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Pyloricidins, Novel Anti-*Helicobacter pylori* Antibiotics Produced by *Bacillus* sp.

I. Taxonomy, Fermentation and Biological Activity

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Novel anti-*Helicobacter pylori* antibiotics, pyloricidins A, A_1, A_2, B, C and D, were discovered in the culture broth of two bacilli strains. Pyloricidins selectively inhibited the growth of *H. pylori*. Pyloricidin B was efficacious in the treatment of gastric infection caused by *H. pylori* in Mongolian gerbils and may be promising for cure of *H. pylori* infection as a single agent.

It has widely been recognized that *Helicobacter pylori* is an etiologic agent for chronic active gastritis, and its sustained infection is closely associated with the recurrence of peptic ulcer and the provocation of gastric cancer[1~3]. Because of clinical evidence that eradication of *H. pylori* results in a significant reduction of ulcer relapse, cure of *H. pylori* infection has become an important subject in the field of gastroenterology[4]. Various antimicrobial agents have been shown to exert activity against *H. pylori in vitro*[5,6], but clinical trials with any of these agents failed to eradicate this pathogen when given as monotherapy[7].

One of the reasons for incomplete eradication of *H. pylori* may be the degradation of antimicrobial agents such as amoxicillin and clarithromycin by gastric acid[8]. In an effort to overcome this problem, concomitant administration of antimicrobial agents and drugs which inhibit gastric acid secretion such as H_2 receptor antagonists and proton pump inhibitors has been introduced with improved efficacy[9,10]. However, the presence of strains resistant to metronidazole and clarithromycin and relatively high incidence of side effects which come from disturbance of gastrointestinal microflora make these regimens far from ideal. In response to the needs for safer and more efficacious stratagems for *H. pylori* infection, we searched for agents with specific activity and promising efficacy among secondary metabolites of microbial origin.

In the course of screening for selective antibiotics against *H. pylori*, we have isolated novel antibiotic pyloricidins (Fig. 1) from culture broths of two bacterial strains belonging to the genus *Bacillus* isolated from soil samples. Pyloricidins were found to have a selective antibacterial activity against *H. pylori*.

In this report, we describe the taxonomy of the producing strains, fermentation, and biological activities of pyloricidins. Isolation from fermentation broth and structure elucidation with absolute configuration of pyloricidins will be reported in the succeeding paper[11].

Materials and Methods

Materials

Brucella broth, Brucella agar, Gas Pak jar and CampyPak were purchased from BBL Becton Dickinson Microbiology

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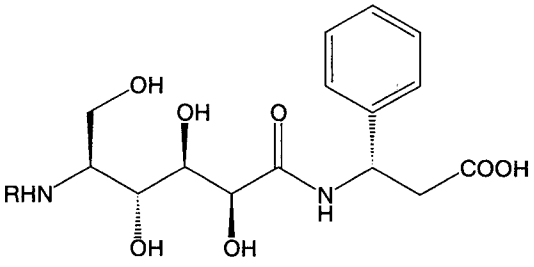
Fig. 1. Structures of pyloricidins.



Pyloricidin A	R=	H-L-Val-L-Val-L-Leu-
Pyloricidin A1	R=	H-L-Val-L-Ile-L-Leu-
Pyloricidin A2	R=	H-L-Val-L-Leu-L-Leu-
Pyloricidin B	R=	H-L-Val-L-Leu-
Pyloricidin C	R=	H-L-Leu-
Pyloricidin D	R=	H-

Systems. GAM Agar Modified was purchased from Nissui Seiyaku Co. Ltd. Fetal bovine serum (FBS) and defibrinated horse whole blood are purchased from BIO WHITTAKER, Maryland and NIPPON BIO-SUPP. CENTER, Tokyo, respectively. Antibiotics medium 3, Yeast Nitrogen Base, Tryptone, yeast extract, and Bacto-oatmeal agar were purchased from DIFCO. Actcol and Silicone were purchased from Takeda Chemical Industries and Shin-Etsu Chemical, respectively. L-[^{35}S]Methionine, filtration plates and scintillator A were purchased from Amersham Pharmacia Biotech UK Ltd., Millipore and Wako Pure Chemical Ltd., respectively.

Microorganisms

H. pylori strain NCTC11637 and strain TN2GF4 were used for *in vitro* test and for *in vivo* test, respectively. The laboratory standard strains of common Gram-positive and Gram-negative bacteria, yeast and anaerobic bacteria were obtained from our collection.

Taxonomic Studies

Morphological and physiological characteristics were determined as described by CLAUS and BERKELEY[12]. Isoprenoid quinones were extracted from freeze-dried cells with chloroform-methanol (2:1, vol/vol) and analyzed by high-performance liquid chromatography (HPLC)[13]. Cellular fatty acids were prepared as described by KOMAGATA and SUZUKI[14]. The fatty acid methyl ester composition was determined by gas-chromatography-mass spectrometry. Diaminopimelic acid in the cell wall was determined by the method of HASEGAWA *et al.*[15]. Guanine plus cytosine (G+C) content of the DNA was determined by the method of MESBAH *et al.*[16].

Fermentation Procedure

A loopful of *Bacillus* sp. HC-70 cells grown on an agar slant at 24°C for 2 days was inoculated into 500 ml of IB medium in a 2000 ml Sakaguchi flask. Agar slant medium (AG medium) was composed of (per liter) 5 g Tryptone, 2.5 g yeast extract, 1 g glucose and 15 g agar. IB medium contained (per liter) 20 g glucose, 30 g soluble starch, 10 g soybean flour, 3 g corn steep liquor, 5 g peptone, 1 g yeast extract, 2 g Bacto-oatmeal agar, 3 g NaCl and 5 g $CaCO_3$. After aerobic cultivation at 28°C for 1 day with reciprocal shaking at 80 spm, culture broth was transferred to 120 liters MA medium in a 200-liter fermentor. MA medium contained (per liter) 50 g dextrin, 5 g glucose, 35 g soybean meal, 5 g yeast extract, 7 g $CaCO_3$, 0.5 g Actcol and 0.5 g Silicone. Fermentation was carried out for 72 hours under the following conditions: temperature, 22°C; agitation rate, 120 rpm; aeration, 1.0 vvm. In the case of *Bacillus* sp. HC-72, a loopful of cells grown on an agar slant was inoculated into 500 ml of IB medium in a 2000 ml Sakaguchi flask. Culture conditions are the same as that of *Bacillus* sp. HC-70. Culture broth was transferred into a 200-liter fermentor containing 120 liters SD medium, which was composed of 20 g glucose, 30 g soluble starch, 10 g soybean flour, 3 g corn steep liquor, 5 g peptone, 1 g yeast extract, 3 g NaCl, 5 g $CaCO_3$, 0.5 g Actcol and 0.5 g Silicone (per liter; pH was adjusted to 7.0 with NaOH). Cultivation was carried out at 24°C for 2 days under the conditions of agitation rate; 120 rpm and aeration; 1.0 vvm. A 10-liter portion of the culture broth was transferred to 1200 liters MB medium in a 2000-liter fermentor. MB medium contained (per liter) 30 g soluble starch, 30 g soybean meal, 5 g corn steep liquor, 3 g NaCl, 5 g $CaCO_3$, 0.5 g Actcol and 0.5 g Silicone (pH 7.0). Fermentation was carried out for 90 hours under the following conditions: temperature, 28°C; agitation rate, 120 rpm; aeration, 0.7 vvm.

Susceptibility Testing

The MICs for *H. pylori* were determined by an agar dilution method. Bacterial suspensions of approximately 10^6 CFU/ml, as prepared with Brucella broth supplemented with 2.5% FBS, were applied to the Brucella agar plates supplemented with 7% defibrinated horse blood containing two-fold serial dilutions of test compounds using a multiple inoculator capable of delivering 5 μl samples. The plates

were incubated at 37°C for 2~3 days in a microaerobic atmosphere consisting of 5% O_2, 10% CO_2, and 85% N_2. MICs for common aerobic and anaerobic bacteria and yeasts were also determined by the agar dilution method. Antibiotic medium 3 supplemented with 0.5% yeast extract and 1.5% agar for aerobic bacteria, GAM Agar Modified for anaerobic bacteria and Yeast Nitrogen Base supplemented with 1.0% glucose and 1.5 % agar for yeasts were used. The inocula were prepared in Antibiotics medium 3 supplemented with 0.5% yeast extract for aerobic bacteria, in GAM broth for anaerobic bacteria and on Yeast Nitrogen Base agar slant for yeasts from 1 day culture and adjusted to give approximately 10^6 CFU/ml. Plates were incubated at 37°C for 1 day in an aerobic atmosphere for aerobic bacteria, in an anaerobic glove box for anaerobic bacteria and at 30°C for 1 day in an aerobic atmosphere for yeasts. MICs were defined as the lowest concentrations for the compounds preventing visible microbial growth.

Assay of Incorporation of L-Amino Acid into Protein

H. pylori NCTC 11637 cells adjusted to the density of OD_{660}=0.290~0.309 were preincubated with varying concentrations of pyloricidin B in Brucella broth supplemented with 2.5% FBS at 37°C in microaerophilic atmosphere under constant horizontal rotation (120 rpm) for 3 hours. Cells were deprived of L-methionine by preparing suspensions of the bacteria in methionine-free chemically defined medium[17,18] and incubating the suspensions at room temperature in microaerophilic atmosphere under constant horizontal rotation (80 rpm) for 90 minutes. Then the pyloricidin B treated cells were labeled with L-[^{35}S]methionine (10 mCi/ml) at 37°C in a 10% CO_2 humidified atmosphere for 10 minutes. The reaction was stopped by adding ice-cold 5% trichloroacetic acid. The precipitates were collected on filtration plates and counted in a liquid scintillation counter (Win Spectra™ α/β 1414, WALLAC) by adding scintillator A. The sample was measured in triplicate.

Gastric Infection in Mongolian Gerbils Caused by *H. pylori*

Five-week-old MON/Jms/Gbs Slc Mongolian gerbils (SLC Japan Inc., Shizuoka, Japan) were inoculated intragastrically with $10^{7.61}$ CFU of *H. pylori* TN2GF4. Two weeks after infection, pyloricidin B, suspended in 0.5% aqueous solution of methylcellulose, was given orally twice daily for 7 days. The animals were killed on the day after the final treatment. Stomachs were aseptically removed and were homogenized with 3 ml of Brucella broth, and the bacterial counts in the homogenates were determined by serial dilution and titration on modified Skirrow's plates. The plates were incubated at 37°C for 4 days in a microaerobic atmosphere prior to counting. No detectable *H. pylori* in the stomach on the day after final treatment was defined as clearance. All animal experiments were conducted in accordance with the Guideline for the Care and Use of Laboratory Animals in Pharmaceutical Research Division, Takeda Chemical Industries, Ltd., and approved by the Ethical Committee for the animal experiments of our division.

Results

Taxonomy of Producing Strains HC-70 and HC-72

Strain HC-70 was isolated from a soil sample collected in Nara prefecture, Japan, and strain HC-72 was isolated from a soil sample collected in Aichi prefecture, Japan. Table 1 shows the morphological, biochemical and chemotaxonomic characteristics of these microorganisms.

The characteristics shown in Table 1 indicate that the strain HC-70 is a facultative anaerobe and belongs to *Bacillus cereus-megaterium* group. However, the strain can be differentiated phenotypically from the previously described species including said group. The strain HC-72 is strictly aerobic and closely related to *Bacillus insolitus* from the data shown in Table 1. The strain may be differentiated from *B. insolitus* on the range of temperature for growth. For identification of these strains at the species level, it is required essentially to clarify the level of DNA relatedness between these strains and previously described *Bacillus* species. These producing strains were designated tentatively as *Bacillus* sp. HC-70 and *Bacillus* sp. HC-72, respectively.

Fermentation

Pyloricidins A, A_1, A_2, B and C were obtained from a fermentation broth of *Bacillus* sp. HC-70, and pyloricidins C and D were obtained from a fermentation broth of *Bacillus* sp. HC-72.

Time course of pyloricidins A, B and C production by strain HC-70 is shown in Fig. 2A. As shown in Fig. 2A, pyloricidin A was accumulated in an early stage of the fermentation. Then, the amount of pyloricidin A accumulated was gradually reduced, followed by increase of pyloricidins B and C. Pyloricidin D was not observed in the culture broth all through the fermentation.

In the case of strain HC-72, accumulation of pyloricidins

Table 1. Morphological, physiological and chemotaxonomic characteristics of strains HC-70 and HC-72.

	HC-70	HC-72
Cells: Width of rod, μm	1.3-1.4	1.3
Length of rod, μm	3.0-4.2	3.0-4.2
Motility	+	+
Spore shape	Ellipsoidal	Round
Sporangia	Not swollen	Not swollen
Parasporal crystal	-	-
Colonies: Pigmentation	Gray	Whitish yellow
Rhizoid colony	-	-
Gram-stain	+	+
Catalase	+	+
Hydrolysis of: Starch	+	-
Casein	+	w
Gelatin	+	-
Reduction of nitrate to nitrite	-	-
Deamination of phenylalanine	+	w
Degradation of tyrosine	-	-
Voges-Proskauer test	+	-
Acid from: D-Glucose	+	-
L-Arabinose	-	-
D-Xylose	-	-
D-Fructose	+	-
Anaerobic growth	+	-
NaCl required	-	-
Organic growth factors required	-	-
Growth at: 5°C	+	-
15°C	+	+
30°C	+	+
Major isoprenoid quinone	MK-7	MK-7
meso-DAP in cell wall	+	-
Major cellular fatty acids	i-C15:0	i-C15:0
	a-C17:0	a-C16:1
	i-C13:0	a-C17:1
	C16:0	
G+C content of DNA, mol%	35.0	36.8

Abbreviations: +, positive; -, negative; w, weakly positive; MK-7, menaquinone 7; meso-DAP, meso-diaminopimelic acid; i-C13:0, 11-methyldodecanoic acid; i-C15:0, 13-methyltetradecanoic acid; C16:0, hexadecanoic acid; a-C16:1, 13-methylpentadecenoic acid; a-C17:0, 14-methylhexadecanoic acid; and a-C17:1, 14-methylhexadecenoic acid.

A and B was not observed. In an early phase of fermentation (at 18 hours of the fermentation), pyloricidin C and pyloricidin D were produced in the culture broth. After this time, pyloricidin C decreased gradually, accompanied by an increase of pyloricidin D accumulation (Fig. 2B). Isolation of pyloricidins from the culture broth is described in the succeeding paper[11].

Antimicrobial Activity of Pyloricidins against Gram-positive and Gram-negative Bacteria and Yeasts

In vitro antibacterial activities of pyloricidins against *H. pylori* NCTC11637 and common bacterial strains and yeasts used as test microorganisms for antibiotics screening were determined by the agar dilution method.

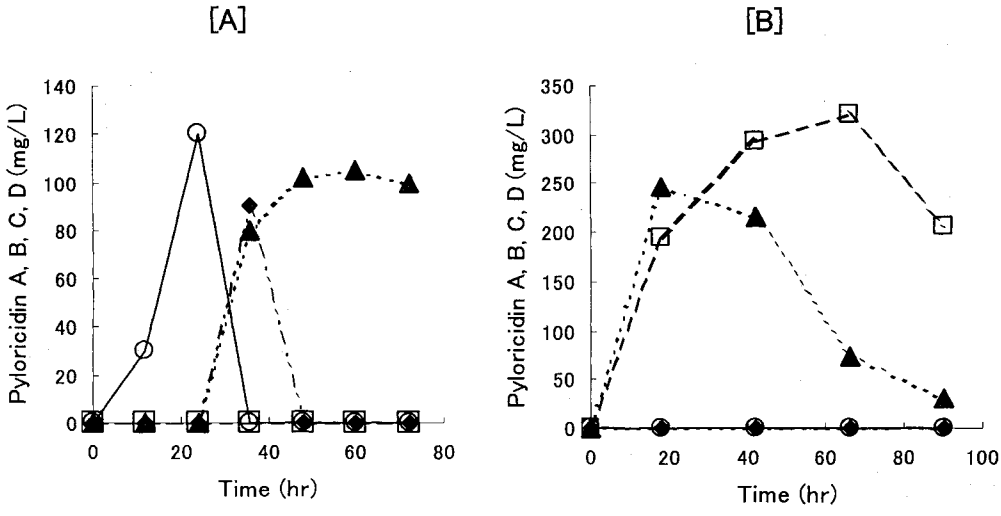
As shown in Table 2, pyloricidins shows antibacterial activity only against *H. pylori* and no activity against other bacterial strains and yeasts.

Among pyloricidins, pyloricidins A, A_1, A_2 and B revealed stronger activity against *H. pylori*. Pyloricidin B was used for further investigations.

Antibacterial Activity of Pyloricidin B, Amoxicillin, Clarithromycin and Metronidazole against Clinical Isolates of *H. pylori*

In vitro antibacterial activity of pyloricidin B against 50 clinical isolates of *H. pylori* was examined and

Fig. 2. Time courses of pyloricidins A, B, C and D production by *Bacillus* sp. HC-70 [Figure 2-A] and *Bacillus* sp. HC-72 [Figure 2-B].



—○—, --◆--, --▲-- and —□— represent pyloricidins A, B, C and D, respectively.

Table 2. Antimicrobial activity of pyloricidins against bacteria and yeasts.

Organisms		MIC(μg/ml)					
		Pyloricidin A	Pyloricidin A1	Pyloricidin A2	Pyloricidin B	Pyloricidin C	Pyloricidin D
Escherichia coli	NIHJ JC-2	>128	>128	>128	>128	>128	>128
Proteus vulgaris	IFO 3045	>128	>128	>128	>128	>128	>128
Morganella morganii	IFO 3168	>128	>128	>128	>128	>128	>128
Salmonella enteritidis	IFO 3313	>128	>128	>128	>128	>128	>128
Pseudomonas aeruginosa	IFO 3080	>128	>128	>128	>128	>128	>128
Alcaligenes faecalis	IFO 13111	>128	>128	>128	>128	>128	>128
Bacillus subtilis	PCI 219	>128	>128	>128	>128	>128	>128
Bacillus megaterium	IFO 12108	>128	>128	>128	>128	>128	>128
Staphylococcus aureus	FDA 209P	>128	>128	>128	>128	>128	128
Micrcoccus luteus	IFO 12708	>128	>128	>128	>128	>128	>128
Candida albicans	IFO 0583	>128	>128	>128	>128	>128	>128
Saccharomyces cerevisiae	IFO 0209	>128	>128	>128	>128	>128	>128
H. pylori	NCTC 11637	0.0625	0.0625	0.125	0.0625	0.5	1

was compared with that of amoxicillin, clarithromycin and metronidazole. The range of the MICs and the concentrations required to inhibit 50% of isolates (MIC_{50}) and 90% of isolates (MIC_{90}) are shown in Table 3. Pyloricidin B inhibited the growth of all the tested isolates at $\leqq 0.5\,\mu g/ml$, and its activity was comparable to that of amoxicillin and superior to that of clarithromycin and metronidazole (MIC_{90}; 0.25 *vs.* 0.125, 64 and 16 $\mu g/ml$,

Table 3. Antibacterial activity of pyloricidin B, amoxicillin, clarithromycin and metronidazole against 50 clinical isolates of *H. pylori*.

Compound	MIC (μg/ml)		
	Range	MIC_{50}[a]	MIC_{90}[b]
Pyloricidin B	0.016 – 0.5	0.063	0.25
Amoxicillin	≦0.008 – 0.5	0.031	0.125
Clarithromycin	0.016 – 128	0.125	64
Metronidazole	2 – 128	4	16

[a]MIC required to inhibit 50% of strains.
[b]MIC required to inhibit 90% of strains.

Table 4. Antimicrobial activity of pyloricidin B against anaerobes.

Organism		MIC(μg/ml) Pyloricidin B
Peptostreptococcus anerobius	B-30	>128
Peptostreptococcus anaerobius	B-38	>128
Clostridium perfringens	CW-2	>128
Clostridium perfringens	0668	>128
Lactobacillus acidophilus	IID-893	>128
Eubacterium aerofaciens	ATCC 25986	>128
Eubacterium alactolyticum	1441	>128
Eubacterium limosum	ATCC 8486	>128
Bifidobacterium adlescentis	15706	>128
Bifidobacterium bifidum	aE-319	>128
Bifidobacterium pseudolongum	Mo2-10	>128
Bacteroides fragilis	2509	>128
Bacteroides fragilis	2537	>128
Bacteroides fragilis	1115	>128
Bacteroides fragilis	1117	>128
Bacteroides vulgatus	W-6	>128
Fusobacterium varium	ATCC 8501	>128
Fusobacterium mortiferum	15	>128
Fusobacterium glutinosum	Ju-21	>128

respectively) (Table 3).

Antimicrobial Activity of Pyloricidin B against Anaerobes

Antimicrobial activity of pyloricidin B against obligatory anaerobes was examined and the result was shown in Table 4. Pyloricidin B did not show any noticeable activity against common anaerobic bacteria. These data indicate that pyloricidin B would not cause the disturbance of normal gastrointesinal microflora.

Effect of Pyloricidin B on Protein Synthesis in *H. pylori*

To elucidate the antibacterial mechanism of pyloricidin B against *H. pylori*, its effect on incorporation of L-[^{35}S]-methionine into protein fraction was examined. As shown in Table 5, pyloricidin B inhibited protein synthesis in *H. pylori* with IC_{50} of 0.42 μM (0.235 μg/ml).

Therapeutic Effect of Pyloricidin B against Experimental Gastric Infection Caused by *H. pylori* in Mongolian Gerbils

Therapeutic effect of pyloricidin B against experimental gastric infection caused by *H. pylori* was examined by using *H. pylori* infected Mongolian gerbil model. All of the vehicle-treated control Mongolian gerbils maintained gastric *H. pylori* at a level of approximately 10^6 CFU. Pyloricidin B, administered orally twice a day for 7 days, decreased the number of the infecting organism in the stomach in a dose-dependent fashion, and clearance was attained at doses of 10 and 30 mg/kg (Table 6).

Table 5. Effect of pyloricidin B on the protein synthesis in *H. pylori* NCTC 11637.

Compound	Concn. (μg/ml)	Relative inhibition (%)	IC_{50} (μg/ml)
Pyloricidin B	0.15	9.02 ± 5.59*	
	0.20	42.84 ± 3.76	0.235
	0.40	91.47 ± 6.81	

*The data were expressed as mean ± SD of the mean for three experiments.

Discussion

Intensive research in the field of gastroenterology led to the recognition that almost all duodenal ulcers and the majority of gastric ulcers are disorders associated with *H. pylori* infection[19]. Moreover, persistent *H. pylori* infection has been shown to be closely related to relapse of peptic ulcer as well as ulcer complications[20]. Therefore, anti-*H. pylori* treatment is a logical consequence promising rapid ulcer healing and a stable remission of ulcer disease.

In contrast with the conventional antibiotics, pyloricidin B, which was isolated as an antibiotic active against *H. pylori* and stable in rat whole blood *in vitro* and in 0.1 N HCl (data not shown), showed potent anti-*H. pylori* activity without exhibiting noticeable effect on common bacteria and yeasts. Because antibiotics currently used in the eradication therapy have broad antibacterial spectra and would therefore affect the natural gut flora, it is likely that their use would be accompanied by gastrointestinal side effects. As the antibacterial spectrum of pyloricidin B is restricted to *H. pylori*, its oral use would not cause the disturbance of normal gastrointestinal microflora. The increasing prevalence of *H. pylori* strains resistant to some

Table 6. Effect of repetitive oral administration of pyloricidin B against gastric infection caused by *H. pylori* TN2GF4 in MON/Jms/Gbs Slc Mongolian gerbils.

Compound	Dose[a] (mg/kg)	Clearance rate Cleared/total(%)	Bacterial recovery Log CFU/gastric wall (Mean ± SE)[b]	
Vehicle control	0	0/5 (0)	6.04 ± 0.19	
Pyloricidin B	1	2/5 (40)	3.05 ± 0.71	(4.10 ± 0.54)
	3	3/5 (60)	1.83 ± 0.29*	(2.37 ± 0.59)
	10	5/5 (100)	ND**	
	30	5/5 (100)	ND**	

ND: Not detected.

[a]b.i.d. for 7 days.

[b]Bacterial counts less than 30 CFU/gastric wall were regarded as 30 CFU to calculate the mean. Figures in parentheses indicate the value for positive cases.

*: $p<0.05$, **: $p<0.01$ vs vehicle control by Dunnett's test on ranked data.

of the most commonly used antibacterial agents is the major cause of failure to eradicate the infection[21,22]. We have found that all the tested clinical isolates were susceptible to pyloricidin B.

Eradication of *H. pylori* has been shown effective not only for preventing recurrence but also for cure of peptic ulcers[23,24]. Furthermore, *H. pylori* infection has been implicated in the pathogenesis of gastric cancer, and the interleukin-1 polymorphisms in host have been shown to be associated with increased risk of gastric cancer[25]. Therefore, application of *H. pylori* eradication therapy for high risk individuals is rationalized to prevent the later development of gastric cancer. In the present study, administration of pyloricidin B resulted in complete clearance of *H. pylori* in experimentally infected Mongolian gerbils. In conclusion, pyloricidin B is able to achieve a high rate of clearance when it is administered as monotherapy.

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