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Properties of Bacteriocin Produced from *Shigella sonnei* 100052

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ABSTRACT: A bacteriocin produced from *Shigella sonnei* 100052 was studied kinetically on its activities. Almost all *S. sonnei* strains seemed to be sensitive to this bacteriocin. Ultrafiltration studies revealed, however, that the bacteriocin had a variety of molecular sizes, whereas their activities seemed to be remain essentially the same. The molecular weight of a minimal unit (monomer) was estimated to be approximately 28,000. The difference in bacteriocin sensitivity among *S. sonnei* strains were revealed by means of kinetic studies; *S. sonnei* strain 17 was more sensitive than *S. sonnei* strain 56, and a resistant variant derived from *S. sonnei* strain 17 grew whether the bacteriocin was present or not. The survivor curve of *S. sonnei* type 7 strain, the producer of the bacteriocin, proved that though the strain was sensitive to the bacteriocin, the killing action was delayed. *S. dysenteriae* strain E7 was far more sensitive to the bacteriocin than *S. sonnei* strains. The bacteriocin was sensitive to KCN, $FeSO_4$ and $CuSO_4$, and resistant to pronase. Its activity was enhanced in the presence of cystein or lysozyme. Strain E7 seemed to be useful to ensure colicin typing of *S. sonnei*.

Bacteriocin produced from *Shigella sonnei* 100052 was reported as the determination factor of colicin type 7 by Abbott and Graham (1). Its characters are unique. In the first place the bacteriocin is not active to *Escherichia coli* K12−Row which is sensitive to the majority of colicins produced by *S. sonnei*, and secondly the bacteriocin is detectable using only one indicator, *S. sonnei* strain 17, among 12 indicator organisms and 6 additional ones, in so far as the standard colicin typing method of *S. sonnei* is used (1, 2, 3, 10, 13, 18, 21). In the course of study, it was found the bacteriocin attack almost all of *S. sonnei* and also some of other organisms were respectively sensitive in a different degree to the becteriocin(11). On this standpoint, we set a hypothesis that *S. sonnei* 100052, which is called as "type 7 strain" afterwards, may be double (major and minor) bacteriocin producers, and that the former may produce a large amount of bacteriocin which is an active factor only on *S. sonnei* strain 17 while the latter may produce a small amount of bacteriocin acting on *S. sonnei* strain 56 and other *S. sonnei* indicators. But the hypothetic factors could not be separated

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by means of transfer of bacteriocin factor(s) (11).

In the present paper, the authors report the fact of the existence of a variety of molecular size of active entities and of a different degree of sensitivities of suffered organisms.

MATERIALS AND METHODS

Bacteria. Nineteen type strains and 12 indicator strains for a standard colicin typing method of *S. sonnei* in Japan (2, 18) and *S. dysenteriae* strain E7 were used. Other organisms used in the present study are shown in the paragraph of Results.

Media. Tryptosoy agar (Eiken) and Bacto nutrient broth (Difco) were prepared from the dehydrated preparations. Soft agar containing 0.8 percent Bacto agar (Difco) in Bacto nutrient broth. Peptone−water containing 1 percent polypeptone (Daigo Eiyo Kagaku) and 0.5 percent NaCl in the distilled water.

Bacteriocin sensitivity tests. The method of Fredericq (9) was used.

Preparation of bacteriocin. Cells of type 7 strain were grown in peptone−water with constant stirring at 37 C for 30 hr. Mitomycin C (Kyowa Hakko Kogyo) was added to a final concentration of 0.5 μg/ml in it and the culture was incubated further for 18 hr. The culture was then centrifuged at 10,000 rpm for 20 min, and the supernatant was collected to be applied to the fractionation study.

For kinetic study, the supernatant was saturated with 90 percent ammonium sulfate. The precipitate was collected, suspended in 0.05 M Tris−HCl buffer (pH 7.5) and filtered through PM−10 Diaflo ultrafilter membrane to remove filterable impurities as stated in the following paragraph.

Fractionation of bacteriocin with ultrafilter. Fractionation was accomplished by forcing the supernatant containing bacteriocin with 0.7 kg/cm^2 of nitrogen gas against in order XM −300, PM−30 and PM−10 Diaflo ultrafilter membranes (Amicon Corp., Lexington, Mass, U. S. A.) mounted in an Amicon 400 ml ultrafilter cell equipped with a magnetic stirring bar.

Gel filtration with Sephadex G−100. A fraction of bacteriocin in 0.05 M Tris−HCl buffer (pH 7.5) was applied to a Sephadex G−100 superfine column (2.5 by 45 cm). Fractions of 3 ml were collected and absorbancy at 280 mμ was read and then assayed for bacteriocin titer.

Bacteriocin assay. Serial dilutions of bacteriocin preparations were spotted on a lawn of *S. sonnei* strain 17 on Tryptosoy agar, and the highest dilution of bacteriocin giving discernible inhibition of growth of strain 17 was defined as containing one arbitrary unit (AU) of activity per ml. One killing unit (KU) was determined as described by Fields and Luria (8), while the incubation time at 37 C was 2 hr in the presence of 2×10^{-3} M 2,4−dinitrophenol.

Isolation of bacteriocin resistant variants. A log−phase culture (0.2 ml) of *S. sonnei* strain 17 were plated on Tryptosoy agar plates. An 0.1 ml amount of bacteriocin fractions was spotted on the plates, which were then incubated for 20 hr at 37 C. From a spot of the fractions that gave near complete inhibition of colony growth, a single colony was picked

and repurified by streaking on plates.

Isolations of bacteriocin resistant variants from other indicator organisms were achieved as follows. Type 7 strain was stabbed in a Tryptosoy agar plate, which was then incubated overnight. The plate was sterilized by chloroform vapor, and a layer of melted top agar, inoculated with the strain to be tested, was poured on the plate surface. Resistant colonies were isolated from within the sterile halo formed by the action of the bacteriocin on the sensitive bacteria.

Kinetics of killing by bacteriocin. Log−phase cells (about 10^6 cells) were exposed to various amounts of bacteriocin in fresh nutrient broth, diluted in saline at the indicated times to stop adsorption, and plated for survival counts. The rates of survivors to the amount of bacteriocin after 2 hr treatment were assayed in the presence of 2×10^{-3}M 2,4−dinitrophenol.

RESULTS

Activity spectrum of bacteriocin

Bacteriocin produced from *S. sonnei* type 7 strain was investigated for its activities towards 12 indicator organisms by the colicin sensitivity test described by Fredericq (9) (Table 1). Among 12 indicator organisms, *S. sonnei* strain 17 was sensitive to high degrees, and *S. sonnei* strains 56, 56/56, 2, R6, 2/7 and R5 were sensitive to low degrees to the bacteriocin. The other 5 indicators, *S. schmitzii* M19 and *E. coli* Row, Row/E, Row/I and K12−30/I were resistant. Six additional indicators were all sensitive to low degrees. Among 19 type strains, 3 strains were sensitive to high degrees and other 16 strains were

Table 1. Sensitivities of various organisms to type 7 colicin

Organisms	No. of organisms investigated	No. of organisms sensitive to the colicin*		
		++	+	−
S. sonnei				
Colicin indicator strains	7	1	6	0
Indicators other than *S. sonnei*	5**	0	0	5
Additional indicators	6	0	6	0
Producers of standard colicin	19	3	16	0
Non-colicinogenic strains	67	0	66	1
Ewing strains	42	5	0	37
Hafnia sp.	6	0	3	3
Citrobacter sp.	6	0	2	4
Vibrio cholerae	121	0	2	119
Arizona sp.	5	0	0	5
Enterobacter sp.	9	0	0	9
Escherichia coli	6	0	0	6
Proteus sp.	5	0	0	5
Salmonella sp.	5	0	0	5
Klebsiella sp.	5	0	0	5
Providencia sp.	4	0	0	4
Rettgerella sp.	5	0	0	5
Staphylococcus sp.	5	0	0	5

* Symbols : ++, + and − are, respectively, clear, weak and no inhibition of growth of the tested organisms.

** This 5 organisms are *S. schmitzii* and *E. coli* strains. See text.

sensitive to low degrees. Among 67 non−colicinogenic strains, only one was resistant but the other 66 strains were sensitive to low degrees. Among other bacterial strains tested, 5 Ewing strains, type strains of genus *Shigella*, were sensitive to high degrees, and 3 strains of *Hafnia*, 2 strains of *Citrobacter* and 2 strains of *Vibrio cholerae* were sensitive to low degrees. Thus almost all of *S. sonnei* strains including type 7 strain, the producer of the bacteriocin, and some of other bacterial strains were revealed to be sensitive to the bacteriocin.

From the indicator strains 17, 56, 56/56, 2, R6, 2/7 and R5 and *S. dysenteriae* strain E7, resistant variants to the bacteriocin were selected and examined on their sensitivity patterns to 19 standard colicins. They showed almost the same sensitivity patterns as those of their parent strains though they lost the sensitivity to type 7 colicin. Consequently the bacteriocin produced from type 7 strain seems to share no common specific receptor with other colicins for the sake of its adsorption to bacterial cells.

Fractionation of bacteriocin with ultrafilter

Three liters of the mitomycin C−induced lysate was ultrafiltered through the Diaflo XM−300, PM−30 and PM−10 ultrafilter membranes in order. Fractions concentrated with XM−300 filter (fraction I), those passed through XM−300 and concentrated with PM−30 (fraction II), those passed through PM−30 and concentrated with PM−10 (fraction III), and those passed through PM−10 (fraction IV) were obtained. The amount of bacteriocin activity of each fraction were estimated approximately 88, 11, 1 and 0 percent of the original lysate respectively.

Activity spectra of fractions I and III

Fractions I and III were spotted on the lawn of 12 indicator organisms, and sensitivities of the indicator organisms were compared each other (Table 2). *S. sonnei* strain 17 was sensitive to high degrees to both the fractions. *S. sonnei* strains 56, 56/56, 2, R6, 2/7 and R5 showed low sensitivity, but other 5 indicators were not sensitive. In this way, the activity spectra of fractions I and III were apparently the same.

Cross−resistance of resistant variants to fractions I and III

Resistant variants to fractions I and III were isolated independently from *S. sonnei* strain 17 and tested for their sensitivities to both the fractions (Table 3). As shown in the Table 3, 29 variants resistant to fraction I were all also resistant to fraction III, and 27 variants resistant to fraction III were all also resistant to fraction I. Two variants sensitive

Table 2. Sensitivities of indicator organisms to fractions I and III

Fractions	Indicator organisms*											
	1	2	3	4	5	6	7	8	9	10	11	12
I	+**	++	+	+	+	−	+	+	−	−	−	−
III	+	++	+	+	+	−	+	+	−	−	−	−

* The indicator organism numbers correspond respectively with: *S. sonnei* 56, 17, 56/56, 2, R6; *S. schmitzii* M19; *S.sonnei* 2/7, R5; *E. coli* Row, Row/E, Row/I and K12−30/I.

** Symbols are the same as Table 1.

Table 3. Sensitivities of resistant variants to fractions I and III to both fractions

Resistant variants		Fractions tested I	III
17/I*	(29***)	−***	−
17/III	(27)	−	−
17/(III)	(2)	+	+

* Symbols: 17/I, 17/III and 17/(III) are correspond to *S. sonnei* strain 17 resistant to fraction I, and III and those sensitive to a low degree to fraction III, respectively.

** Number of organisms tested.

*** Symbols are the same as Table 1.

in low degrees to fraction III were all sensitive in low degrees to fraction I. Thus, cross−resistance to fractions I and III was certainly recognizable.

Molecular weight estimation by chromatography

Fraction III was chromatographed on Sephadex G−100 column with bovine serum albumin, ribonuclease and insulin as markers (Fig. 1). Bacteriocin activity was eluted at the point indicating the molecular weight of arround 28,000.

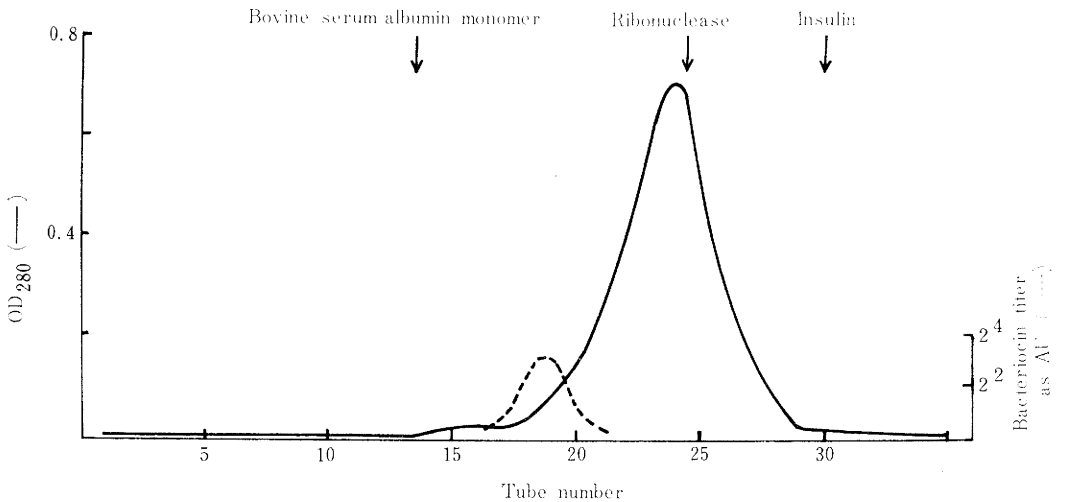

Fig. 1. Sephadex G−100 chromatogram of fraction III

Antibacterial action of bacteriocin to S. sonnei strain 17

In the preceding studies, mitomycin C−induced lysate seemed to contain the same species of bacteriocin regardless of their various sizes. As the bacteriocin did not dissociate in the monomer in so far as using the routine methods, the partially purified bacteriocin preparation as described in "Materials and Methods" was used in the following studies.

Log−phase cultures of strain 17 were mixed with different concentrations of bacteriocin and incubated at 37 C, and each sample thus obtained was plated at intervals to determine the survivors (Fig. 2). The viable count decreased exponentially with time and the curves were represented near slope with bacteriocin concentrations adopted.

Antibacterial action of bacteriocin to other S. sonnei strains

S. sonnei strains sensitive in a different degree respectively to the bacteriocin were compared one another regarding their behavior toward the bacteriocin (Fig. 2). The amount of bacteriocin used in the present studies was 128 AU.

The survivor curve of strain 56 had decreased slowly in 40 min and then increased slowly. The curve of strain 17/I, the resistant variant to fraction I derived from strain 17,

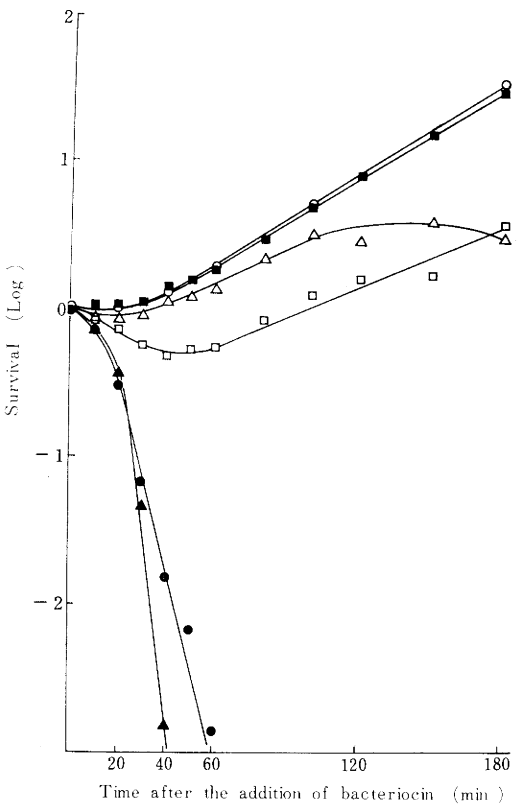

Fig. 2. Survivals of *S. sonnei* strains 17, 56, 17/I and 7. Log−phase cells (about 10^6 cells) growing in nutrient broth were treated with 128 and 1,280 (only strain 17) AU of bacteriocin. Samples (0.1 ml) were removed at intervals, diluted in cold saline and plated for survival counts. Symbols: 17 (●—●), 17 with 1,280 AU of bacteriocin (▲—▲), 56 (□—□), 17/I (■—■) and 7 (△—△). No difference was observed among the strains in control experiments (○—○).

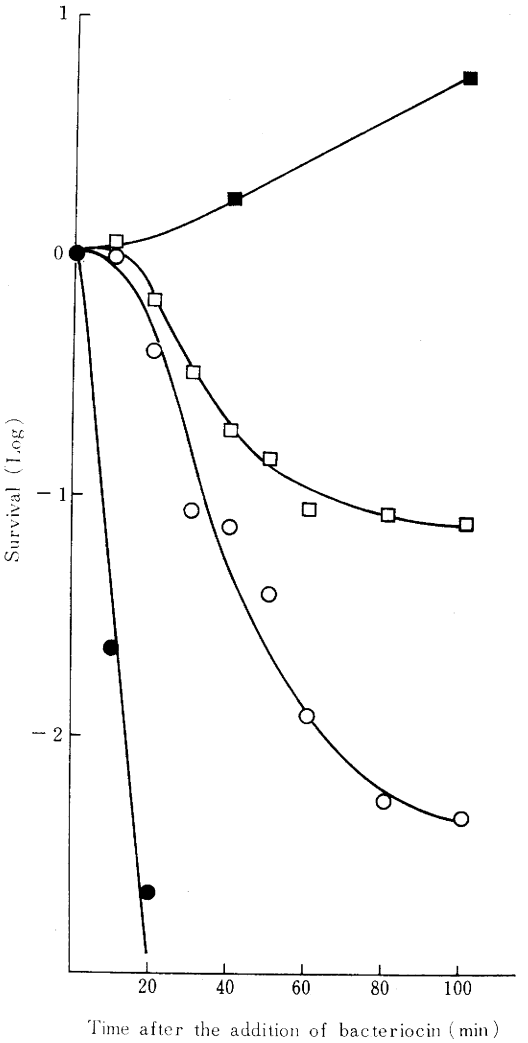

Fig. 3. Survivals of *S. dysenteriae* strain E7 at different bacteriocin concentrations. Log−phase cells (about 10^6 cells) growing in nutriant broth were treated with different concentrations of bacteriocin. Samples (0.1 ml) were removed at intervals, diluted in cold saline and plated for survival counts. Symbols: 1.28×10^{-1} AU (●—●), 2.56×10^{-2} AU (○—○), 1.28×10^{-2} AU (□—□) and control (■—■).

had increased without being affected by the bacteriocin. Type 7 strain, a producer of the bacteriocin, had also grown, but a rate of growth had decreased gradually and then minimized in 100 min.

Antibacterial action of bacteriocin to S. dysenteriae strain E7

At the bacteriocin concentration of 128 AU, the viable counts of *S. dysenteriae* strain E7 were 10^{-2} times lower than those without bacteriocin even at zero time and decreased rapidly afterward. Then the concentration of the bacteriocin was needed to be further lowered (Fig. 3). Even if the concentration of bacteriocin was lowered to 1.28×10^{-1} AU, the curve with steeper slope than that given by the survivor tests using strain 17 versus bacteriocin of 1.28×10^{3} AU was seen.

Comparison of the sensitivity to bacteriocin between strains E7 and 17

S. dysenteriae strain E7 and *S. sonnei* strain 17 grown in nutrient broth were treated with various concentrations of the bacteriocin in nutrient broth containing 2×10^{-3} M 2,4-dinitrophenol for 2 hr at 37 C. The drug has been known to stop cell growth without prevention of the initial interaction of bacteriocin and bacteria, or reduction of the viable count (7, 19). The number of viable cells was then determined (Fig. 4). When percentages of survivors after exposure of strain E7 cells to the bacteriocin for 2 hr were plotted on a logarithmic scale against the concentration of the bacteriocin, the curve was obtained the slope of which became gentle. However, a straight line was given at the concentrations of the bacteriocin less than 10^{-1} AU, and in this case one killing unit (KU) was calculated to be approximately 6.7×10^{-2} AU. Using crude bacteriocin preparations, however, the number of bacteriocin molecules corresponding to one killing unit could not be determined. Strain 17 gave a straight line, but it was more than 160 times resistant to the bacteriocin than strain E7.

Stability of bacteriocin to chemicals and enzymes

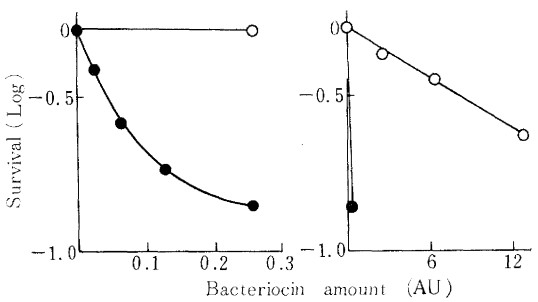

Fig. 4. Comparison of the sensitivities to bacteriocin between strains E7 and 17. Log-phase cells (about 10^6 cells) were treated with the amounts of bacteriocin for 2 hr in the presence of 2×10^{-3} M 2,4-dinitrophenol and plated for survivals. Symbols: E7 (●—●) and 17 (○—○).

Bacteriocin activity was completely destroyed by the treatment with 0.1N KCN for 120 min at 37 C. It was partially (90%) destroyed by treatments with 10^{-3} M $FeSO_4$ or 10^{-2} M $CuSO_4$ for 120 min at 37 C. It was, on the other hand, enhanced three times as much as that of the original activity by the treatment with cystein (10 mg/ml) or lysozyme (1 mg/ml) for 120 min at 37 C. The killing action was resistant to the treatment with pronase (0.1 mg/ml) for 120 min at 37 C, but largely (>95%) destroyed in 16 hr.

Table 4. Sensitivities of strains E7 and 17 to colicins produced by *S. sonnei**

Colicin	Strains used E7	17
1A	+	+
1B	−	+
2	−	+
3	−	+
3A	+	+
4	−	+
4A	+	+
5	−	+
6	+	+
7	+	+
8	+	+
9	−	+
9A	−	+
10	−	+
11	−	+
12	−	−
13	+	+
13A	+	+
14	+	+

* A method of Fredericq (9) was used.

Sensitivity of S. dysenteriae strain E7 to other colicins

S. dysenteriae strain E7 was examined on its sensitivity to colicins produced by *S. sonnei* using the method of Fredericq (9). The results are shown in Table 4. As was shown in the Table, strain E7 was sensitive to the colicins produced by *S. sonnei* strains of types 1A, 3A, 4A, 6, 7, 8, 13, 13A and 14, while strain 17 was sensitive to the colicins other than that originated from type 12 culture.

DISCUSSION

The research on the bacteriocin was started from the previous findings by the authors (11) of the fact that the activity spectrum was large and that different degrees of sensitivities in suffered organisms had been observed by the use of the present bacteriocin. Almost all *S. sonnei* strains seemed sharing a common receptor to the bacteriocin, which appeared rather specific to *S. sonnei* with a few exceptions.

The bacteriocin had a wide variety of molecular size; however, the activity spectrum of its fraction I was the same as that of fraction III. Cross−resistance between fractions I and III was observed so far as resistant variants to the two fractions were concerned. From these data, active entities of various molecular sizes seemed as if they were essentially the same in their activities and the particle having molecular weight of 28,000 seemed to be the minimal unit (monomer). The bacteriocin, therefore, is considered to be an aggregate composed diversely of this sort of monomer. But, the amount of free monomer in crude preparations was small (1%), and the majority of active material seemed to constitute the aggregate. On account of such characters the authors felt it difficult to purify the bacteriocin. Then the authors were compelled to go on working with the crude preparations. The analysis of structure of the bacteriocin is an interesting problem, which will become possible when purification of the bacteriocin is achieved.

Strain 17 was highly sensitive to the bacteriocin among *S. sonnei* strains used, but in the present paper it is stated to be far resistant compared with *S. dysenteriae* strain E7. As discussed by Nomura (15) and Levisohn *et al.* (12), two kinds of explanation are possible. The necessity for an increased number of bacteriocin unit to kill a strain 17 cell may reflect the mechanism requiring the cooperation of bacteriocin particles at many sites. Alternatively, it may reflect the situation in which the probability of successful killing by one bacteriocin particle at individual sites is greatly reduced, and yet, when successful, one particle is

sufficient to achieve the entire killing effect.

It is known that the killing action of most colicins is a single hit process as in the killing action of phage particles (16), that colicinogenic cells are immune to the lethal effect of the colicin which they produce, and that in the presence of very high concentrations of colicin, colicinogenic cells are no longer immune to the homologous colicin (12). We found that it was necessary to apply more than 160 times the amount of bacteriocin to strain 17 to obtain the same degree of the killing effect as to strain E7. All indicator strains of *S. sonnei* origin are known to be colicinogenic (21), but strain E7 does not produce any colicin detectable using the mentioned 12 indicator organisms. It seems that strain 17 produces colicin E, and strain 56 colicin E2 (21). Nomura (15) reported that the presence of colicin E2 factor rendered the cells about 8−fold more resistant to colicin E3. Therefore, it is impossible tc exclude the possibility that the presence of colicin E factor in the strain 17 cell decreases the efficiency of the killing effect of adsorbed bacteriocin particle.

On the other hand, the authors showed that a large number of non−colicinogenic *S. sonnei* strains were sensitive to the bacteriocin in low degrees. In regard to these strains, we can not attribute the lowering of sensitivity to their immunity. Many investigators (14, 17, 20) reported of the tolerant mutants in which the resistance was various in degrees. The degree of sensitivities observed in non−colicinogenic *S. sonnei* strains suggested that *S. sonnei* strains might be tolerant to the bacteriocin regardless their colicinogenicity.

It was found that type 7 strain, the producer of the bacteriocin, was sensitive to the bacteriocin in low degrees (11). In the present study, in addition, it was observed that the killing effect of type 7 strain was delayed to some extent. Type 7 strain could be grown in the presence of the bacteriocin, but the rate of growth was decreased gradually and then stopped. Colicinogenic cells which produce colicin E3 were known to be immune to their homologous colicin, because each of them produced the immunity substance inhibiting the 16 S ribosomal RNA cleavage reaction of colicin E3 (4,5,6). Our results thus obtained suggest that type 7 strain of *S. sonnei* also produces the substance which inhibits the killing effect of the bacteriocin, and that, when whole of the active substance is neutralized by the bacteriocin, the cell may become susceptible to the killing effect which may derive the curve showing some delay of the killing effect.

The sensitivity spectrum of *S. dysenteriae* strain E7 to standard colicins produced by *S. sonnei* strains is more limited than that of *S. sonnei* strain 17. Strain E7 was sensitive to 9 colicin types, whereas strain 17 was sensitive to the colicins other than type 12 colicin. Type 7 colicin, which is difficult to be detected using indicator strains other than strain 17, certainly is to be detected by strain E7. These data will be useful to ensure colicin typing of *S. sonnei*.

Biochemical events associated with the killing action of cells by the bacteriocin are left to be studied in future.

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Shigella sonnei 100052 の産生するバクテリオシンの性状

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Shigella sonnei 100052 の産生するバクテリオシンの活性を検討した．用いた *S. sonnei* のほとんどすべての菌株がこのバクテリオシンに感受性であった．限外濾過膜による分画実験の結果，バクテリオシンの分子量は不均一であるがその活性スペクトルは同一であることが明らかになった．バクテリオシンの最小単位の分子量は約 28,000であった．バクテリオシンに対する *S. sonnei* strain 17 の感受性は *S. sonnei* strain 56 の感受性よりも高かった．strain 17 から分離したバクテリオシン耐性株はバクテリオシンの影響を受けずに増殖した．バクテリオシン産生株 *S. sonnei* 100052 はバクテリオシンに感受性であったが，この菌株に対する殺菌作用の発現に要する時間は他の菌株に比較して長時間を要した．*S. dysentheriae* strain E7 は *S. sonnei* のどの菌株よりもはるかに感受性が高かった．バクテリオシンは KCN，$FeSO_4$，$CuSO_4$ に感受性，プロナーゼに耐性であった．バクテリオシンの活性はシステインまたはリゾチームによって増強された．strain E7 は *S. sonnei* のコリシン型別法の確立に有用であろうと考えられる．

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