Erwinia carotovora による抗細菌性物質の生成およびその諸性質

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Studies on the Production of Antibacterial Agent by *Erwinia carotovora* and its Properties*

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遠藤頼嗣**・津山博之***・仲谷房治***: *Erwinia carotovora* による抗細菌性物質の生成およびその諸性質

Abstract

The production of antibacterial agents (AAs) by isolates of *Erwinia carotovora*, the properties and their activity spectra were studied. The bacterial cell inductively produced the AA, when irradiated with UV or treated with mitomycin C at their early exponential growth phase, corresponding to the lysis of bacterial cells observed during 3-5 hrs of culture after the treatment. The AAs obtained from 5 hrs cultures of each isolate after UV irradiation differed from each other in their activities. In the case of one isolate, however, the activity did not vary with the difference in the induction method. All of the 17 isolates were found to have the ability to produce the agents, which were classified tentatively into 4 groups according to the similarity of their activity spectrum. The agent produced by each isolate indicated that it cannot be reproduced in the cell of each indicator. The activity of AA was found to be maintained for at least 20 days at 4 C; it was retained in dialized solution but was lost by heat treatment; and it was found in the precipitate obtained by salting out the lysate. UV absorption spectra of the partially purified preparation exhibited the highest absorption at 275 nm and the lowest at 260 nm. The AAs appeared to be protein characterized by a large molecule. It was concluded that AAs are a kind of bacteriocin.

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Introduction

In our laboratory, Fukuda[2] has shown that some isolates of *Erwinia carotovora* can produce bactericidal principle in their lysate after induction by UV ray or mitomycin C. Nakatani and Tsuyama[7] confirmed that antagonistic phenomenon observed between isolates of *E. carotovora* was caused by two kinds of agents. One of these diffused rapidly through agar and its activity was lost by trypsin treatment, while the other diffused very slowly and its activity was not lost by trypsin treatment. It has been assumed that these two agents indicate some common properties toward small molecular bacteriocin and large molecular bacteriocin according to the classification by Bradley[1].

The present paper is concerned with the second type of AAs and reports the

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results of its inductive production, tentative grouping of the agents according to the similarity in activity spectrum and some chemical and biological properties.

Materials and Methods

Bacteria Some or all of 17 isolates classified as *E. carotovora* were used. They were isolated at and obtained from various parts of Japan. Four isolates (Ar, Ec 3, Ec 6 and Eall), 4 isolates (P 7, P 14, CP and CPT), 2 isolates (3057 and 3830) and 1 isolate (310) were kindly furnished by Dr. Tominaga of Nat. Inst. Agr. Sci., Tokyo, Dr. Goto of Shizuoka Univ., Inst. Fermentation, Osaka, and Dr. Sakamoto of Agr. Inst. Tohoku Univ., respectively. Six isolates (Ar 5, Ar 13, Ar IT, 645 Ar, ET and 1) were isolated at the Iwate University Farm, from diseased chinese cabbage and tobacco plant. Most of these retained virulence for fresh vegetable tissues, however, isolates Ec 3 and Ec 6 had lost their virulence in the course of successive trnasfers.

Culture and induction methods The bacterial cells grown on nutrient agar slant (NAS) at 28 C for 18 hrs were suspended in sterilized saline and its concentration was adjusted to about 2×10^9 cells/ml. One volume of the suspension was added to 99 volumes of nutrient glucose broth (NGB) consisting of: nutrient broth, 1 liter; glucose, 10 g; pH, 7.0. The culture was incubated on a reciprocal shaker (120 str/min) for 3 hr, when the growth of bacterial cells was confirmed to be in early exponential phase. The cells collected by low speed centrifugation (3,000 rpm, 30 min, 4 C), were suspended again in sterilized saline at the concentration of 5×10^8 cells/ml. Ten ml of the suspension transferred into a dish (diam. 14 cm) was then irradiated with UV. The irradiation was carried out in a dark box at a distance of 30 cm from the germicidal lamp (Toshiba Co., Lmt., GL 15, 15 w). During the irradiation the dish was continuously shaken by hand. The lethal rate at a 30 sec UV irradiation was about a 95% average.

After irradiation, the cells were cultured further in NGB for 5 hr at 28 C away from light. At the end of culture, chloroform was added to make the concentration of 3% (v/v) and shaken for 30 sec. The lysate obtained as the supernatant liquid by centrifugation (15,560 $\times g$, 30 min, 4 C) was submitted for investigation of AA and other factors.

Mitomycin C (Kyowa Hakko Co., Lmt., Tokyo, Japan) was also used as inducer. It was added to the culture (5×10^8 cells/ml) at an exponential phase at a concentration of 1.0 μg/ml, unless otherwise mentioned. Ten min later, the culture was diluted with sterilized water in order to stop the reaction and the cells collected by centrifugation were cultured further for 5 hrs. The method for obtaining lysate was the same as mentioned above.

Activity assay Nutrient agar (NA) plate was spread with 0.07 ml of the culture of indicator isolate grown in advance in nutrient broth (NB) for 18 hrs at 28 C, using a glass spreader. After 3 hrs, the plate was spotted with 0.03 ml of the lysate obtained by the method mentioned above. Inhibition zone (IZ) formation was noted after a 12 hrs incubation at 28 C. In this experiment, the AA activity of each isolate was examined with 17 isolates as indicators.

Activity measurement of AA (spot method) The activity was quantitatively determined by the following procedures: 0.03 ml of a series of twofold diluted lysate was spotted on the plate inoculated in advance with an indicator isolate and the end-point of IZ

formation was noted. The activity was expressed by the reciprocal of dilution end-point at which the IZ was formed.

Purification procedures 60 ml of 1 M $MnCl_2$ solution was added to 1 liter of lysate and the pH of the solution was adjusted to 7.0 by adding 1 N NaOH solution. 500 g of ammonium sulphate was added to the supernatant liquid obtained by centrifugation (13,180 × *g*, 30 min, 4 C) of the solution mentioned above and allowed to stand overnight at 4 C. The pellet obtained by centrifugation was suspended in 10 ml of 0.01 M Tris-buffer. Five ml of the suspension was deionized and fractionated by passing through a column (26.5 × 350 mm) of Sephadex G-25 into 50 fractions (5 ml/fraction). Each of the fractions was further centrifuged (150,000 × *g*, 60 min, 4 C). UV absorption spectra were measured with a spectrophotometer (Shimadzu Co., Lmt., Type QB 50).

Results

1. Growth of bacteria after UV irradiation and the activity of AA

a. Growth of bacteria after UV irradiation The growth of 4 isolates (P7, 3057, Ar 13 and Ec 3) after irradiation was compared with one of the non-irradiated control isolate. The growth was followed in terms of optical density at 660 nm. One of the results of these experiments is shown in Fig. 1.

The growth curve of irradiated cells indicates a slight reduction from the beginning of the culture, a sharp drop after about 3 hrs, lowest density at 5 hrs and a subsequent gradual rise. The drop in the optical density is assumed to be caused by the lysis of bacterial cells during culture. The curve of the control cell indicated no sign of reduction or drop, as shown in Fig. 1.

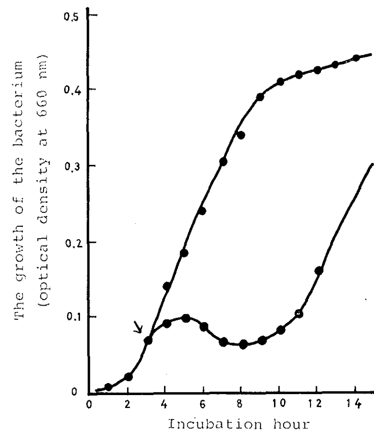

Fig. 1. Growth of the isolate P7 after UV induction. The upper curve shows the growth of control cells and the lower one shows the growth of induced cells. The arrow indicates UV irradiation.

b. The activity of AA and UV irradiation time. The variation in AA activity with change in irradiation time was determined. Isolates P7 and 3057 were irradiated for 0, 15, 30 and 50 sec, respectively, and then cultured for 5 hrs at 28 C. The activity was established by the spot method and the result is shown in Table 1.

As clearly shown in the table, isolate P7 shows the highest activity with a 30 sec irradiation, and isolate 3057 shows high activity at a 15

Table 1. The relation between the time of UV irradiation and activity of antibacterial agent

Isolates	P7				3057			
Time of irradiation (sec)	0	15	30	50	0	15	30	50
Lethal rate (%)	0	71.2	94.8	99.0	0	77.8	96.5	99.6
Activity[a)]	4	16	32	8	0	16	16	8

a) Activity was expressed as the reciprocal of dilution end-point determined by spot method using the isolate 645 Ar as indicator.

and 30 sec irradiation. It is remarkable that isolate P 7 showed weak activity without induction, but that isolate 3057 did not show any activity without induction. In both cases, the activity was clearly increased by a 30 sec UV irradiation.

c. Effet of mitomycin C treatment on the growth of bacteria. Mitomycin C was added to the culture of isolate P 7 in its exponential growth at concentrations of 0.1, 0.2, 0.5, 1.0 and 1.5 μg/ml. After a 10 min treatment the cells collected by centrifugation were further cultured in NGB for 14 hrs at 28 C. The growth is shown in Fig. 2.

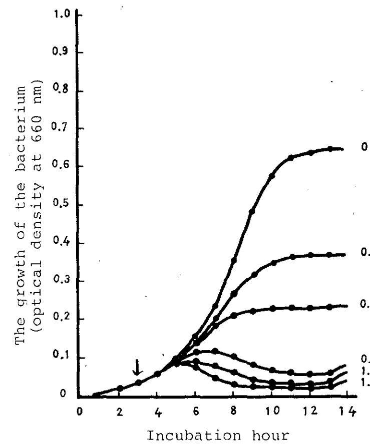

Fig. 2. Growth of the isolate P 7 after treatment with mitomycin C of different concentration. The figures on the right side of each curve show the concentrations of mitomycin C (μg/ml). The arrow indicates the time of treatment.

It appears that low concentration (0.1 and 0.2 μg/ml) treatment causes growth retardation, but there is no drop in the optical density during the course of culture. High concentration (0.5, 1.0 and 1.5 μg/ml) treatment, on the other hand, causes growth inhibition from the beginning of culture and a sharp drop beginning 3 hrs after treatment. The lowest optical density was observed at about 5–7 hrs after treatment.

d. AA activity induced by mitomycin C treatment. The variation in AA activity induced by several concentrations of mitomycin C was determined. The result is shown in Table 2. It shows that low concentration of mitomycin C did not induce production of AA, while high concentration induced it and highest activity was obtained by treatment at a concentration of 1.0 μg/ml. The activity of AA seems to be correlated with the lysis of bacterial cells as was observed in Fig. 2.

Table 2. The relation between the concentration of mitomycin C used as inducer and activity of antibacterial agent

Isolate	P 7					
Concentration of mitomycin C (μg/ml)	0	0.1	0.2	0.5	1.0	1.5
Activity[a]	4	4	4	16	32	16

a) Activity was expressed as the reciprocal of dilution end-point determined by spot method using the isolate 645 Ar as indicator.

Table 3. Difference in activity of antibacterial agent between isolates

Isolates	Methods of induction	Indicator isolates	Activity[a]
P 7	UV irradiation (30 sec)	645 Ar	32
do.	Mitomycin C (1 μg/ml)	do.	32
3057	UV irradiation (30 sec)	do.	16
1	do.	do.	512
645 Ar	do.	Ar 5	16

a) Activity was expressed as the reciprocal of dilution end-point determined by spot method.

e. Difference in AA activity between isolates. Four isolates (P 7, 3057, 1 and 645 Ar) were used as the producers and 2 isolates (645 Ar and Ar 13) as the indicators. UV or mitomycin C treatment was conducted and the results are shown in Table 3.

The lysate of isolate 1 showed the highest activity, but significant differences were not observed among the other isolates. Probably, considerable difference would be found in the activity between isolates if more isolates were examined. As far as isolate P 7 is concerned, no differences were found according to the induction methods used.

2. The grouping of AAs by their activity spectra and the reproductive ability of AAs within indicator cell

The activity spectra of AAs produced by 17 isolates were examined by spotting the lysates using 17 isolates also as indicators. The results are shown in Table 4.

All of the IZs formed on the plate were clear and small colonies (most of them were shown to be resistant to the action of the AA) grew on the surface of IZ after 24 hrs incubation. It is also evident from Table 4 that no IZ was formed when an AA produced by an isolate was spotted on the plate inoculated with the same isolate used as the indicator in advance.

As is clearly shown in the table, the AAs produced by 10 isolates may be provi-

Table 4. Activity spectra of antibacterial agents produced by each isolate and their groups

Sensitivity and resistance of indicator	Activity of antibacterial agent produced by isolate*									
	Ar 5	CP	Ea 11	P 7	3057	310	ET	Ar	1	645 Ar
Ar 5	−	−	−	−	+	+	+	−	−	+
CP	−	−	−	+	+	+	−	−	−	−
P 14	−	−	−	+	+	+	−	−	−	−
Ea 11	−	−	−	+	+	+	−	−	−	−
3830	−	−	−	+	+	+	−	−	−	+
Ar 13	−	−	−	+	+	+	−	−	−	+
Ar IT	−	−	−	+	+	+	−	−	−	+
CPT	−	−	−	+	+	+	−	−	−	+
P 7	+	+	+	−	−	−	−	−	−	−
3057	+	+	+	−	−	−	−	−	−	−
Ec 3	+	+	+	−	−	−	−	−	−	−
Ec 6	+	+	+	−	−	−	+	−	−	−
310	−	−	−	+	+	−	−	−	−	−
ET	−	−	−	−	−	−	+	−	−	−
Ar	−	−	−	+	+	−	+	−	−	−
1	−	−	−	−	−	−	−	−	−	−
645 Ar	+	+	+	+	+	+	+	−	+	−
Group		I		II			III			IV

* The AAs liberated from the isolates P 14, 3830, Ar 13, Ar IT and CPT and those from the isolates Ec 3 and Ec 6 were omitted in this table, as they showed identical activity ranges respectively with the ones produced by isolates Ar 5 (Group I) and P 7 (Group II).

sionally divided into 4 groups in accordance with the similarities and differences in activity spectrum.

In the first group, the AAs produced by 8 isolates (Ar 5, Cp, P 14, Ea 11, 3830, Ar 13, Ar IT and CPT) are involved and they have exactly the same activity spectrum. In the 2nd group, The AAs produced by 4 isolates (P 7, 3057, Ec 3 and Ec 6) are involved. They show almost the same activity spectrum, with the exception of the AA produced by isolate P 7, which shows no activity against isolate Ar 5. In the 3rd group, the AAs produced by 4 isolates (310, ET, Ar and 1) are tentatively involved. They have different activity spectrums but indicate a common activity toward isolate 645 Ar. By further investigation they will be separated into different groups using more isolates as producers. In the 4th group, only one AA produced by isolate 645 Ar is involved. The activity spectrum is clearly different from those of the other groups mentioned above. Isolate 645 Ar is of interest because of its character of having sensitivity toward most of the AAs, so far as the present investigation is concerned.

All of the IZs produced on a plate inoculated with an incator cell after spotting the lysate of each isolate were examined by the following 3 methods in order ascertain whether each AA contained in an IZ and lysate can reproduce in an indicator cell or not.

(1) AA adhered to a needle by touching on an IZ was suspended in 1 ml of NB. A part of the suspension (0.1 ml) was dropped on a plate inoculated in advance with indicator cells. After incubation at 30 C for 24 hrs, the IZ formation was monitored. (2) AA adhered to a needle by touching on an IZ, was suspended in 2.5 ml of soft agar (peptone, 10 g ; NaCl, 2.5 g ; deionized water, 1 liter ; agar, 5 g ; pH, 7.2) in which an indicator had been inoculated in advance. The soft agar was poured on an agar plate. After incubation for 24 hrs at 30 C, the IZ formation was monitored. (3) A series of tenfold dilute solutions were made using the lysate which was prepared for the spot method as the original solution. A part (0.1 ml) of each dilute solution was mixed with the soft agar containing indicator and then was poured on an agar plate. After incubation at 30 C for 24 hrs, the IZ formation was monitored.

As the result of examination by these 3 methods, it was found that the IZ had not formed in all cases. This indicates that the AA contained in an IZ and the lysate does not have the ability to reproduce in the indicator cells.

3. Some properties of AAs

a. Loss of activity during preservation. The lysates of 5 isoaltes (P 7, 3057, Ec 3, Ar 13 and 1) were stored in a refrigerator (4 C) and their activities were examined every 10 days. The lysate of four isolates lost their activity within 30 days of preservation. The lysate of isolate 1, on the other hand, retained its activity even after 60 days of preservation.

b. Thermostability. The lysates of 5 isolates (P 7, 3057, Ec 3, Ar 13 and 1) were examined for their thermostability and were found to lose their activity after a 10 min treatment at 50 C.

c. Dializability. The lysates of 4 isolates (P 7, 3057, Ar 13 and 1) were respectively dialized through cellulose membrane (Visking Co., Lmt., USA) against 500 ml of deionized water in a beaker for 12 hrs at 4 C. The activity was completely retained in the dialized solution and no ativity was found in the diffusate.

Table 5. Salting out of the lysate produced by the isolate P 7 and the activity of antibacterial agent

Saturation grades by ammonium sulphate	0-0.1	0.2-0.4	0.4-0.6	0.6-0.8
Activity[a)]	2	512	32	0

a) Precipitant was collected by centrifugation and suspended in 2 ml of Tris-buffer solution. Activity was expressed as the reciprocal of dilution end-point determined by spot method using isolate 645 Ar as indicator.

d. Salting out. The lysate of isolate P 7 was salted out with ammonium sulphate. Precipitates collected at four grades of saturation were respectively suspended into 2 ml of Tris-buffer solution. As shown in Table 5, the active principle was completely salted out at 60% of saturation with ammonium sulphate.

e. Gel chromatography and AA activity. Five hundred gramme of ammonium sulphate was added to 1 liter of crude AA containing liquid obtained from culture lysate of isolate P 7 (activity 32). In this case, some floating material was observed on the surface of the solution. The material has shown AA activity in a preliminary test. Ten ml of a suspension of the pellet obtained by ultra-centrifugation (150,000×*g*, 60 min, 4 C), including the floating material, showed high activity (activity 512).

Five ml of the suspension of the pellet was separated into 50 fractions by passing it through a Sephadex G-25 column in order to desalt the lysate. Optical density at 280 nm was measured for each fraction. As shown in Fig. 3, 3 peaks were clearly indicated on the curve. AA activity was found in the bottom peak ranging from the 14th to 17th fractions. UV absorption spectra of these four fractions are shown in Fig. 4. Each of these showed a similar pattern having the lowest limit at 260 nm and the highest at 275 nm. The mixed solution of the 4 fractions gave an activity of 128.

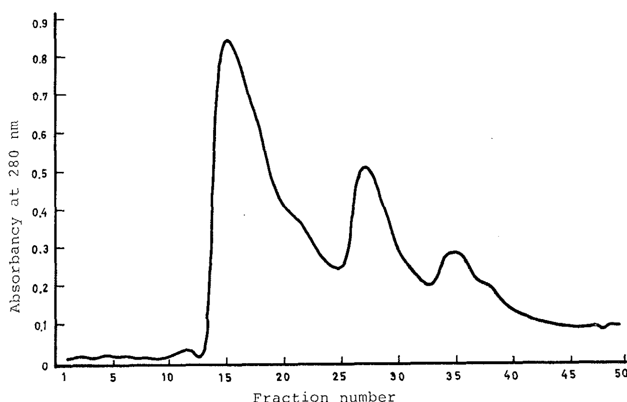

Fig. 3. Separation of active principle from the lysate obtained from the culture filtrate of the isolate P 7 using Sephadex G-25 column. Antibacterial activity was found in the bottom peak from 14th to 17th fraction.

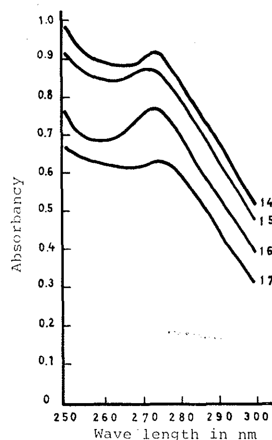

Fig. 4. UV rays absorbancy spectra of active fractions obtained by gel-chromatography (Fig. 3). The figures on the right side of each curve indicate the number of fractions shown in Fig 3.

Discussion

Nakatani and Tsuyama[7] confirmed that two kinds of AAs were produced by isolates of *E. carotovora*. One was concerned with the formation of a large inhibition zone and was inactivated by trypsin treatment. The other was concerned with the formation of a small inhibition zone and was not inactivated by trypsin. In the present study, the authors used mainly the isolate which have the ability to produce only the latter type of AA, with the exception of one isolate (CP) which have the ability to produce both kinds of AAs[7].

The activity of the latter types of AA was evidently increased by the induction with UV or mitomycin C. The increase occurred in accordance with the lysis of bacterial cells. The AA is assumed to be liberated from lysed cells after induction, as already reported by Jacob et al[3]. It is known that bacteriocin and temperate phage are the antibacterial agents which are liberated from a bacterial cell and inhibit the growth of the other isolates or strains of the same species and related genera. The production of these agent has been known to be controlled by an episomic or plasmid gene[6]. The bacterial strain of *E. carotovora* will liberate bacteriocin or temperate phage by induction in the lysate or in IZ, if it has genetic material for producing them in a latent state.

Is it the bacteriocin or temperate phage that shows an inhibition of growth on the indicator? The AA present in IZ or in lysate was examined for the ability to reproduce within a sensitive cell using 3 methods which have been used for the detection of temperate phage[9]. However, none of these showed signs of reproduction.

The activity of AAs is lost by heat treatment, but is retained in dialized solution and in the precipitate obtained by salting out with ammonium sulphate. The AAs reported in the present paper were not affected by trypsin treatment[7,10].

The AA was fractionated by column chromatography using Sephadex G-25 as the bottom peak of protein and showed a UV absorption pattern peculiar to protein. The AA was sedimented by ultra-centrifugation. From the facts mentioned above, it is thought that the AA produced from isolates of *E. carotovora* is a kind of large molecule bacteriocin according to the classification by Bradley[1].

Hamon and Péron[4] have reported at first that 7 out of 9 strains of *Erwinia* spp. showed the ability to produce bacteriocin by induction, and they[5] have given the name "caratovoricin" to these. Some of the "caratovoricin" were also active against other genera, for example, *Escherichia coli* K 12 and B, some strains of *Pseudomonas fluorescens*, of *Sordaria* spp. and a strain of *Xanthomonas pruni*[4]. They have also shown that some strains of *E. carotovora* are sensitive to some types of colicins[4]. The authors have evidence that some AAs have bactericidal activity toward *E. coli* K 12 (unpublished data), but do not have the evidence concerning the activity toward strains of other genera. It is not clear whether the AAs discussed in the present article are identical to "caratovoricin" or not.

As a result of examination of the activity spectrum of the 17 AAs, they were divided into 4 groups based on the similarity of their activity spectrum. The ability of bacteriocin formation is said to be controlled by an episomic or plasmid gene[6]. Consequently, the isolates of each group have different kinds of genes corresponding

to the different kind of bacteriocin production. Further investigation using more isolates of the same species and other related species may enable us to classify them into distinct groups as in the case with *Pseudomonas solanacearum* reported by Okabe[8].

It is interesting to note that some isolates showed spontaneous AA production in low activity and induced production in high activity, although most of the other isolates have shown production of AA only after induction. Such a difference seems to be an ostensible phenomenon. It is possible that the spontaneous production of AA resulted from a small population of cells of the isolates. Some of them originally contain a sufficient number of cells producing AA in detectable amount without induction, while the others may not. In both cases, the induction treatment increases the population of cells producing AA.

Acknowledgement

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和文摘要

Erwinia carotovora による抗細菌性物質の生成およびその諸性質

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Erwinia carotovora 諸菌株による抗細菌性物質の生成，作用域およびその諸性質について研究した。この物質の生成は，対数増加初期の細菌細胞に紫外線照射あるいはマイトマイシンC処理を施すと著しく誘発され，その活性は，処理後3～5時間の培養期間におこる溶菌に対応して増加する。抗細菌性物質の活性は菌株によって異なるが，誘発法による差は認められなかった。供試17菌株はすべてこれらの物質を生成するが，これらの物質はそれを生成する菌株に対して活性を示さない。これらの物質はそれぞれの作用域の異同を比較した結果，暫定的に4群に分類された。各菌株によって生成されたこれらの物質は，それぞれの感受性菌細胞内で増殖できない。これらの物質の活性は4Cで保存した場合少なくとも20日間は維持される。また透析されないが，熱処理によって失活する。活性物質は硫安塩析によって沈殿する。この物質の部分的精製標品の紫外部吸収曲線は275nmに最大吸収値をもち，260nmで最少吸収値を示した。これらの物質は分子量の比較的大きな蛋白質と考えられる。これらの諸事実から，これら物質はバクテリオシンの一種であろうと結論した。