japanese soy bean fermented food, Natto, against *Streptococcus pneumoniae* and *Bacillus subtilis* group strains (納豆由来ペプチドの肺炎球菌およびサブチリスグループに属するバチラス属細菌に対する新奇な抗菌活性)
Author(s) 著　者	北川，学
Degree number 学位記番号	甲第 2954 号
Degree name 学位の種別	博士（医学）
Issue Date 学位取得年月日	2017-03-31
Original Article 原著論文	札幌医学雑誌　第 86 巻　第 1 号
Doc URL	
DOI	
Resource Version	

Novel antimicrobial activities of a peptide derived from a Japanese soybean fermented food, Natto, against *Streptococcus pneumoniae* and *Bacillus subtilis* group strains

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Keywords: Natto, antimicrobial peptide, *Streptococcus pneumoniae*, *Bacillus subtilis*, subtilisin

ABSTRACT

We recently isolated a tumoricidal peptide from Natto, a Japanese traditional fermented food. In the present study, antimicrobial activity of the Natto peptide was examined. The peptide consisted of 45 amino acid residues, and its structure was predicted to be rich in α-helix. It excreted antimicrobial activity only against *Streptococcus pneumoniae* and *Bacillus subtilis* group (*B. subtilis*, *Bacillus pumilus*, and *Bacillus licheniformis*). Lesser antimicrobial activity was observed for *Streptococcus* species other than *S. pneumoniae*. Hemolysate or hemin was required for the antimicrobial activity of the peptide. The Natto peptide damages the cell membrane of *B. subtilis*. On the other hand, chain morphology was induced in *S. pneumoniae*, which is naturally diplococcus, during the early phases of the Natto peptide treatment; following that the cells were rapidly lysed. This suggested that the Natto peptide displayed a novel narrow spectrum of bactericidal activity and inhibited cell separation during cell division of *S. pneumoniae*.

1. Introduction

Natto is a traditional Japanese food obtained after fermentation of boiled soybeans by *Bacillus subtilis*. It is recognized as a healthy food, and a rich source of vitamins, calcium, and various nutrients. Isoflavone, an analogue of estrogen, is derived from soybeans, and it is shown to prevent cerebral and myocardial stroke, breast cancer and prostate cancer (Zaheer and Akhtar, 2015). Lecithin is also derived from soybeans; it is a phospholipid containing unsaturated fatty acids. Lecithin has shown to prevent coronary artery diseases by preventing the deposition of cholesterol and other fats on the vascular endothelium and to prevent liver damages (Nicolosi et al., 2001). Nattokinase is a subtilisin family serine protease derived from *B. subtilis*. It exhibits a pronounced fibrinolytic activity, and is known as a blood thinner (Sumi et al., 1990; Dabbagh et al., 2014). Dipicolinic acid, which is contained in *B. subtilis* spores with a high content (approximately 5-15% of dry weight) contributing to the dormancy and germination of spore-forming bacteria (Murrel and Warth, 1965), shows a broad spectrum of antimicrobial activity against bacteria and yeasts (Sumi and Ohsugi, 1999). Natto is the only food containing high levels of

64 water-soluble vitamin K_2 (menaquinone-7). The high vitamin K_2 content of
65 Natto results from fermentation; consequently, vitamin K_2 content in Natto
66 is over 100 times higher than in soybeans (Sakano et al., 1988). Natto,
67 therefore, contains numerous useful substrates for human health derived
68 from the soybeans, *B. subtilis,* and soybean fermentation products. We were
69 interested in the new beneficial effects of the multifunctional food, Natto, on
70 human health.

71 Recently, we discovered a new peptide (termed "Natto peptide") that
72 has cytotoxicity toward tumor cell lines but not toward normal cell lines
73 (Hatakeyama et al., 2016). Injury of the cell membrane was suggested as a
74 mechanism of this cytotoxicity. Such tumoricidal activity is also displayed by
75 various antimicrobial peptides. One family of such antimicrobial peptides is
76 the defensin family (Suarez-Carmona et al., 2015; Mattar et al., 2016). The
77 defensin family peptides are disulfide-bond-rich cationic peptides found in
78 both vertebrates and invertebrates. In human, α-defensins are mainly
79 produced by neutrophils, and β-defensins are produced by leukocytes and
80 epithelial cells. Another antimicrobial peptide is the cathelicidin family
81 (Wang et al., 2014; Xhindoli et al., 2016). They are α-helix-rich peptide, with

82 basic amino acids present every few residues. They are found in mammals,
83 and are produced by neutrophils, macrophages, and keratinocytes. These
84 cationic peptides display tumoricidal activity like as the Natto peptide and
85 antimicrobial activity against various types of microorganisms, including
86 Gram-positive bacteria, Gram-negative bacteria, and fungi (Sang and Blecha,
87 2008; Suarez-Carmona et al., 2015; Mattar et al., 2016; Xhindoli et al., 2016).
88 Given the above, we were interested in the antimicrobial activity of the
89 Natto peptide.
90
91
92 **2. Materials and Methods**
93 *2.1. Preparation of the Natto peptide*
94 Natto, which is commercially available, was donated by Yamada
95 Foods (Misato, Akita Prefecture, Japan). Six Natto preparations were
96 obtained, generated by different *B. subtilis* strains. Natto was homogenized
97 with Polytron PT4000 (Kinematica AG, Luzern, Switzerland) and
98 centrifuged at 20,000 x *g* for 15 min at 4°C. Saturated ammonium sulfate
99 was added to the supernatant at a concentration of 30%, and the mixture

was stirred for 30 min at 4°C and centrifuged at 20,000 x *g* for 15 min at 4°C. The supernatant was collected, and then saturated ammonium sulfate was added at a concentration of 50%. The mixture was stirred for 30 min at 4°C and centrifuged at 20,000 x *g* for 15 min at 4°C. The resulting precipitate [30-50% saturated ammonium sulfate precipitated fraction] was dialyzed against 10 mM Tris-HCl (pH 7.4) for 24 h. The resulting material was precipitated with 25% saturated ammonium sulfate at 4°C, and the supernatant was filtered through a 0.45 μm membrane filter. The filtrate was applied to a column of Butyl-Sepharose High Performance (GE Healthcare Life Sciences, Chalfont St Giles, UK) equilibrated with 10 mM Tris-HCl (pH 7.4) containing 25% saturated ammonium sulfate. The column was successively eluted with 25%, 20%, 15%, 10%, 5% and 2% saturated ammonium sulfate in 10 mM Tris-HCl (pH 7.4). The eluent with 10% saturated ammonium sulfate was dialyzed in 10 mM Tris-HCl (pH 7.4), lyophilized, and used as a Natto peptide preparation. The Natto peptide was yielded approximately 1 g from 500 g of Natto.

The purity of the peptide was confirmed by Tris/Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tris/Tricine-SDS-PAGE)

(Schagger and von Jagow, 1987) and Coomassie Brilliant Blue (CBB) staining.

2.2. Amino acid sequence of the Natto peptide

For amino acid sequence determination, the Natto peptide preparation was further purified by column chromatography on Wakosil-5C18HG column (Wako Pure Chemical, Tokyo, Japan). The peptide was eluted with acetonitrile [linear gradient of 0 to 60% (v/v)]. The fraction eluted with approximately 30% acetonitrile was pooled and lyophilized. The resulting material was analyzed. Amino acid sequence of the peptide was determined using the protein sequencer PPSQ-31B/33B (Shimadzu, Kyoto, Japan).

2.3. Bacterial strains

B. subtilis, AHU 1035, AHU 1037, AHU 1604, AHU 1615, AHU 1708^{T} and AHU 1722, *Bacillus cereus* AHU 1358, *Bacillus licheniformis* AHU 1371, *Bacillus megaterium* AHU 1373, and *Bacillus pumilus* AHU 1386 were obtained from the Research Faculty of Agriculture, Hokkaido University

(Sapporo, Japan). *B. cereus* JCM 2152^T, *Streptococcus agalactiae* JCM 5671^T, *Streptococcus mitis* JCM 12971^T, *Streptococcus mutans* JCM 5705^T, *Streptococcus pyogenes* JCM 5674^T, *Streptococcus salivarius* JCM 5707^T, and *Streptococcus sanguinis* JCM 5708^T were obtained from the Japan Collection of Microorganisms, Riken BioResource Center (Tsukuba, Japan). *Acinetobacter baumannii* ATCC 19606, *Escherichia coli* ATCC 25922 and ATCC 35218, *Lactobacillus fermentum* ATCC 9338^T, *Lactobacillus rhamnosus* ATCC 7469^T, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Streptococcus pneumoniae* ATCC 49619, and *Haemophilus influenzae* ATCC 51907 were obtained from the American Type Culture Collection (Manassas, VA). *Lactobacillus plantarum* NRIC 1067^T was obtained from the NODAI Culture Collection Center, Tokyo University of Agriculture (Tokyo, Japan). *A. baumannii* SRAC2, *S. aureus* SUNA1 [hospital-acquired methicillin-resistant *S. aureus* (MRSA)] and SR-581 [community-acquired MRSA], *Enterococcus faecium* M2483, *Enterococcus faecalis* HU1 [vancomycin-resistant *Enterococcus* (VRE)] and M2486, *E. coli* 7249 (ST131), *P, aeruginosa* PA103 and 9728, *Serattia marcescens* SRSM2, *S. pneumoniae* SR11, MH101 [penicillin-resistant *S.*

154 *pneumoniae* (PRSP)], 237 [PRSP] and 442 [mucoid], and *Candida albicans*
155 SRCA1 and SRCA4 were clinical isolates stocked in our laboratory. *S.*
156 *pneumoniae* R6 and P103 [*lytA*-deficient mutant derived from R6 (Δ*lytA*)]
157 were kindly provided by Dr. Ernesto Garcia (Centro de Investigaciones
158 Biológicas, CSIC, Madrid, Spain) (Moscoso et al., 2014).
159
160 *2.4. Minimum inhibitory concentration*
161 Antimicrobial activity was estimated by minimum inhibitory
162 concentration (MIC) determinations using a broth microdilution procedure
163 basically according to a guideline of the Clinical Laboratory Standards
164 Institute (2015). Briefly, cell suspensions (1 x 10^4 cells/mL) and serially
165 diluted solutions of the Natto peptide were mixed in a 96 well microplate,
166 and cultured at 37°C for 24 h. The basal medium was Mueller-Hinton (MH)
167 broth (Becton Dickinson, Franklin Lakes, NJ) or Todd Hewitt (TH) broth
168 (Becton Dickinson) supplemented with 5% (v/v) horse hemolysate (Nippon
169 Bio-Test Laboratories, Tokyo, Japan).
170
171 *2.5. The combined effect of the Natto peptide and other antibacterial*

172 *agents*

173 Cefditoren and tebipenem were provided by Meiji Seika Pharma (Tokyo,
174 Japan). Clarithromycin was provided by Taisho Pharmaceutical (Tokyo,
175 Japan). Amoxicillin, amikacin, tobramycin, and gentamicin were purchased
176 from Wako Pure Chemical. Levofloxacin was purchased from LKT
177 Laboratories (St. Paul, MN). A combined effect was defined according to the
178 fractional inhibitory concentration (FIC) index deduced from MIC values, as
179 follows: synergistic effect, FIC ≤0.5; additive effect, FIC 0.6－1.0; no effect,
180 FIC 1.1－2; and antagonistic effect, FIC >2.

181

182 *2.6. The effect of supplements on the antimicrobial activity of the Natto*
183 *peptide*

184 Hemin was purchased from Wako Pure Chemical. Horse serum was
185 from Thermo Fisher Scientific (Waltham, MA). Heat-treated horse
186 hemolysate was prepared by heating at 100°C for 10 min; various
187 concentrations were suspended in TH broth, and then centrifuged at 9,000 x
188 *g* for 5 min. The supernatant was filtered (0.22 μm) and used in culture
189 media for MIC measurements. The effect on antimicrobial activity was

190 determined by MIC measurements using the broth microdilution method.
191 Briefly, suspensions of *S. pneumoniae* R6 cells (1 x 10^4 cells/mL) in TH broth
192 containing serial dilutions of the Natto peptide and serial dilutions of the
193 supplements were cultured at 37°C for 24 h.
194
195 *2.7. The effect of the Natto peptide on bacterial cell growth and survival*
196 *S. pneumoniae* and *B. subtilis* cells were precultured on TH agar
197 containing 5% (v/v) horse hemolysate at 37°C for 12 h. The cells were then
198 suspended in saline, and used to inoculate TH broth containing 5% (v/v)
199 horse hemolysate at an optical density at 625 nm of 0.000625 (approximately
200 1 x 10^5 cells/mL). The Natto peptide was added to the cell cultures at the
201 time of inoculation or 4 h after the inoculation (approximately 1 x 10^7
202 cells/mL). Cell numbers were determined by plating aliquots of the cultures
203 sampled at various times on TH agar containing 5% (v/v) horse hemolysate,
204 and culturing under microaerophilic conditions; the resulting colonies were
205 then counted.
206
207 *2.8. Morphological observations of the Natto peptide treated cells*

208 *S. pneumoniae* and *B. subtilis* cells (control cells or cells treated with
209 the Natto peptide) were observed by transmission electron microscopy (TEM)
210 after negative staining as previously described (O'Connell Motherway et al.,
211 2011), with minor modifications. The bacterial cells were cultured in TH
212 broth containing 5% (v/v) horse hemolysate at 37°C for 4 h or 8 h. in the
213 presence or absence of the Natto peptide. Cells were fixed with 1% (v/v)
214 glutaraldehyde for 30 min at 4°C. Copper grids (150 mesh; Veco B.V.,
215 Eerbeek, the Netherlands) were floated on the fixed cell suspensions for 10
216 min at room temperature. The grids were washed once for 5 min in 0.02 M
217 glycine-phosphate-buffered saline, and three times for 5 min in ultra-pure
218 water. Negative staining was performed using an EM stainer (Nisshin EM,
219 Tokyo, Japan). The grids were observed using JEOL JEM-1400 TEM (JEOL,
220 Tokyo, Japan) operated at 80 kV.
221
222
223 **3. Results**
224 *3.1. Characterization of the Natto peptide*
225 The Natto peptide was successfully isolated from the active fraction

226 of homogenate of Natto by successive ammonium sulfate precipitation steps
227 and hydrophobic interaction chromatography (Hatakeyama et al., 2016). The
228 resulting peptide migrated as a single band of approximately 5 kDa, as
229 estimated by Tris/Tricine-SDS-PAGE and CBB staining. The Natto peptide
230 consisted of 45 amino acid residues and had the following sequence;
231 NH_2-SMATPHVAGAAALILSKHPTWTNAQVRDRLESTATYLGNSFYYGK-
232 COOH. The sequence shared extremely high homology with a sequence near
233 the C-terminal side of subtilisin family proteins, which are serine proteases
234 produced by *Bacillus* species. High, 97.8%, homology with nattokinase of *B.*
235 *subtilis* (GenBank ACJ48969.1) (Supplemental Fig.) and 80.0% homology
236 with keratinase of *B. licheniformi*s (GenBank AID16241.1) were observed.
237
238 *3.2. Antimicrobial activity of the Natto peptide*
239 The antimicrobial activity of the Natto peptide was determined by
240 MIC measurements with various microorganisms. The MH medium was first
241 used in the assays, according to the CLSI recommendations. The Natto
242 peptide showed antimicrobial activity only *S. pneumoniae* (data not shown).
243 MIC for *S. pneumoniae* was determined using MH broth supplemented with

5% (v/v) hemolysate (CLSI, 2015). Next, MIC values were determined using TH broth, a medium more rich than MH broth, supplemented with 5% (v/v) horse hemolysate (Table 1). The Natto peptide displayed antimicrobial activity against *S. pneumoniae*, including PRSP and mucoid strains, and *B. subtilis*, *B. pumilus*, and *B. licheniformis*, at a concentration of 80 or 160 μg/mL. Lesser activity was observed against strains of *Streptococcu*s species other than *S. pneumoniae* and against *L. fermentum* at a concentration of 640 or 1,280 μg/mL. The Natto peptide did not display any antimicrobial activity against other Gram-positive bacteria, including *B. cereus* and *B. megaterium*, any of the Gram-negative bacteria examined, and *C. albicans*.

Natto peptides obtained from various Natto preparations that were prepared using different soybeans and different *B. subtilis* strains also displayed specific antimicrobial activity against *S. pneumoniae* and *B. subtilis*, however, the activities were different in some instances (Table 2). This indicated that the Natto peptide was typically and commonly present in Natto.

3.3. The combined effect of the Natto peptide and other antimicrobial

agents

Next, the combined effect of the Natto peptide and antimicrobial agents was examined. The tested antimicrobials were aminoglycosides (that injure the cell membrane in addition to inhibition of protein synthesis) and drugs frequently used in pneumococcal infections. The antimicrobial agents examined did not affect (synergistically, additively or antagonistically) to the Natto prptide activity, as assessed by the FIC index analysis (Table 3).

3.4. Hemin is required for antimicrobial activity of the the Natto peptide

Horse hemolysate was required for the occurrence of antimicrobial activity of the Natto peptide in a dose-dependent manner (Table 4). Heat treatment (100°C, 10 min) of horse hemolysate resulted in the aggregation of many proteins present in the hemolysate. The aggregates were removed by centrifugation and filtration. The resulting supernatant retained the ability to support the antimicrobial activity of the Natto peptide. This suggested that heat-stable substance(s) present in the horse hemolysate were required for the antimicrobial activity of the peptide. Horse serum did not alter the antimicrobial activity of the peptide, therefore, blood cell component(s) were

280 subsequently evaluated. Hemin is used as a supplement in MIC
281 measurements with *H. influenzae* as the X factor (White and Granick, 1963).
282 The antimicrobial activity of the Natto peptide against *S. pneumoniae* was
283 supported by hemin in a concentration-dependent manner (Table 4). The
284 amount of hemin required for the occurrence of the antimicrobial activity
285 was comparable with the amount of heme (5-6 mg/L) in horse hemolysate
286 required for the occurrence of the antimicrobial activity.
287
288 *3.5. The effect of the Natto peptide on bacterial cell growth and survival*
289 Survival curves of *S. pneumoniae* and *B. subtilis* cell cultures in the
290 presence or absence of the Natto peptide were examined. The growth of *S.*
291 *pneumoniae* was similar during the early phases of growth in the presence
292 and absence of the Natto peptide (Fig. 1). When high cell numbers were used
293 in the inoculum (approximately 10^7 cells/mL), the cultures reached
294 maximum cell density (approximately 10^8 cells/mL) regardless of the
295 presence of the Natto peptide; the number of viable cells then decreased (Fig.
296 1-A). The rate of the decrease of viable cell numbers was markedly higher in
297 the presence of the Natto peptide than in the absence of the peptide. When

298 low cell numbers were used in the inoculum (approximately 10^5 cells/ml), in
299 the absence of the peptide, the culture reached maximum cell density
300 (approximately 10^8 cells /ml) after 8 h, following which the viable cell
301 numbers decreased. On the other hand, in the presence of the peptide, the
302 culture did not reach maximum cell nombers and the number of viable cells
303 rapidly decreased 2 h after the addition of Natto peptide (Fig. 1-B). These
304 results suggested that *S. pneumoniae* cells initially grew normally in the
305 presence of the Natto peptide; the growth was then terminated and the cells
306 rapidly lysed. The rapid decrease of viable *S. pneumoniae* cells seemed to be
307 associated with autolysis. Consequently, the contribution of LytA, the main *S.*
308 *pneumoniae*-specific autolysin, to the decrease of viable cells in late phase
309 was examined, using the *lytA*-deficient mutant P103. The rate of viable cell
310 number decrease in the presence of the Natto peptide was lower in P103
311 than in the parent strain R6 (Fig. 1-B and C). This indicated that the
312 autolysis caused by LytA enhanced the bactericidal rate, but it was not
313 essential for the antimicrobial activity of the Natto peptide.
314 The survival curves of *B. subtilis* cells also indicated that viable cell
315 numbers rapidly decreased in the presence of the Natto peptide (Fig. 2). The

316 decrease occurred immediately after the addition of the Natto peptide, and
317 seen when both high (approximately 10^7 cells /mL; Fig. 2-A) and low
318 (approximately 10^5 cells /mL; Fig. 2-B) inocula were used. This suggested
319 that the antibacterial action of the Natto peptide differed in *S. pneumoniae*
320 and *B. subtilis*.
321
322 *3.6. Morphological observations of cells treated with the Natto peptide*
323 *S. pneumoniae* cell morphology was examined by TEM after negative
324 staining (Fig. 3). In the absence of the Natto peptide, *S. pneumoniae* cells
325 looked like typical diplococci (Fig. 3-A). The cells formed chain-like structure
326 4 h after the addition of the Natto peptide (Fig. 3-B). These results suggested
327 that the cells failed to separate after cell division in the presence of the
328 peptide. After 8 h of culture, the observed cell lysis was more pronounced in
329 the presence than the absence of the Natto peptide (Fig. 3-C and D).
330 In the case of *B. subtilis* (Fig. 4), localized cell membrane damages
331 and cell swelling were observed 4 h after that Natto peptide addition (Fig. 4
332 C). After 8 h when no viable cells were seen, the cells completely lysed (Fig. 4
333 D).

4. Discussion

Recently, we reported a tumoricidal activity of the Natto extract; approximately 5 kDa peptide was identified as the active component (Hatakeyama et al., 2016). As shown here, the peptide consisted of 45 amino acid residues, and shared extremely high homology with the C-terminal region of a subtilisin family serine protease, Nattokinase produced by *B. subtilis*. The structure of the peptide is probably rich in α-helix deduced from secondary structure predictions and the presence of basic amino acid residues appearing every few residues. Such structural characteristics are found in several types of antimicrobial peptides (Yount et al., 2006). Typically, the characters were found in cathelicidin family antimicrobial peptides (Wang et al., 2014; Xhindoli et al., 2016). The cathelicidin antimicrobial peptides generally have broad spectrum of antimicrobial activity and are effective against multidrug-resistant bacteria (Zanetti et al., 2002; Sang and Blecha, 2008). They also show cytotoxicity toward tumor cell lines, but not toward normal cell lines, similarly to the Natto peptide. Thus, we anticipated

352 that the Natto peptide possesses a broad-spectrum antimicrobial activity
353 with broad spectrum, similarly to the antimicrobial peptides.
354 Unexpectedly, we found a novel antimicrobial activity of the Natto
355 peptide. The spectrum of activity was very narrow, namely, only against *S.*
356 *pneumoniae* and several *Bacillus* strains (Table 1). Little antimicrobial
357 activity was observed against *Streptococcus* spp., including group A (*S.*
358 *pyogenes*) and group B (*S. agalactiae*) streptococci and oral viridans
359 streptococci. The Natto peptide seemed to potentially possess an
360 antimicrobial activity against streptococci, and most pronouncedly against *S.*
361 *pneumoniae*. On the other hand, the Natto peptide showed antimicrobial
362 activity against *B. subtilis*, *B. licheniformis*, and *B. pumilus*, no
363 antimicrobial activity was observed for *B. cereus* and *B. megaterium*. *B.*
364 *subtilis*, *B. pumilus*, and *B. licheniformis* are phylogenetically classified into
365 the *B. subtilis* group (Bhandari et al., 2013; Turenne et al., 2015); *B. cereus*
366 and *B. megaterium* are phylogenetically distant from the *B. subtilis* group.
367 The results indicated that the antimicrobial activity of the Natto peptide was
368 specific for *S. pneumoniae* and the *B. subtilis* group of the genus *Bacillus*.
369 The specific antimicrobial action point(s) of the Natto peptide is very

370 interesting, i.e., the early growth phase after the addition of the peptide.
371 Comparable *S. pneumoniae* cell growth was observed at early time points,
372 regardless of whether the peptide was added. The *S. pneumoniae* cells
373 formed long chains in the presence of the Natto peptide, although *S.*
374 *pneumoniae* is naturally diplococcus as seen in the absence of the peptide
375 (Fig. 3). The results suggested that the Natto peptide caused a failure of cell
376 separation after cell division. At late growth phases, viable cell numbers
377 rapidly decreased in the presence of the peptide (Fig. 1). We proposed that
378 the cells were dead by autolysis. The rapid and pronounced cell death was
379 attributable to LytA, which is a major autolysin specific for *S. pneumoniae*
380 (Maestro and Sanz, 2016); however, LytA was not a direct target of the anti-*S.*
381 *pneumoniae* bactericidal activity of the Natto peptide. Similar long chain
382 morphology of *S. pneumoniae* is found in cells treated with choline (Maestro
383 et al., 2007). Choline interacts with several pneumococcal proteins and
384 affects autolysis and cell division/separation (Maestro and Sanz, 2016).
385 Chain morphology is also observed in a defective mutant of LytB, a
386 peptidoglycan hydrolase contributing to cell separation (De Las Rivas et al.,
387 2002; Bai et al., 2014). However, choline did not affect the antimicrobial

388 activity of the Natto peptide (data not shown).

389 The current results suggest that the Natto peptide might be a
390 fragment of subtilisin family protein, namely Nattokinase. It might be
391 generated by a proteolytic cleavage; however, the responsible protease was
392 not identified. The bactericidal activity against *B. subtilis* was immediately
393 apparent following the addition of the Natto peptide, and the cells died
394 because of a direct membrane injury (Fig. 4). This means that the production
395 of the Natto peptide constitutes a suicide mechanism of *B. subtilis* group
396 bacteria. Its mode of action has not been characterized and should be the
397 focus of future research.

398 Hemolysate or hemin was required for the antimicrobial activity of
399 the Natto peptide against both *S. pneumoniae* and *B. subtilis* group strains
400 (Table 4). Heme/hemin could alter the secondary structure of the peptide;
401 however, further examination is necessary to verify this hypothesis.

402 In conclusion, we identified a unique antimicrobial peptide with a
403 narrow spectrum of activity in Natto extract. *S. pneumoniae* has great
404 impact in the clinic. It causes acute otitis media and sinusitis mainly in
405 children, community-acquired pneumonia mainly in elders, and meningitis

406 as an invasive infection (Hamborsky et al., 2015). Vaccines against *S.*
407 *pneumoniae* are used for the prevention of invasive infection in children and
408 community-acquired pneumonia in elders. Furtermore, antimicrobial
409 resistant *S. pneumoniae*, such as PRSP, are increasing, and their treatment
410 is now problematic. *S. pneumoniae*-specific antimicrobials would be
411 promising therapeutic agents, as such agents would not disturb the normal
412 flora; however, peptides are usually difficult to employ as therapeutic agents
413 because of their potentially low stability and bioavailability. Thus the
414 molecular mechanisms of the anti-pneumococcal activity of the Natto peptide
415 should be evaluated for application in therapeutic agents for *S. pneumoniae*
416 infection.
417
418
419 **Acknowledgements**
420 This study was supported, in part, by a Grant-in-Aid for Scientific Research
421 (15K08103) from the Japan Society for the Promotion of Science.
422
423 **Competing Interests**

The authors declare no competing interests.

References

Bai, X.H., Chen, H.J., Jiang, Y.L., Wen, Z., Huang, Y., Cheng, W., Li, Q., Qi, L., Zhang, J.R., Chen, Y., Zhou, C.Z., 2014. Structure of pneumococcal peptidoglycan hydrolase LytB reveals insights into the bacterial cell wall remodeling and pathogenesis. J Biol Chem 289, 23403-23416.

Bhandari, V., Ahmod, N.Z., Shah, H.N., Gupta, R.S., 2013. Molecular signatures for *Bacillus* species: demarcation of the *Bacillus subtilis* and *Bacillus cereus* clades in molecular terms and proposal to limit the placement of new species into the genus *Bacillus*. Int J Syst Evol Microbiol 63, 2712-2726.

Clinical and Laboratory Standards Institute, 2015. M100-S25. Performance standards for antimicrobial susceptibility testing; 25th informational supplement. Clinical and Laboratory Standards Institute, Wayne, PA.

Dabbagh, F., Negahdaripour, M., Berenjian, A., Behfar, A., Mohammadi, F., Zamani, M., Irajie, C., Ghasemi, Y., 2014. Nattokinase: production and

444 application. Appl Microbiol Biotechnol 98, 9199-9206.

445

446 De Las Rivas, B., Garcia, J.L., Lopez, R., Garcia, P., 2002. Purification and

447 polar localization of pneumococcal LytB, a putative

448 endo-β-N-acetylglucosaminidase: the chain-dispersing murein hydrolase. J

449 Bacteriol 184, 4988-5000.

450

451 Hamborsky, J., Kroger, A., Wolfe, S., 2015. Pneumococcal disease, in:

452 Hamborsky, J., Kroger, A., Wolfe, S. (Eds.), Centers for Disease Control and

453 Prevention. Epidemiology and prevention of vaccine-preventable diseases.

454 13th ed. Public Health Foundation, Washington D.C., pp. 279-295.

455

456 Hatakeyama, S., Kafuku, M., Okamoto, T., Kakizaki, A., Shimasaki, N., N.,

457 F., Takahashi, S., Nakayama, M., Tagawa, H., Komatsuda, A., Grave, E.,

458 Wakui, H., Itoh, H., 2016. Studies on the anticancer mechanisms of the Natto

459 extract. J Soc Mater Eng Resour Jpn 27, 15-19 (in Japanese).

460

461 Maestro, B., Gonzalez, A., Garcia, P., Sanz, J.M., 2007. Inhibition of

462 pneumococcal choline-binding proteins and cell growth by esters of bicyclic
463 amines. FEBS J 274, 364-376.
464
465 Maestro, B., Sanz, J.M., 2016. Choline binding proteins from *Streptococcus*
466 *pneumoniae*: A dual role as enzybiotics and targets for the design of new
467 antimicrobials. Antibiotics 5, 21.
468
469 Mattar, E.H., Almehdar, H.A., Yacoub, H.A., Uversky, V.N., Redwan, E.M.,
470 2016. Antimicrobial potentials and structural disorder of human and animal
471 defensins. Cytokine Growth Factor Rev 28, 95-111.
472
473 Moscoso, M., Esteban-Torres, M., Menendez, M., Garcia, E., 2014. *In vitro*
474 bactericidal and bacteriolytic activity of ceragenin CSA-13 against
475 planktonic cultures and biofilms of *Streptococcus pneumoniae* and other
476 pathogenic streptococci. PLoS One 9, e101037.
477
478 Murrel, W.G., Warth, A.D., 1965. Composition and heat resistance of
479 bacterial spores, in: Campbell, L.L., Halvorson, H.O. (Eds.), Spores III.

480 American Society for Microbiology, Washington, D.C., pp. 1-24.

481

482 Nicolosi, R.J., Wilson, T.A., Lawton, C., Handelman, G.J., 2001. Dietary
483 effects on cardiovascular disease risk factors: beyond saturated fatty acids
484 and cholesterol. J Am Coll Nutr 20, 421S-427S.

485

486 O'Connell Motherway, M., Zomer, A., Leahy, S.C., Reunanen, J., Bottacini, F.,
487 Claesson, M.J., O'Brien, F., Flynn, K., Casey, P.G., Munoz, J.A., Kearney, B.,
488 Houston, A.M., O'Mahony, C., Higgins, D.G., Shanahan, F., Palva, A., de Vos,
489 W.M., Fitzgerald, G.F., Ventura, M., O'Toole, P.W., van Sinderen, D., 2011.
490 Functional genome analysis of *Bifidobacterium breve* UCC2003 reveals type
491 IVb tight adherence (Tad) pili as an essential and conserved
492 host-colonization factor. Proc Natl Acad Sci USA 108, 11217-11222.

493

494 Sakano, T., Notsumoto, S., Nagaoka, T., Morimoto, A., Fujimoto, K., Masuda,
495 S., Suzuki, Y., Hirauchi, K., 1988. Measurement of K vitamins in food by
496 high-performance liquid chromatography with fluorometric detection.
497 Vitamins (Tokyo) 62, 393-398 (in Japanese).

498

499 Sang, Y., Blecha, F., 2008. Antimicrobial peptides and bacteriocins:

500 alternatives to traditional antibiotics. Anim Health Res Rev 9, 227-235.

501

502 Schagger, H., von Jagow, G., 1987. Tricine-sodium dodecyl

503 sulfate-polyacrylamide gel electrophoresis for the separation of proteins in

504 the range from 1 to 100 kDa. Anal Biochem 166, 368-379.

505

506 Suarez-Carmona, M., Hubert, P., Delvenne, P., Herfs, M., 2015. Defensins:

507 "Simple" antimicrobial peptides or broad-spectrum molecules? Cytokine

508 Growth Factor Rev 26, 361-370.

509

510 Sumi, H., Hamada, H., Nakanishi, K., Hiratani, H., 1990. Enhancement of

511 the fibrinolytic activity in plasma by oral administration of nattokinase. Acta

512 Haematol 84, 139-143.

513

514 Sumi, H., Ohsugi, T., 1999. Anti-bacterial component dipicolic acid measured

515 in natto and natto bacilli. Nippon Nogeikagaku Kaishi 73, 1289-1291 (in

516 Japanese).

517

518 Turenne, C.T., Snyder, J.W., Alexander, D.C., 2015. *Bacillus* and other

519 aerobic endspore-forming bacteria, in: Carroll, K.C., Funke, G., Landry, M.L.,

520 Richter, S.S., Warnock, D.W. (Eds.), Manual of Clinical Microbiology, 11th

521 edition. ASM Press, Washington, DC.

522

523 Wang, G., Mishra, B., Epand, R.F., Epand, R.M., 2014. High-quality 3D

524 structures shine light on antibacterial, anti-biofilm and antiviral activities of

525 human cathelicidin LL-37 and its fragments. Biochim Biophys Acta 1838,

526 2160-2172.

527

528 White, D.C., Granick, S., 1963. Hemin biosynthesis in *Haemophilus*. J

529 Bacteriol 85, 842-850.

530

531 Xhindoli, D., Pacor, S., Benincasa, M., Scocchi, M., Gennaro, R., Tossi, A.,

532 2016. The human cathelicidin LL-37-A pore-forming antibacterial peptide

533 and host-cell modulator. Biochim Biophys Acta 1858, 546-566.

534

535 Yount, N.Y., Bayer, A.S., Xiong, Y.Q., Yeaman, M.R., 2006. Advances in

536 antimicrobial peptide immunobiology. Biopolymers 84, 435-458.

537

538 Zaheer, K., Akhtar, M.H., 2015. An updated review of dietary isoflavones:

539 Nutrition, processing, bioavailability and impacts on human health. Crit Rev

540 Food Sci Nutr, doi: 10.1080/10408398.2014.989958.

541

542 Zanetti, M., Gennaro, R., Skerlavaj, B., Tomasinsig, L., Circo, R., 2002.

543 Cathelicidin peptides as candidates for a novel class of antimicrobials. Curr

544 Pharm Des 8, 779-793.

545

546

547 Table 1. MICs of the Natto-peptide tested against various Gram-positive

548 bacteria, Gram-negative bacteria, and fungi

549

Strain	MIC (μg/mL)
Gram-positive bacteria	
B. cereus JCM 2152^T	>1,280
B. cereus AHU 1358	>1,280
B. licheniformis AHU 1371	80
B. megaterium AHU 1373	>1,280
B. pumilus AHU 1386	80
B. subtilis AHU 1708^T	80
B. subtilis AHU 1035	160
B. subtilis AHU 1037	80
B. subtilis AHU 1604	80
B. subtilis AHU 1615	160
B. subtilis AHU 1722	160
E. faecalis HU1 (VRE)	>1,280
E. faecalis M2486	>1,280
E. faecium M2483	>1,280
L. fermentum ATCC 9338^T	640
L. plantarum NRIC 1067^T	>1,280
L. rhamnosus ATCC 7469^T	>1,280
S. aureus ATCC 25923	>1,280
S. aureus SUNA1 (HA-MRSA)	>1,280
S. aureus SR-581(CA-MRSA)	>1,280

S. agalactiae JCM 5671^T	640
S. mitis JCM 12971^T	640
S. mutans JCM 5705^T	640
S. pneumoniae ATCC 49619	80
S. pneumoniae R6	80
S. pneumoniae P103 (Δ*lytA*)	80
S. pneumoniae SR11	160
S. pneumoniae MH101 (PRSP)	160
S. pneumoniae 237 (PRSP)	80
S. pneumoniae 442 (mucoid)	80
S. pyogenes JCM 5674^T	1,280
S. salivarius JCM 5707^T	1,280
S. sanguinis JCM 5708^T	640
Gram-negative bacteria	
A. baumanni ATCC 19606	>1,280
A. baumanni SRLAC2	>1,280
E. coli ATCC 25922	>1,280
E. coli ATCC 35218	>1,280
E. coli 7249 ST131	>1,280
H. influenza ATCC 51907	>1,280
P. aeruginosa ATCC 27853	>1,280
P. aeruginosa PA103	>1,280
P. aeruginosa 9728 (mucoid)	>1,280
S. marcescens SRSM2	>1,280
Fungi	
C. albicans SRCA1	>1,280

C. albicans SRCA4	>1,280

550

551 Table 2. Antimicrobial activity of the Natto peptide from various Natto

552 preparations obtained using different soybeans and *B. subtilis* strains

553

Natto preparation	MIC(μg/mL)	
	S. pneumoniae R6	*B. subtilis* AHU 1708^{T}
160	640	1,280
170	80	160
180	80	160
200	80	80
300	320	320

554

555

556 Table 3. Synergistic antimicrobial effect (FIC index) of the Natto peptide and

557 other antimicrobial agents

558

Antibiotic	FIC index
Amoxicillin	2
Cefditoren	2
Tebipenem	2
Levofloxacin	2
Clarithromycin	1.38
Amikacin	1.09
Tobramycin	2
Gentamicin	2

559

560

561 Table 4. The effect of medium supplemented with horse hemolysate on the.

562 MIC of the Natto peptide with *S. pneumoniae* R6 strain

563

Supplement	Concentration	MIC（μg/mL）
None	-	>1,280
Horse hemolysate	5%*	80
	1%	40
	0.32%	40
	0.10%	80
	0.032%	320
	0.010%	640
	0.0032%	1,280
	0.0010%	>1,280
Supernatant of heat-treated horse hemolysate	20%	40
	5%	320
Horse serum	5%	>1,280
Hemin	6.9 μg/mL	80
	3.4 μg/mL	320
	1.7 μg/mL	640
	0.86 μg/mL	1,280
	0.43 μg/mL	>1,280

564

565 *The concentrations are (v/v).

566

567 Figure Legends;

568

569 Fig. 1. Survival curves of *S. pneumoniae* strains in the presence and absence

570 of the Natto peptide.

571 A: *S. pneumoniae* R6 cells were pre-cultured to a cell density of

572 approximately 10^7 cells/mL in TH broth containing 5% (v/v) horse

573 hemolysate. The Natto peptide was then added at a concentration of 640

574 μg/mL.

575 B and C: *S. pneumoniae* R6 (B) and P103 (*lytA*-deficient mutant derived

576 from R6) (C) cells were suspended at a density of approximately 10^5 cells/mL

577 in TH broth containing 5% (v/v) horse hemolysate. The Natto peptide was

578 then added at a concentration of 640 μg/mL.

579 Throughout the cultivation, the cultures were sampled, and culture aliquots

580 were spread on a TH agar supplemented with 5% (v/v) horse hemolysate, and

581 cultured for 24 h. Grown colonies were then counted. Dashed lines indicate

582 cell numbers in the presence of the Natto peptide. Solid lines indicate cell

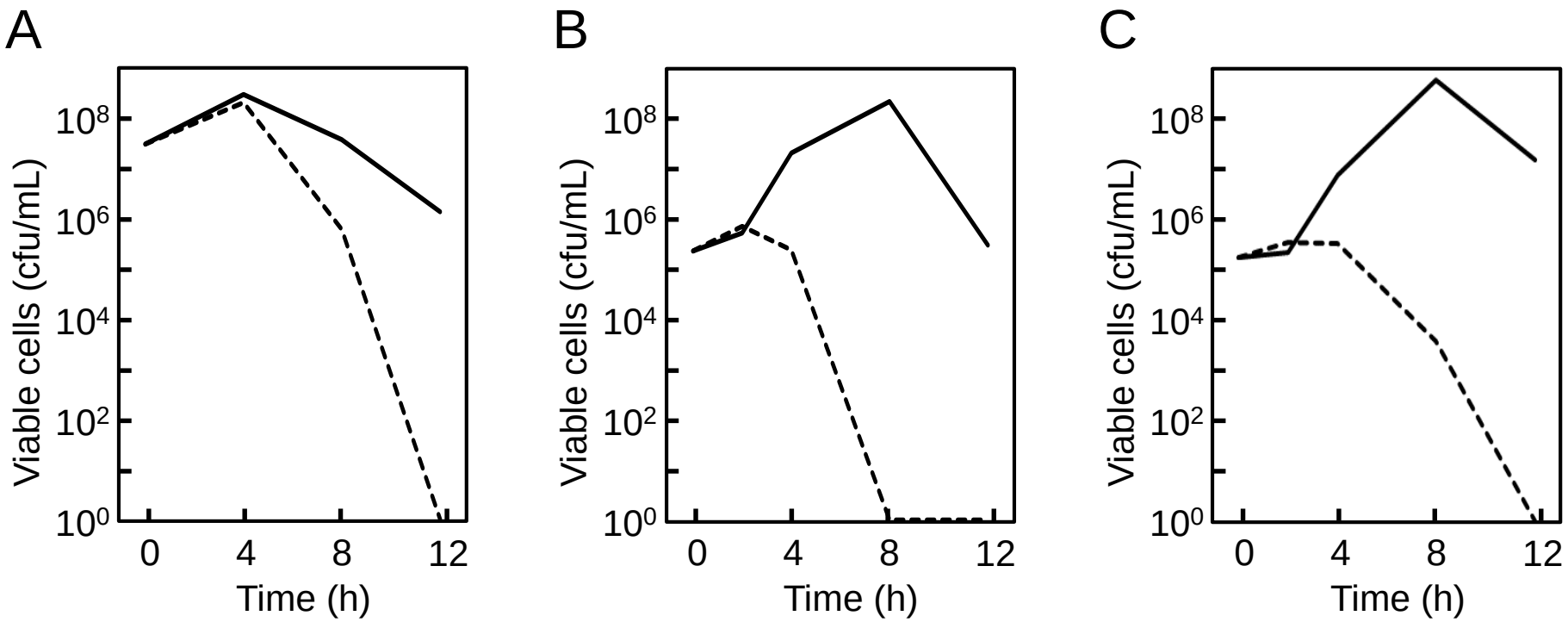
583 numbers in the absence of the Natto peptide.

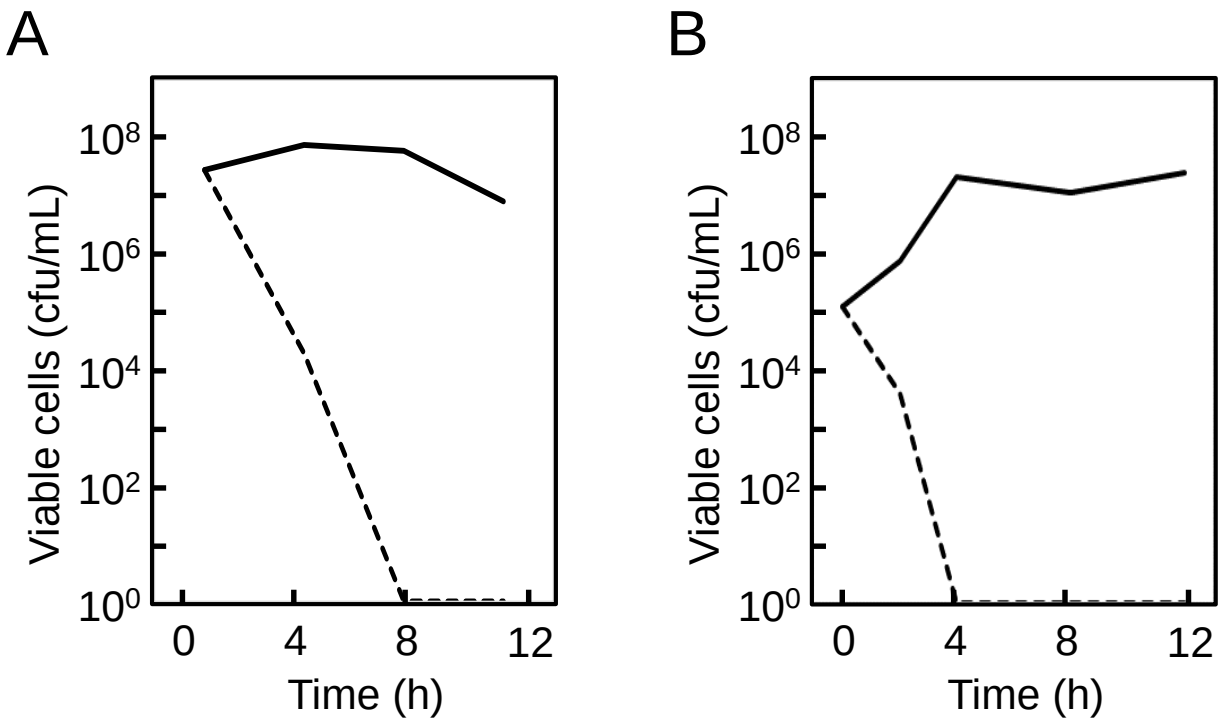
584

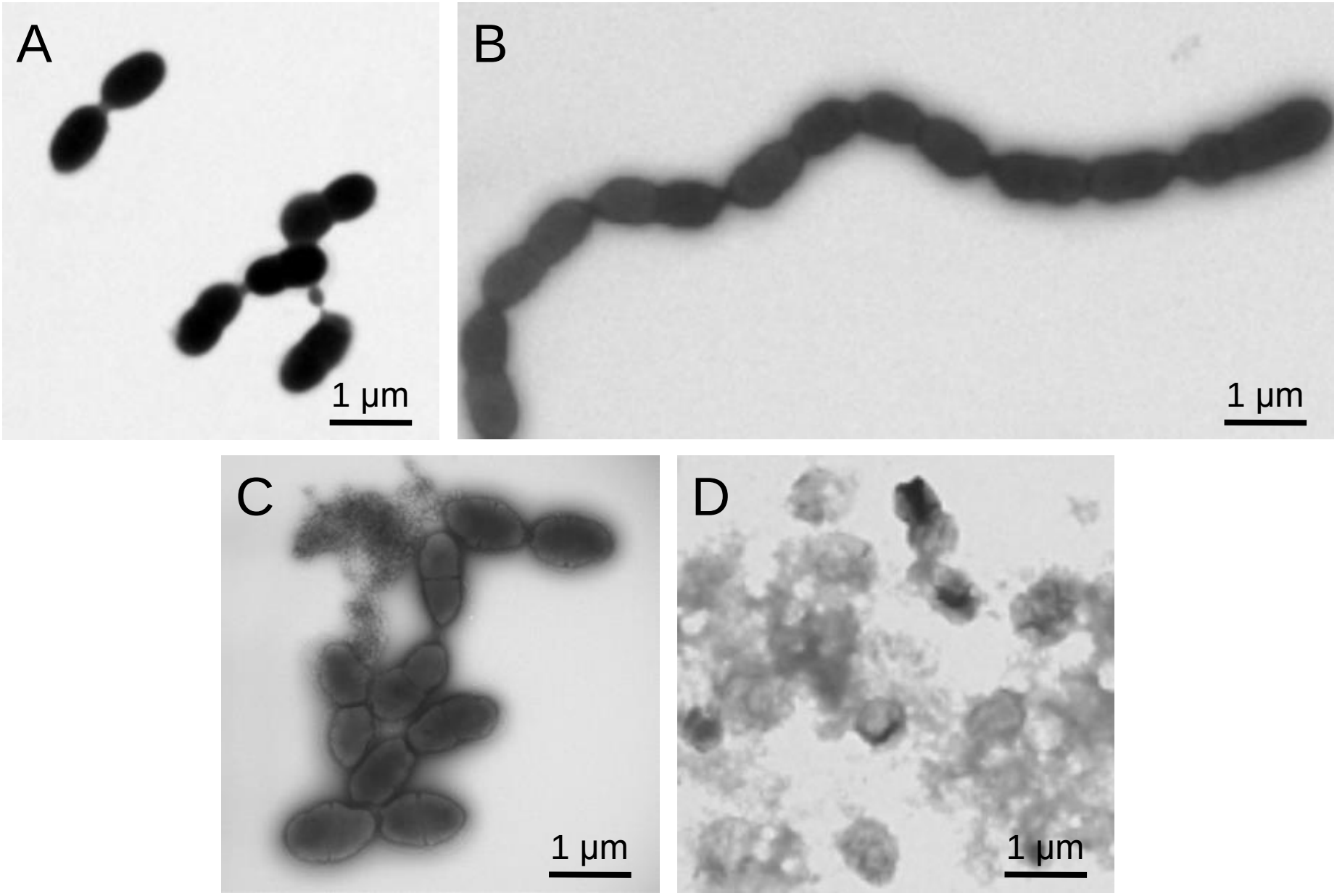
585

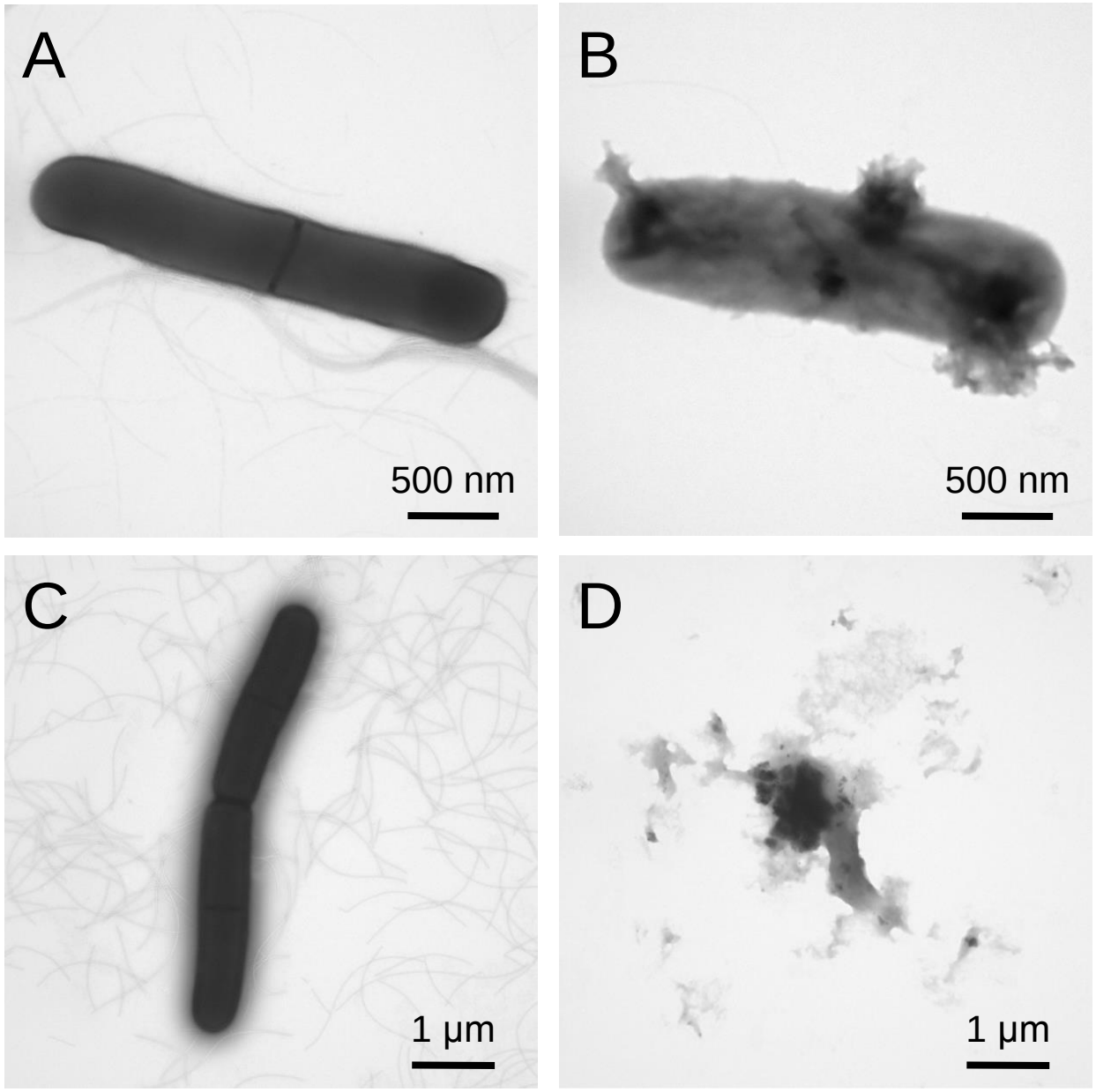
586 Fig. 2. Survival curves of *B. subtilis* strain in the presence and absence of the
587 Natto peptide.
588 A: *B. subtilis* AHU 1708^{T} cells were pre-cultured to a cell density of
589 approximately 10^{7} cells/mL in TH broth containing 5% (v/v) horse
590 hemolysate. The Natto peptide was then added at a concentration of 640
591 μg/mL.
592 B: *B. subtilis* AHU 1708^{T} cells were suspended at a cell density of
593 approximately 10^{5} cells/mL in TH broth containing 5% (v/v) horse
594 hemolysate. The Natto peptide was then added at a concentration of 640
595 μg/mL, and the cells were cultured.
596 Throughout the cultivation, the cultures were sampled, and culture aliquots
597 were spread on TH agar supplemented with 5% (v/v) horse hemolysate, and
598 cultured for 24 h. Grown colonies were then counted. Dashed lines indicate
599 cell numbers in the presence of the Natto peptide. Solid lines indicate cell
600 numbers in the absence of the Natto peptide.
601
602

603 Fig. 3. TEM analysis with negative staining of *S. pneumoniae* R6 cells
604 cultured in the presence or absence of the Natto peptide.
605 The cells were cultured in TH broth containing 5% (v/v) horse hemolysate in
606 the absence (A and C) or presence (B and D) of the Natto peptide (640 μg/mL).
607 After 4 h (A and B) or 8 h (C and D) of incubation, the cells were harvested
608 and observed TEM after negative staining.
609
610
611 Fig. 4. TEM analysis with negative staining of *B. subtilis* AHU 1708^{T} cells
612 cultured in the presence or absence of the Natto peptide.
613 The cells were cultured in TH broth containing 5% (v/v) hemolysate in the
614 absence (A and C) or presence (B and D) of the Natto peptide (640 μg/mL).
615 After 4 h (A and B) or 8 h (B and D) of incubation, the cells were harvested
616 and observed using TEM after negative staining.
617


A
B
C
Viable cells (cfu/mL)
10^8
10^6
10^4
10^2
10^0
0
4
8
12
Time (h)


A
B
Viable cells (cfu/mL)
10^8
10^6
10^4
10^2
10^0
0
4
8
12
Time (h)


A
1 μm
B
1 μm
C
1 μm
D
1 μm


A
500 nm
B
500 nm
C
1 μm
D
1 μm

```
            10         20         30         40         50
NK   MAFSNMSAQA AGKSSTEKKY IVGFKQTMSA MSSAKKKDVI SEKGGKVQKQ
NP   ---------- ---------- ---------- ---------- ----------

            60         70         80         90        100
NK   FKYVNAAAAT LDEKAVKELK KDPSVAYVEE DHIAHEYAQS VPYGISQIKA
NP   ---------- ---------- ---------- ---------- ----------

           110        120        130        140        150
NK   PALHSQGYTG SNVKVAVIDS GIDSSHPDLN VRGGASFVPS ETNPYQDGSS
NP   ---------- ---------- ---------- ---------- ----------

           160        170        180        190        200
NK   HGTHVAGTIA ALNNSIGVLG VAPSASLYAV KVLDSTGSGQ YSWIINGIEW
NP   ---------- ---------- ---------- ---------- ----------

           210        220        230        240        250
NK   AISNNMDVIN MSLGGPTGST ALKTVVDKAV SSGIVVAAAA GNEGSSGSTS
NP   ---------- ---------- ---------- ---------- ----------

           260        270        280        290        300
NK   TVGYPAKYPS TIAVGAVNSS NQRASFSSAG SELDVMAPGV SIQSTLPGGT
NP   ---------- ---------- ---------- ---------- ----------

           310        320        330        340        350
NK   YGAYNGTSMA TPHVAGAAAL ILSKHPTWTN AQVRDRLEST ATYLGSSFYY
NP   -------SMA TPHVAGAAAL ILSKHPTWTN AQVRDRLEST ATYLGNSFYY

           360
NK   GKGLINVQAA AH
NP   GK
```

Supplement Figure. Comparison of amino acid sequence of nattokinase (NK) and Natto peptide (NP).

The amino acid sequence of NK is cited from ACJ48969.1.