STUDIES ON THE MYXOBACTERIUM *CHONDROCOCCUS COLUMNARIS*[1,2]

II. BACTERIOCINS

ROBERT L. ANACKER[3] AND ERLING J. ORDAL

Department of Microbiology, University of Washington, Seattle, Washington

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Chondrococcus columnaris, an aquatic myxobacterium with a complex developmental cycle, has been found to be pathogenic to fishes. It was first observed by Davis (1922) as an agent of disease in warm water fishes in the Mississippi Valley. The myxobacterial nature of *C. columnaris* was first recognized by Ordal and Rucker (1944) who found the organism to be the etiological agent in outbreaks of disease in young and adult salmonid fishes in the region of the upper Columbia River. In other studies, *C. columnaris* has been found in epizootics among both warm and cold water fishes in various regions of the United States. (Nigrelli and Hutner, 1945; Davis, 1949; Johnson and Brice, 1952).

C. columnaris has been found to be an important agent of disease among salmonid fishes in waters of the Pacific Northwest (Rucker *et al.*, 1953). Outbreaks of columnaris disease ordinarily occur only during the summer months and damage to populations of salmonid fishes is usually directly related to water temperature. Although the main stem of the upper Columbia River is normally a relatively cold stream, evidence has been obtained which indicates that columnaris disease represents a serious hazard to runs of salmonid fishes in the Columbia River and its tributaries. In earlier studies (Rucker *et al.*, 1953) it was found that strains of *C. columnaris* isolated from fishes taken in various waters of the State of Washington differed greatly in virulence. Of particular consequence was the finding that some of the strains of *C. columnaris* isolated from adult and young salmon from the upper Columbia River and its tributaries exhibited extraordinary virulence as compared to other strains from this region, and to the strains from Western Washington.

A study on *C. columnaris* and its role as an agent of disease in fishes was undertaken, with special emphasis given to evaluating the effects of columnaris disease on runs of salmonid fishes in the Columbia River System. Since a method of identification of specific strains of *C. columnaris* was needed, an attempt was made by Anacker and Ordal (1959) to develop an adequate typing system for *C. columnaris*. In this study it was found that all available strains of *C. columnaris* possessed a common antigen and one or more type antigens, and that the strains could be separated into four general serological groups on the basis of several type antigens. On application of the typing system to the available strains of *C. columnaris*, it was found that the origin and virulence of the strains could not be correlated with serological type. However, the serological groups were large and provided relatively little information on the interrelationships of the strains. Although there was evidence that the serological system could be refined, it was decided instead to explore the possibility of subdividing the serological groups by bacteriophage typing.

A bacteriophage which lysed a strain of *C. columnaris* had been discovered earlier (Anacker and Ordal, 1955), and it seemed likely that temperate phages could be obtained from lysogenic organisms. On testing a number of filtrates of liquid cultures of various strains of *C. columnaris*, it was unexpectedly found that these filtrates did not contain bacteriophages, but contained instead specific bactericidal substances probably similar to colicins (Frédéricq, 1957; Hartman, 1957). Studies on the bacteriocins

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[3] Present address: Department of Bacteriology, Montana State University, Missoula, Montana.

produced by strains of *C. columnaris* and the application of these substances to the problem of classification are described in the present report.

MATERIALS AND METHODS

Sources of strains. Most of the strains of *C. columnaris* mentioned in the present study were isolated from fishes taken at a number of different locations in the Columbia River or its tributaries. All of these strains were isolated before 1957 and were recovered either from external lesions or from the internal organs of fish. Separate isolates of *C. columnaris* were labeled and identified by numbers or other codes and were preserved by the lyophile process.

Cultivation of strains. For this study the organisms were grown either in a tryptone broth medium or on tryptone agar. The liquid medium contained tryptone (Difco), 0.4 per cent; yeast infusion, 3 per cent; and Tween 80, 0.1 per cent. When the organisms were grown in a solid medium for test purposes, the double agar layer technique was used. The base agar consisted of tryptone (Difco), 0.4 per cent; and agar (Difco) 1.5 per cent. The overlay agar contained tryptone (Difco), 0.4 per cent; NaCl, 0.06 M; and agar (Difco) 0.7 per cent. All media were adjusted to pH 7.2 to 7.4.

Preparation of the bacteriocins. Stocks of the bacteriocins were prepared from the supernatants of 16-hr broth cultures of the bacteriocin-producing strains. The supernatants were sterilized before use by storing them 1 to 2 days at 4 C with an excess of chloroform.

Testing of the bacteriocins. The activities of the bacteriocins of *C. columnaris* were determined in essentially the same manner as Craigie and Yen (1938) determined the lytic ranges of typing phages for *Salmonella typhi.* Two-tenths ml of log-phase cultures in the optical density range 0.08 to 0.12, as determined with the Coleman Nepho-Colorimeter, were incorporated into 3 ml of melted and cooled overlay agar, and the mixture was poured over 30 ml of the hardened base agar in a petri dish. After the overlay agar had solidified, the bacteriocin preparations were spotted on the surface of the overlay agar with a platinum loop 2 mm in internal diameter. The plates were examined for inhibition of the bacterial growth after approximately 18 hr of incubation at 28 C.

RESULTS

Discovery of bacteriocins. The bacteriocins of *C. columnaris* were first observed during attempts to isolate temperate phages by spotting culture supernatants on heterologous strains (Fisk, 1942). It was found that the supernatants of 10 different mixtures each containing 5 strains of *C. columnaris* inhibited growth of many of the 50 constituent strains which were tested individually. In a few instances individual plaques were noted in the areas spotted with the supernatants. Some of the data from this experiment are presented in table 1.

TABLE 1

Reactions of certain strains of Chondrococcus columnaris in overlay agar to supernatant fluids from broth cultures of mixtures of strains of this organism

Indicator Strain	Supernatant Fluid No.*									
	1	2	3	4	5	6	7	8	9	10
1-U-8	−	+	−	−	−	++	±	−	−	−
1-M-7	−	+	−	−	−	++	+	−	−	−
1-HR-1a	−	++	−	−	−	++	−	−	−	−
1-S-13b1	−	−	−	−	−	++	−	−	−	−
1-R-7	−	−	++	−	++	+++	−	−	−	−
2-M-5	−	−	−	−	−	++	−	−	−	−
2-S-1a	−	−	++	++	++	++	−	−	−	−
1-R-13	−	−	−	−	−	++	−	−	−	−
2-O-3	P	+	−	P	P	+++	+	−	−	P
4-O-2	−	+	−	−	−	++	−	−	−	−

* +++ = Zone of inhibition clear or with little growth; ++ = considerable growth in distinct zone of inhibition; + = zone of inhibition barely perceptible; ± = zone of inhibition doubtful; and P = plaques.

Since individual plaques were observed in a few instances, as recorded in table 1, it seemed probable, at this point in the investigation, that all the zones of inhibition were phage-induced. However, evidence from subsequent experiments proved this first assumption to be incorrect in most cases. First, it was found that the growth-inhibiting agents could not be transmitted with the broth eluates of portions of the agar removed from areas of inhibition on 11 different plate cultures. Second, plaques were not produced when dilutions of several growth-inhibiting supernatants were spotted on sensitive cells. Finally, phage could not be observed when pseudo-replicates of the surface of zones of inhibition on several plates were examined with an electron microscope using the method of Hillier and Baker (1946). A bacteriophage of *C. columnaris* had already been successfully demonstrated by this method (Anacker and Ordal, 1955). It was apparent from these and other observations presented in a later section of this report that specific, lethal, and nontransmissible substances resembling the colicins of *Escherichia coli* were produced by strains of *C. columnaris*.

Bacteriocin typing of strains of C. columnaris. Several reports which suggested that bacteriocins could be used to type members of a bacterial group have appeared in the literature. Frédéricq and Levine (1947) found that all of the serotypes of *Salmonella schottmuelleri* and *Shigella sonnei* tested were susceptible to the colicins of specific cultures. Fastier (1949), however, found that 1 of 4 strains of *Shigella paradysenteriae* type XI was not sensitive to an antibiotic substance produced by a strain of *Shigella paradysenteriae* type III. In addition, Fastier obtained antibiotic-resistant mutants which were serologically indistinguishable from the parent organism. These reports indicated that not only could representatives of specific serological types be identified with bacteriocins but also that strains of a given serological type could be further subdivided with bacteriocins.

Before a bacteriocin typing study of *C. columnaris* could be undertaken, it was first necessary to select strains which produced bacteriocins which differed in their spectrum of activity. Fifty strains of *C. columnaris* were screened for activity by spotting chloroform-treated supernatant fluids of broth cultures on a number of strains on solid media. Then each of 14 supernatant fluids, selected from the 50 which were screened, were spotted on the 50 strains. A portion of the results obtained with the supernatant fluids of 7 strains are presented in table 2; the patterns of activity of the supernatant fluids of the remaining 7 strains did not differ from those which are recorded. (The activities of supernatant fluids 2-O-20 and 1-S-9a2 listed in table 2 are identical; but differences in specificity of these bacteriocins will be demonstrated later in this report (table 3).

It may be observed from the data of table 2 that there is a partial correlation between the

TABLE 2

Response of strains of Chondrococcus columnaris in overlay agar to supernatant fluids from broth cultures of this organism

Indicator Strain	Serological Group	Supernatant Fluid (Strain No.)/(Serological Type)						
		2-O-20/(I)	3-O-28/(I)	1-S-9a2/(I)	2-S-1a/(II)	3-O-15/(III)	1-T-12/(III)	2-M-7/(IV)
1-HR-1a	I	−	−	−	++	−	−	−
2-O-20	I	−	−	−	++	−	−	−
1-M-7	I	−	−	−	++	−	++	−
2-M-13	I	−	−	−	++	−	++	−
1-S-9a2	I	−	−	−	++	++	−	−
1-R-13	I	−	−	−	++	++	−	−
2-M-2	II	++	+++	+++	−	+++	+++	++
2-S-1a	II	++	++	++	−	++	++	++
3-O-15	III	++	++	++	++	−	−	−
1-T-12	III	−	++	−	+++	+	−	−
4-O-10a	III	−	++	−	+++	−	−	−
2-M-7	IV	−	+	−	+	−	−	−

TABLE 3

Response of representative strains of Chondrococcus columnaris in overlay agar to selected bacteriocin preparations

Strain	Serological Group	Source of Bacteriocin (Strain No.)/(Serological Type)							
		2-O-20/(I)	3-O-28/(I)	1-S-9a2/(I)	2-S-1a/(II)	3-O-15/(III)	1-T-12/(III)	2-M-7/(IV)	Bacteriocin Type
1-M56-11	I	−	−	−	++	−	−	−	A
1-Ro56-6	I	−	−	P	+++	−	−	−	A
2-S56-1b	I	−	−	−	++	−	++	−	B
1-O56-1a	I	−	−	−	+++	−	++	−	B
1-R56-3	I	−	−	P	+++	++	−	−	C
3-R56-15	I	−	−	−	+++	++	−	−	C
3-M56-8	II	++	++	++	−	++	++	++	D
1-Ro56-3	II	+++	+++	+++	−	+++	+++	+++	D
1-Ro56-26	III	++	++	++	+++	++	++	++	E
4-R56-30	III	++	++	++	++	++	++	++	E
3-R56-31	II	++	++	++	++	−	++	++	F
4-R56-15	I	+++	+++	++	++	−	+++	++	F
1-B56-14	III	−	++	−	+++	−	−	−	G
4-R56-14	IV	−	++	−	++	−	−	−	G
1-Ro56-4	IV	++	++	−	++	−	−	P	H
2-R56-10	IV	++	++	−	++	−	−	−	H
1-O56-5c	III	++	++	++	++	−	−	−	I

antigenic nature of a test organism and resistance to bacteriocins produced by organisms of the same serological group. For example, 1-HR-1a, a serological group I strain, is susceptible to the action of the bacteriocin produced by 2-S-1a, a serological group II strain, but strain 1-HR-1a is resistant to the bacteriocins of the 3 strains of serological group I. Similarly, strain 2-M-2, a group II strain is inhibited by all the bacteriocins listed in table 2 except the one produced by a strain of group II. However, exceptions to this general rule have been noted, and the mechanism of the immunity of certain strains to the bacteriocins produced by serologically related strains has yet to be elucidated.

Because the sample strains listed in table 2 varied in their response to the action of the bacteriocins, it seemed feasible to type strains of *C. columnaris* on the basis of their sensitivity to selected bacteriocins. Cells from each of the strains of *C. columnaris* isolated during 1956 were plated and spotted with the same 7 bacteriocin preparations mentioned above. As a control each of the strains was spotted with chloroform-treated tryptone broth. Distinct patterns of sensitivity of the strains to the bacteriocins were again observed, and the reactions of strains representative of the 134 strains examined are presented in table 3. One interesting fact learned from this experiment is that a single strain may produce both a bacteriophage and a bacteriocin which exhibit different host ranges for their respective activities. Strains 1-S-9a2 and 2-M-7 produced phages which attacked only strains of the same serological group as the lysogenic host and bacteriocins which attacked primarily strains which differed antigenically from the bacteriocin-producing strains. Figure 1 shows the typical zones of growth inhibition produced by the bacteriocins.

The strains in table 3 have been grouped according to the response of the strains to the bacteriocins. For example, the strains which are sensitive to the bacteriocin of 2-S-1a are placed in bacteriocin type A; bacteriocin type B strains are susceptible to the bacteriocins of strains 2-S-1a and 1-T-12. In this manner each of the strains isolated in 1956 was classified into 1 of 9 bacteriocin types. A summary of the bacteriocin typing of strains of *C. columnaris* isolated in 1956 is presented in table 4. Again the partial correlation between serological group and bacteriocin type may be noted.

Properties of bacteriocins of C. columnaris. Some

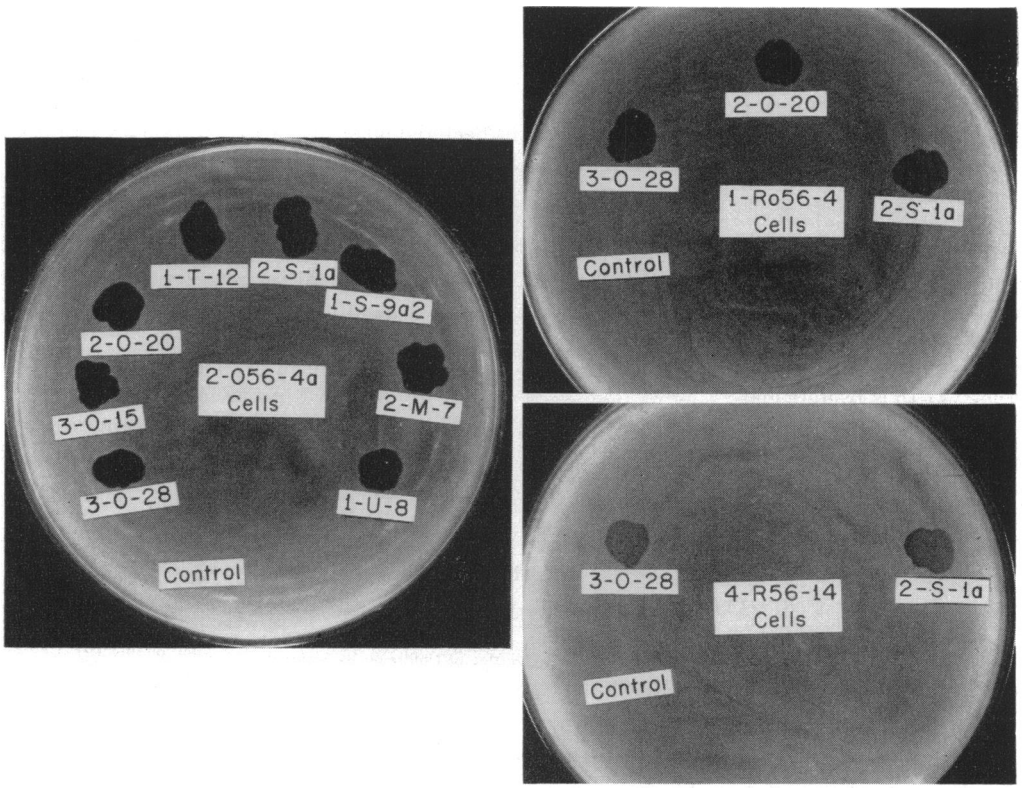

Figure 1. Several patterns of growth inhibition produced by typing bacteriocins spotted on overlay agar plates of several strains of *Chondrococcus columnaris*. Each of the plates was spotted with 8 bacteriocins and the control mixture, but only those areas spotted with bacteriocins which inhibited growth have been labeled.

TABLE 4

Relationship between serological group and bacteriocin type of strains of Chondrococcus columnaris isolated in 1956

Serological Group	Bacteriocin Type								
	A	B	C	D	E	F	G	H	I
I	54	17	6	0	3	1	1	0	0
II	2	0	0	23	8	1	0	0	0
III	1	0	0	0	3	0	7	0	1
IV	0	0	0	0	0	0	2	4	0
Total. . . .	57	17	6	23	14	2	10	4	1

TABLE 5

Bactericidal effect of the supernatant of a broth culture of strain 3-O-15 on the viable count of strain 4-R56-1

Time	Avg Colony Count		*A* (corrected)/*B*
	A, Cells plus supernatant (10^{-5})	*B*, Cells plus sterile broth (10^{-6})	
min			
0	—	222	—
30	55	276	0.020
60	63	270	0.023

of the properties which characterize colicins are specificity, nontransmissibility, diffusibility, lethality, and heat-stability (Frédéricq, 1957). Several of the characteristics of the bacteriocins of *C. columnaris* have been found to be similar to those of the colicins. The properties of specificity and nontransmissibility of the bacteriocins of *C. columnaris* have been discussed above; other properties which have been studied will now be presented.

Several experiments were performed in order to demonstrate that the substances found in cultures of *C. columnaris* were actually bactericidal and not merely inhibitory. In one of these experiments 2 ml of a bacteriocin preparation of strain 3-O-15 were added to 8 ml of a log-phase culture of a sensitive strain, strain 4-R56-1. Two ml of sterile broth were added to the control tube also containing 8 ml of the same culture. At 0, 30, and 60 min, pour plates were made in duplicate from dilutions of aliquots removed from the cultures. The plate counts, presented in table 5, provide strong evidence that the substance produced by strain 3-O-15 is bactericidal.

The diffusibility of the bacteriocins was not determined, but the filterability of these substances was proved. Dilutions of chloroform-treated bacteriocin preparations were spotted on indicator strains before and after the supernatants had been filtered through Chamberland L3 filters. It may be concluded from the data of table 6 that the bacteriocins pass through an ordinary bacterial filter, although filtration does reduce the concentration of the bacteriocins.

Colicins vary considerably in their sensitivity to heat. Jacob *et al.* (1952) reported that the colicin produced by *Escherichia coli* strain ML was reduced to 20 per cent activity after 10 min at 100 C. Gardner (1950) found that the activity of colicin D was markedly reduced after 30 min at 70 C, but colicin V, on the other hand, was little affected by autoclaving 20 min at 121 C.

The heat-stability of 2 bacteriocins of *C. columnaris* was examined. There was a negligible loss in activity of bacteriocins 2-S-1a and 3-O-15 after a heat treatment of 52 C for 32 min. How-

TABLE 6

Effect of filtration upon the activity of several bacteriocins

Indicator Strain	Bacteriocin Donor	Dilution of Unfiltered Bacteriocin					Dilution of Filtered Bacteriocin			
		10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^0	10^{-1}	10^{-2}	10^{-3}
1-Ro56-21	2-S-1a	+++	++	±	−	−	+++	++	−	−
3-R56-4	2-S-1a	+++	++	−	−	−	+++	++	−	−
4-R56-1	3-O-15	+++	+++	++	+	−	+++	++	+	−
4-O56-5c	3-O-15	+++	++	+	−	−	++	+	−	−

TABLE 7

Relationship between phase of growth of broth cultures of Chondrococcus columnaris and concentration of bacteriocin

Source of Bacteriocin	Age of Culture	Optical Density	Indicator Strain	Dilution of Bacteriocin				
				10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}
	hr							
2-S-1a	1	0.036	1-Ro56-21	±	−	−	−	−
	4	0.120		++	+	−	−	−
	6	0.360		+++	++	−	−	−
	8	0.580		+++	++	−	−	−
	10	0.652		+++	++	−	−	−
	12	0.670		+++	++	±	−	−
	24	0.720		+++	++	−	−	−
3-O-15	1	0.018	4-R56-1	+++	+	−	−	−
	4	0.038		+++	++	+	−	−
	6	0.168		+++	++	++	−	−
	8	0.400		+++	+++	++	−	−
	10	0.536		+++	+++	++	+	−
	12	0.590		+++	+++	++	±	−
	24	0.620		+++	+++	++	−	−

ever, a heat treatment of 1 min at 100 C completely inactivated these same bacteriocins. From this limited experimental evidence it appears that the heat resistance of the bacteriocins of *C. columnaris* resembles that of colicin D much more more than it does that of colicin V.

In order to harvest the bacteriocin preparations at the optimal time it was necessary to determine when the concentration of the bacteriocins in broth cultures was highest. Flasks containing 150 ml of tryptone broth plus 0.1 per cent Tween 80 were inoculated with 12 ml of 24-hr broth cultures of strains 2-S-1a and 3-O-15. At intervals aliquots of the flask cultures were removed, the optical densities of the samples were recorded, the cells were removed by centrifugation, and the supernatants were treated with chloroform. Then serial 10-fold dilutions of the bacteriocins were spotted on sensitive cultures in overlay agar. The data presented in table 7 indicate that maximal bacteriocin concentration of these cultures was attained by the end of the log phase of growth and that this level remained fairly constant until at least the end of the first 24 hr. It may be noted that the culture of 3-O-15 possessed measurable bacteriocin activity at 1 hr, even though growth was barely detectable. Presumably this activity was carried over with the inoculum from the 24-hr culture.

DISCUSSION

An intraspecific antagonism among myxobacteria has also been reported by Tchan and Giuntini (1950) who discovered that a strain of *Sporocytophaga myxococcoides* produced an antibiotic substance which inhibited the growth of 2 other strains of the same species. Perhaps the ability to produce intraspecific inhibitory substances has survival value for individual strains in an ecological environment physiologically suitable for closely related organisms.

The mechanism for the specificity of the bacteriocins of *C. columnaris* has not been explained, but the mechanism is probably similar to the one demonstrated by Frédéricq (1946) for the colicins of *E. coli*. Frédéricq showed that cells of *E. coli* possess specific receptors for each of several different colicins. It may be postulated that (a) cells of *C. columnaris* also possess multiple specific receptors for the bacteriocins, and, as a corollary, (b) cells are sensitive only to those bacteriocins for which the cell possesses receptors. For example, strains of bacteriocin type B may have at least 2 distinct receptors, one for bacteriocin 2-S-1a and a second for bacteriocin 1-T-12. Strains of bacteriocin type A, however, may have lost through mutation the receptor for bacteriocin 1-T-12 and therefore are sensitive only to bacteriocin 2-S-1a.

Perhaps some relationship exists between the number and type of bacteriocin receptors and the antigens characteristic of the serological groups. With only 5 exceptions, all of the strains of serological group I are placed in bacteriocin types A, B, and C. In addition, 23 of the 32 strains of serological group II are classified as bacteriocin type D. It may be suggested from this evidence that certain of the antigenic determinants and bacteriocin receptors are either closely related or identical.

Even though all of the factors involved in the specificity of the bacteriocins are not completely understood, bacteriocins have been applied to the typing of strains of *C. columnaris*. The 134 strains under investigation were separated into only 4 groups by the serological methods available, but, with the use of selected bacteriocins, these same 134 strains were subdivided into 9 bacteriocin types. It is probable that additional bacteriocin types could be characterized after further investigation.

It is of interest to note that in so far as the numbers of strains typed are of significance, the results of bacteriocin typing support the conclusions reached on the basis of serological typing. Strains of specific serological or bacteriocin type are not localized in any particular region of the Columbia River System, but instead representative types have been found in the several district geographical areas where cultures of *C. columnaris* have been obtained.

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SUMMARY

Many strains of the myxobacterial fish pathogen, *Chondrococcus columnaris* have been found to produce bacteriocins which are filterable, nontransmissible, and lethal for certain other strains of *C. columnaris*. The bacteriocins of *C. columnaris* appear to be similar to the colicins produced by the enteric bacteria.

The 134 strains of *C. columnaris* isolated during the summer of 1956 were divided into 9 bacteriocin types on the basis of their sensitivity to 7 selected bacteriocin preparations. By serological methods these same 134 strains could be divided into only 4 groups. Some correlation exists between resistance to specific bacteriocins and the antigenic nature of the organisms.

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