[原著] Production of bacteriocins by Clostridium scindens and some of their properties

Production of bacteriocins by *Clostridium scindens* and some of their properties

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ABSTRACT

Twenty-three strains of *Clostridium scindens* isolated from 11 human feces samples and one reference strain were examined for both bacteriocin production and bacteriocin susceptibility. Out of these, 10 strains were found to produce bacteriocins, and another 10 strains were sensitive to the bacteriocins. The bacteriocin-producing strains were resistant to their own bacteriocin as well as bacteriocin produced by other strains. Six strains were sensitive to all tested bacteriocins. Four of the strains tested were insensitive to all bacteriocins and produced no bacteriocin. The physicochemical properties of three bacteriocins produced by strains O-51, Y-1113 and O-161 were similar, and were mitomycin C inducible, trypsin resistant and heat-labile. Sensitivity to the bacteriocins was confined to other strains of *C. scindens*, *Eubacterium* species VPI 12708, *Eubacterium* species strains C-25 and *Peptostreptococcus anaerobius* PL-9; the other gram-positive and gram-negative bacteria tested for sensitivity were not affected by the bacteriocins. Crude bacteriocins killed sensitive cells rapidly but cell lysis did not appear to be involved. *Ryukyu Med. J., 20(2)47~52, 2001*

Key words: *Clostridium scindens*, bacteriocin, anaerobes, bacteriocin production, bacteriocin susceptibility

INTRODUCTION

Bacteriocins are usually defined as bactericidal proteins with activity restricted to species closely related to the producer strain, and have been described for many bacteria, including *Clostridium*[1, 2, 11, 16-19]. However, bacteriocins produced by *Clostridium scindens* have not been reported.

C. scindens is a gram-positive, spore-forming, nonmotile, obligate anaerobe which has been detected at approximately 10^7 cfu per g of human feces[4]. This bacterium has been shown to degrade bile acids by 7 α-dehydroxylation and 7 β-dehydrogenation[21], and to cleave the side-chain of cortisol and 17 α-hydroxyprogesterone[13]. Secondary bile acids, deoxycholic and lithocholic acids formed, are considered to be important in the etiology of intestinal carcinogenesis[6, 14, 15] and cholesterol gallstone formation[3].

Through screening for antagonistic activity between intestinal bacteria in vitro, bacteriocins were found in strains of *C. scindens* isolated from human feces. In this report, we describe the production of bacteriocins by *C. scindens* and some of their properties.

MATERIALS AND METHODS

Bacterial strains and media All strains, including the reference strain used in this study, are listed in Table 1. Twenty-three strains were originally isolated in our laboratory from 11 samples of feces from healthy humans from 1983 to 1998. Multiple isolates from the same individual were included in the study. All the isolates included were identified as *C. scindens* by physiological and biochemical properties, DNA base composition, DNA-DNA homology and 16S rDNA sequences analysis[12]. A strain of *C. scindens* ATCC 35074 was kindly given by Dr. P.B. Hylemon, Virginia Commonwealth University. Cultures used daily were maintained in GAM semisolid medium without dextrose (Nissui Pharmacy Corp. Tokyo). Stock cultures were kept at -80℃.

Screening for bacteriocin production and susceptibility

One loop of an overnight culture was inoculated onto GAM agar (Modified "Nissui") plates, and then the plates were incubated at 37℃ for 3-4 days under anaerobic conditions. The cells that grew were then killed by exposure to air for 1 day and the plates were overlayered with 3 ml of 0.3% brain heart infusion broth soft agar

Table 1 The susceptibility of strains of *C.scindens* to ten cultured supernatant fluids

Indicator strain	Cultured supernatant fluid obtained from bacteriocin-producing strain									
	O 5 1	O 7 1	Y 9 8	Y 1 1 2	Y 1 1 3	O 2	O 1 4	O 1 6	O 2 8	O 3 9
							3	1	1	1
35074	+	+	+	+	+	+	+	+	+	+
36S	−	−	−	−	−	−	−	−	−	−
M-18	−	−	−	−	−	−	−	−	−	−
O-51	−	−	−	−	−	−	−	−	−	−
O-71	−	−	−	−	−	−	−	−	−	−
Y-98	−	−	−	−	−	−	−	−	−	−
Y-1112	−	−	−	−	−	−	−	−	−	−
Y-1113	−	−	−	−	−	−	−	−	−	−
O-22	−	−	−	−	−	−	−	−	−	−
O-143	−	−	−	−	−	−	−	−	−	−
O-161	−	−	−	−	−	−	−	−	−	−
O-281	−	−	−	−	−	−	−	−	−	−
O-391	−	−	−	−	−	−	−	−	−	−
I-6	+	+	+	+	+	+	+	+	+	+
I-10	+	+	+	+	+	+	+	+	+	+
RK-1	+	+	−	−	−	−	−	−	−	−
RK-4	+	+	−	−	−	−	−	−	−	−
MK-251	+	+	+	+	+	+	+	+	+	+
TH-101	+	+	+	+	+	+	+	+	+	+
TH-104	+	+	+	+	+	+	+	+	+	+
YN-3	−	−	−	−	−	−	−	−	−	−
YN-8	−	−	−	−	−	−	−	−	−	−
NA-31	−	−	+	+	+	−	−	−	−	−
NA-35	−	−	+	+	+	−	−	−	−	−

+; Sensitive to bacteriocin, −; Resistant to bacteriocin The activities against strain I-10 of the bacteriocins produced by each strain were all 2 A.U.

(BHI, Eiken Kagaku Co. Ltd.,) containing approximately 10^7 cfu/ml indicator cells. The plates were incubated at 37℃ for 18-24 h and then examined for zones of inhibition in the bacterial lawn surrounding the colonies. To determine whether zones of inhibition were due to phage activity, blocks of agar were cut from these zones, crushed in BHI broth, and incubated at 37℃ overnight. The supernatant was spotted onto GAM agar plates seeded with an indicator strain. After incubation, the plates were examined for the presence of phage plaques. A lack of phage plaques was considered presumptive evidence that the inhibition of the indicator strain was due to a bacteriocin.

Producer and indicator strains were also identified by the cross culture spot method. Briefly, each prospective bacteriocinogenic BHI broth culture supernatant and cell extract was spotted using a syringe with a tuberculin needle onto GAM agar plates seeded with a prospective indicator strain. The plates were incubated overnight anaerobically at 37℃, and then examined as described above for zones of inhibition.

Determination of optimal conditions for bacteriocin production in liquid media *C. scindens* O-51, Y-1113 and O-161 were incubated under anaerobic conditions in the following media to determine the optimal conditions for bacteriocin production: BHI broth, GAM (Nissui) broth, heart infusion (HI, Eiken) broth, and peptone yeast extract supplemented with 0.1% glucose (PY).

Induction of bacteriocin Overnight BHI broth cultures of bacteriocin- producing strains were harvested by centrifugation. Aliquots of the cells were inoculated in 10 volumes of fresh BHI broth and reincubated at 37℃ anaerobically. Mitomycin C (MC, Sigma) was added to BHI cultures at concentrations of 0.5, 1.0, and 2.0 μg/ml after 1, 2, 4, 6, and 8 h of incubation to determine the inducibility of the bacteriocin. After 1 h, the cells were harvested by centrifugation, suspended in fresh BHI broth and incubated for an additional 18-24 h. Bacteriocins were then prepared as follows: The cultures were centrifuged at 2,270 × g for 30 min and the supernatant fluids were then sterilized through a membrane filter (Millipore, pore size 0.45 μm) and stored at 4℃. The pellets were washed two times with 0.02 M Tris-HCl buffer (pH 7.5) and suspended in the original volume. The suspension was subjected to ultrasonic treatment at 200W for a total of 4 min, with pauses for 30 sec each 1 min in an ice box and centrifuged at 20,000 × g for 30 min at 4℃. The supernatant fluids were filtrated through membrane filters to remove intact cells or cell debris.

Sedimentation of bacteriocins A sample of each bacteriocin solution was centrifuged at 105,000 × g for 2 h. The supernatant was removed and the pellets were vigorously mixed after adding fresh BHI broth to achieve the original volume. Both fractions were then assayed for bacteriocin activity.

Dialysis of bacteriocin Cellulose casing dialysis tubing (Visking Company, Chicago) containing 3 ml of 0.02M Tris-HCl buffer (pH 7.5) was suspended in 30 ml of bacteriocin solution. After this solution was stirred for 24 h at 4℃, the solution inside the dialysis tubing was assayed for bacteriocin activity.

Bacteriocin assay Drop of two-fold serial dilutions of the sample in nutrient broth was spotted onto the indicator strain-seeded agar plates. The bacteriocin production was expressed in terms of arbitrary units (A.U.) representing the reciprocal of the highest dilution that gave clear zone of growth inhibition of the indicator strain.

Treatment with various reagents and heat The effect of five enzymes at a final concentration of 100 μg/ml on bacteriocin activity was individually examined. All enzymes were purchased from Sigma Chemical., St, Louis, Mo. Trypsin, proteinase K, catalase, DNase and RNase were suspended in 0.02M Tris-HCl (pH 7.5). Bacteriocin

(32 A.U.) -enzyme mixtures were incubated for 1 h at 37℃. The effect of chloroform and ethyl ether on bactriocin was examined by mixing the solvent (50% v/v) with bacteriocin. Then the solvent was removed by aeration, and the remaining bacteriocin activity was assayed. Also, bacteriocin was treated for 5 to 30 min at various temperatures.

Bactericidal effect of bacteriocin Overnight BHI cultures of bacteriocin-sensitive strain I-10 were harvested by centrifugation. The cells were incubated in fresh BHI broth for 3-4 h until they reached early log phase, at which time bacteriocin was added. Viability was determined by standard colony count procedures on GAM agar plates.

Sucrose fermentation test Five drops of overnight cultures grown in semisolid GAM without dextrose medium (Nissui) were inoculated into semisolid GAM medium with or without 1 % sucrose. After incubation for 7 days, the pH in the culture was measured. Strains in which the difference of pH between the cultures without and with sucrose was over 0.35 were considered to be positive for sucrose fermentation[20].

RESULTS

Bacteriocin production and susceptibility Twenty-three fecal isolates of *C. scindens* and one reference strain were tested for both bacteriocin production and susceptibility using the solid media and cross culture spot methods. Ten strains isolated from three persons were found to produce bacteriocin. Six strains (35074, I-6, I-10, MK-251, TH-101 and TH-104) isolated from four persons were sensitive to all of the bacteriocins produced by *C. scindens*. Four strains (36S, M-18, YN-3 and YN-8) neither produced nor were sensitive to the bacteriocins. The growth of all of the bacteriocin-producing strains was inhibited not only by their own bacteriocin, but also by the bacteriocins produced by the other strains. Bacteriocin-producing and -susceptible strains are summarized in Table 1. All strains that originated from the same person had identical bacteriociogeny or bacteriocin susceptibility. The bacteriocins could be arranged in three groups with identical activity spectra. Three strains, O-51, O-161 and Y-1113 were therefore selected as bacteriocin producers and were used for further study. Strain I-10 was also selected as the indicator strain since it had relatively high susceptibility. Table 2 shows diameters of inhibition zones of the three bacteriocins produced by the three strains on solid media. The diameter of inhibition zones varied among producer strains. An incubation period of 120 h or longer for production usually resulted in larger zones in strains O-51 and Y-1113, but no change was observed in strain O-161.

Table 2 The differences in diameter of the inhibition zone among three bacteriocins produced by *C. scindens* grown on solid media

Cultivation time	Diameter of inhibition zone		
	O-51	Y-1113	O-161
72hr	15mm	12mm	11mm*
120hr	37mm	27mm	11mm

*: The results shown are the average of the inhibition zones formed on three plates. Strain I-10 was used as indicater strain. The details are described in Materials and Methods.

Table 3 Bacteria sensitive to three bacteriocins produced by *C. scindens*

	Bacteriocins		
	O-51	Y-1113	O-161
Eubacterium species VPI 12708	+	−	−
Eubacterium species C-25	+	+	+
Peptostreptococcus anaerobius PL-9	−	+	+

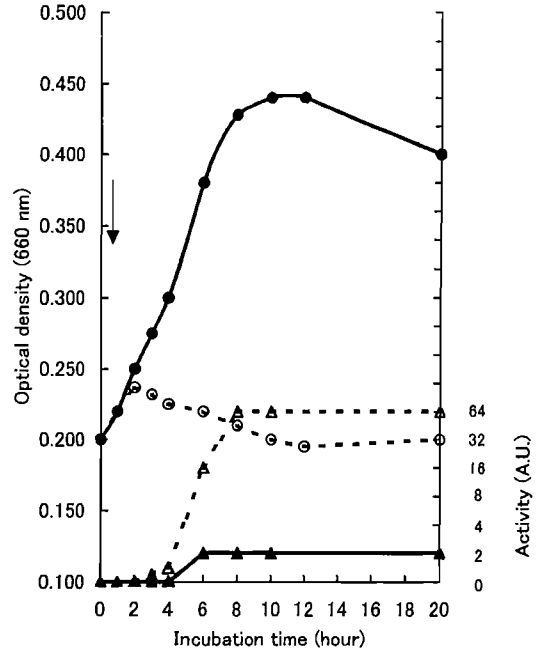

Fig. 1 Induction of bacteriocin of *C. scindens* strain O-161 by mitomycin C (MC)

MC (1 μg/ml) was added to cultures of strain O-161 as indicated by the arrow. After 1h, MC was removed by centrifugation. The cells were suspended in fresh BHI broth, and then further incubated overnight. The culture was obtained at the indicated time and centrifuged. The bacteriocin activity in the supernatant was measured. Growth of bacteria: MC treated (○), untreated (●). Bacteriocin activity: MC treated (△), untreated (▲).

Host range against other bacterial strains

The activity of the three bacteriocins against 46 different bacterial species was examined. *Eubacterium sp.* VPI 12708 (VPI 12708), *Eubacterium sp.* strain C-25, and *Peptostreptococcus anaerobius* (PL-9) were found to be

Table 4 Properties of bacteriocin produced by *C. scindens* strains O-51, Y-1113 and O-161

Treatment	Conditions	Bacteriocins O-51	Y-1113	O-161
None (control)		32	32	32 A.U.
Heat	37℃, 30 min	32	32	32
	45℃, 30 min	32	32	32
	55℃, 30 min	4	4	4
	60℃, 5 min	0	0	0
pH 3.5	4℃, 18 h	0	0	0
pH 7.2	4℃, 18 h	32	32	32
pH 11.0	4℃, 18 h	0	0	0
Trypsin (100μg/ml)	37℃, 1 h	32	32	32
Proteinase K (100μg/ml)	37℃, 1 h	0	0	0
DNase (100μg/ml)	37℃, 1 h	32	32	32
RNase (100μg/ml)	37℃, 1 h	32	32	32
Chloroform (50%)	37℃, 1 h	8	8	4
Ethyl ether (50%)	37℃, 1 h	32	32	32
Dialysis against distilled water	4℃, 18 h	32	32	32
Sedimentation	105,000×g, 2 h	0	0	0

The bacteriocin was treated with various reagents under the indicated conditions, and the residual activity was measured with indicator strain I-10. The details are described in Materials and Methods.

sensitive (Table 3), while other gram-positive bacteria (24 species, 41 strains; *Staphylococcus, Streptococcus, Enterococcus, Corynebacterium, Bacillus, Peptococcus, Peptostreptococcus, Bifidobacterium, Eubacterium, Lactobacillus, Propionibacterium, Actinomyces, Clostridium* and gram-negative bacteria (19 species, 23 strains; *Escherichia, Klebsiella, Proteus, Providencia, Enterobacter, Salmonella, Shigella, Serratia, Yersinia, Aeromonas, Vibrio, Pseudomonas, Veillonella, Bacteroides, Fusobacterium*) tested were not affected by the bacteriocins.

Bacteriocin production and location Strains were grown in several media to determine optimal conditions for bacteriocin production (data not shown). The highest titers (32 to 64) of bacteriocins of the strains were obtained with the addition of MC at a final concentration of 1 μg/ml under anaerobic conditions in BHI broth and in GAM broth. Culture supernatants of BHI broth and cell extracts were prepared from 20 strains and assayed against *C. scindens* strain I-10. The bacteriocin activities were recovered in the culture supernatants, but not in the cell extracts. Fig. 1 shows the time course of bacteriocins produced by *C. scindens* strain O-161. The bacteriocin O-161 was released from the cells during the logarithmic phase of growth. Also, bacteriocin production stopped when the cells entered the stationary phase of growth. When MC (1μg/ml) was added to the exponentially growing culture of *C. scindens* strain O-161, a significant amount (64 A.U.) of bacteriocin was detected in the culture supernatant. On the other hand, the amount of spontaneously synthesized bacteriocin O-161 was 2 A.U. in the absence of MC. The inducible synthesis of bacteriocin O-161 was accompanied with cell lysis. Turbidity of the culture began to decrease at about 2 h after the addition of MC and the bacteriocin activity began to increase from this time and reach the maximum (64 A.U.) after 7h as shown in Fig.1.

Several properties of bacteriocins Several properties of bacteriocins produced by strains O-151, Y-1113 and O-161 are shown in Table 4. The activities of bacteriocins were inactivated by treatment for 5 min at 60℃. Treatment with proteinase K caused complete inactivation, but trypsin, nuclease and catalase had no effect on the activity. The activity was reduced to 1/4-1/8 by chloroform treatment. Centrifugation at 105,000 × g for 2 h did not sediment the bacteriocins. They were not dialyzable.

Bactericidal effect of bacteriocins Within 5 min after addition of the bacteriocin (32 A.U.) to a sensitive culture (strain I-10) in the logarithmic phase of growth, the viability of the culture decreased by approximately one hundred fold (Table 5). At a cell concentration of 3.4×10^7 cfu/ml, bacteriocin concentrations of 2.0 A.U. or higher were bactericidal, but concentrations of 1.0 A.U. or lower did not kill the cells (data not shown).

Sucrose fermentation It was noted that bacteriocins were produced by 10 (59 %) of 17 sucrose-fermenting strains (36S, O-51, O-71, Y-98, Y-1112, Y-1113, M-18, O-22, O-143, O-161, O-281, O-391, RK-1, RK-4, MK-251, NA-31 and NA-35), but there were no bacteriocin-producing strains among the sucrose-non-fermenting strains (35704, I-6, I-10, TH-101, TH-104, YN-3 and YN-8), as shown in Table 1. On the other hand, five (71.4 %) of seven sucrose-fermenting strains, not including any bacteriocin-producing strains, and five (71.4 %) of seven sucrose-non-fermenting strains were sensitive to *C. scindens* bacteriocins.

Table 5 Bactericidal effect of the bacteriocin O-161 on sensitive cells, *C. scindens* strain I-10

	Time (minutes)			
	5	10	15	30
Control	3.4×10^7	3.0×10^7	3.3×10^7	3.6×10^7
Treated	5.0×10^5	7.2×10^4	1.3×10^4	$<\times10^4$

Cells from exponential phase were harvested, washed, and resuspended in fresh BHI broth. Samples of this suspension were treated with bacteriocin (32 A.U.) or with 0.025M Tris-HCl (pH 7.4) and were incubated at 37℃. The number of viable cells was determined at the indicated times.

DISCUSSION

The bacteriocinogeny of 24 strains of *C. scindens*, in cluding one reference strain, was investigated. Ten strains from the feces of three persons were bacteriocinogenic. To our knowledge, this is the first report on bacteriocin production by this organism. The production of bacteriocins by the strains of *C. scindens* was consistently detected either in broth or on solid media. The activity of the bacteriocins was also increased by exposure of producer cultures to MC, indicating that synthesis of bacteriocins is inducible, although bacteriocins produced by other species of *Clostridium* were not inducible by this reagent[1, 17]. Most bacteriocins are extracellular, and found in culture supernatants[7, 8], whereas others are surface bound or intracellular[9, 16]. The bacteriocins produced by *C. scindens* were found in culture supernatants, and appeared to be extracellular.

The possibility that the bacteriocins produced by these strains was a phage particle or lytic enzyme can be excluded based on the following evidence. (i) A part of the inhibition zone was incubated overnight, and then spotted onto sensitive cells. No plaques were observed. (ii) Bacteriocins were not precipitated from the MC-induced supernatant by ultracentrifugation at 105,000 × g. (iii) When indicator cells were treated with the bacteriocin, the number of viable cells were decreased, but the turbidity of the mixture was not reduced.

The bacteriocins produced by *C. scindens* were inactivated by heating at 60℃ and by proteinase K treatment, although trypsin did not appear to affect the bacteriocins. This suggests that they are at least partially protein in nature, but no explanation can be provided presently for the reduction of bacteriocin activity by chloroform treatment.

The inhibitory effect of the bacteriocins produced by *C. scindens* is not restricted to other strains among the same species, since three strains of gram-positive bacteria, *Eubacterium sp.* VPI 12708, *Eubacterium sp.* strain C-25 and *P. anaerobius* PL-9, were sensitive to them. However, the activity spectra of a number of bacteriocins produced by *Clostridium* were narrow, since the bacteriocins inhibited only other strains of the same species or the same genus[1, 2, 17, 18]. Frederique[8] and Hamon and Peron[10] reported that bacteriocins produced by gram-positive organisms had a much wider action spectrum than bacteriocins produced by gram-negative organisms. None of the gram-negative bacteria tested were affected by bacteriocins produced by *C. scindens*.

Bradley[5] suggested that bacteriocins fall into two large groups, a low molecular weight group comprising trypsin-sensitive and thermo-stable bacteriocins, and a high molecular weight group characterized by trypsin-resistance and thermolability. The bacteriocins produced by *C. scindens* may belong to the high molecular weight group of bacteriocins since they are trypsin resistant and thermolabile.

From these results the bacteriocins produced by *C. scindens* were divided into at least three groups, O-51, Y-1113 and O-161, based on the differences of their activity spectra (Tables 1, 3) and diameter of inhibition zones by bacteriocins produced on solid media (Table 2), although their physicochemical properties (Table 4) were similar. A number of bacteriocins were also grouped by bactericidal specificity (host ranges).

It is interesting that only atypical sucrose-fermenting *C. scindens* strains produced bacteriocins, although no differences of the bacteriocin susceptibility between sucrose-fermenting and sucrose-non-fermenting strains was observed. This raises the question of the possible genetic transfer of a bacteriocinogenic factor plus sucrose-fermentation-coding factor to sucrose-non-fermenting *C. scindens*. Experiments are currently underway in our laboratory to search for such plasmids and to purify the bacteriocins.

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