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# Purification and Genetic Determination of Bacteriocin Production in *Enterobacter cloacae*

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*Enterobacter cloacae* (strain DF13) was found to produce a bacteriocin which could be induced by mitomycin C. In the supernatant fluid of the induced culture phagelike particles were found. The bacteriocin was partially purified from induced cultures by ammonium sulfate precipitation and gel-filtration on Sephadex G-150. Ultraviolet-absorbing material was eluted from the Sephadex column in three fractions. The biological activity was mainly present in the second fraction and is associated with a protein with a molecular weight of about 61,000. The phagelike particles were found in the first fraction and show no biological activity. Upon conjugation of *E. cloacae* strain DF13 with another strain of the same species and with *Escherichia coli* K-12S, the ability to produce bacteriocin was transferred. The new bacteriocinogenic strain produced bacteriocin, which could not be distinguished from that produced by *E. cloacae* strain DF13. Although transfer of the bacteriocinogenic factor often occurred together with transfer of the ability to produce phagelike particles, it was shown that these two factors are two separate genetic entities. In addition to a bacteriocinogenic factor, *E. cloacae* strain DF13 was found to carry two other transferable plasmids: one determining resistance against streptomycin and sulfanilamide and another determining resistance against penicillin.

Bacteriocins are highly specific antibiotics produced by many bacteria and are active against strains of the same or related species. Many bacteriocins have been described and classified (26), but only a few of them have been studied in detail. The characteristic property of bacteriocins is their ability to kill sensitive cells, but not to multiply within them. Bacteriocins are produced spontaneously or after induction. The ability to produce colicins is determined by a genetic factor (colicinogenic factor) which behaves as a cytoplasmic element and which can be transferred from colicinogenic to noncolicinogenic bacteria by conjugation (6, 25). Many bacteriocins have been found in lysogenic or defect lysogenic strains. Fredericq (12) and Ivánovics (16) suggested that bacteriocins may represent the products of defective lysogeny and therefore may vary from simple proteins to complete phage particles.

In an electron microscopic examination of colicinogenic strains, Kellenberger and Kellenberger (19) found many phagelike particles but could not identify the structural components as being the bacteriocinogenic principle. Partially purified preparations of colicin 15, an inducible bacteriocin from *Escherichia coli* WT15, show an abundance of phagelike particles and intact

phages (9, 27). Thus, *E. coli* WT15 is a defective lysogenic strain, and colicin 15 is a bactericidal component of its phage. A purified pyocin isolated from *Pseudomonas aeruginosa* strain R, homogeneous in ultracentrifugal analysis (18), consisted of headless tails of contractile bacteriophages (17). Other phagelike particles, which are apparently identical to bacteriocins, have been isolated from several *Bacillus* species (3, 29, 30). More recently, Bradley and Dewar (4) described the structure of phagelike particles from bacteriocinogenic strains of *E. coli*, *P. aeruginosa*, and *Listeria monocytogenes*. They concluded that some of these phagelike particles are responsible for the bacteriocinogenic activity. On the other hand, some bacteriocins are known which are apparently proteins with relatively low molecular weight. Megacin, a bacteriocin produced by *B. megaterium*, has a molecular weight of about 51,000 (15), and colicin A, a bacteriocin produced by *Citrobacter freundii* CA31A, has a molecular weight of 50,000 (2). These preparations have not been investigated under an electron microscope.

If bacteriocins represent the products of defective lysogeny (12, 16), it will be necessary to accomplish isolation and purification of bacteriocins by use of electron microscopy; if bacteriocinogeny is associated with the production

of phagelike particles, the relation between both should be clarified. This was the purpose of our study. In this paper, the purification of a previously detected (31) bacteriocin produced by *Enterobacter cloacae* is reported. In addition to the bacteriocin of relatively low molecular weight, this strain also produces phagelike particles without bacteriocinogenic activity. By conjugation experiments, it could be shown that bacteriocinogeny can be transferred with or without the ability to produce phagelike particles. It is concluded that in this case the bacteriocin is not a product of the multiplication of the phage-like particles.

#### MATERIALS AND METHODS

**Organisms.** The characteristics of the organisms used are presented in Table 1. *E. cloacae* strain DF13 was found to be resistant to 2,400 µg of penicillin per ml and to 500 µg of streptomycin per ml in Brain Heart Infusion medium and to 800 µg of sulfanilamide per ml in minimal medium supplemented with 1% Casitone. The strain is sensitive to tetracycline, kanamycin, and chloramphenicol. Strain S478 was isolated from strain S458 after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by the method of Adelberg, Mandel, and Chen (1).

**Media.** Cultures were usually grown in dialyzed

3.7% Brain Heart Infusion (Oxoid), designated as BHI medium. Brain Heart Agar contained 3.7% BHI and 1.2% agar (Davis Gelatine LTD, Christchurch, New Zealand). Soft agar contained 3.7% BHI and 0.8% agar (Difco). The composition of the minimal medium was as described by Hadjipetrou et al. (14).

**Chemicals.** Mitomycin C and trypsin were obtained from Sigma Chemical Co., St. Louis, Mo. Beef liver catalase (analytical grade, about 27,000 units/mg) was obtained from C. F. Boehringer & Soehne GmbH, Mannheim, Germany. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England.

**Buffer solutions.** Tris buffer was 0.05 M tris(hydroxymethyl)aminomethane chloride (pH 7.0) containing 10<sup>-3</sup> M MgSO<sub>4</sub>. Phosphate buffer was 0.02 M potassium phosphate (pH 7.5).

**Turbidity.** The turbidity (*E*) of growing cultures or suspensions of bacteria was measured at a wavelength of 660 mµ with an Unicam SP 600 spectrophotometer with 1-cm glass cells (Unicam Instruments Ltd., Cambridge, England).

**Chemical analysis.** Protein was determined by the method of Lowry (21) with the use of Folin's reagent; deoxyribonucleic acid (DNA), by the method of Burton (5); ribonucleic acid (RNA), by the method of Mejbaum (23); and sugars, by the method of Scott and Melvin (28).

**Assay of the killing activity of bacteriocin.** Samples

TABLE 1. *Characterization of the bacterial strains used*

Collection no.	Biotype	Strain	Properties <sup>a</sup>	Origin	Reference
S 458	<i>Enterobacter cloacae</i>	DF13	(Bac DF13)( <i>str-r sul-r</i> )( <i>pen-r</i> )		(13)
S 165		DF13	<i>arg thy</i> (Bac DF13)( <i>str-r sul-r</i> )( <i>pen-r</i> )	S 458	(13)
S 478		DF13	<i>ura arg</i> (Bac DF13)( <i>str-r sul-r</i> )( <i>pen-r</i> )	S 458	
H 456		O2			(13)
H 475		O2	<i>met</i>	H 456	(13)
H 478		O2	<i>his</i>	H 456	(13)
S 89		O2	<i>his bac</i> DF13-r	H 478	(31)
S 231		O2	<i>his</i> (Bac DF13)( <i>str-r sul-r</i> )( <i>pen-r</i> )	S 165 × S 89	
S 232		O2	<i>his</i> (Bac DF13)	S 165 × S 89	
S 237		O2	<i>met</i> (Bac DF13)( <i>str-r sul-r</i> )	S 231 × H 475	
S 238		O2	<i>his</i> ( <i>str-r sul-r</i> )	S 237 × S 89	
S 239		O2	<i>met</i> (Bac DF13)	S 165 × H 475	
S 15		<i>Klebsiella edwardsii</i> var. <i>edwardsii</i>			NTCC 7761
H 447	<i>Escherichia coli</i>	K-12S		KMBL	— <sup>b</sup>

<sup>a</sup> The following abbreviations are used: *arg*, *thy*, *ura*, *met*, and *his* indicate strains which grow on minimal medium supplemented with arginine, thymine, uracil, methionine, or histidine respectively (all at 20 µg/ml except thymine and uracil, which are provided at 10 µg/ml), no designation indicates strains able to grow without supplement; symbols placed in parentheses represent plasmids or properties which are located on a plasmid; *str-r*, *sul-r*, and *pen-r* indicates resistance to streptomycin (200 µg/ml), sulfanilamide (800 µg/ml), and penicillin (2,400 µg/ml), respectively. Bac DF13 is a genetic factor determining the production of bacteriocin DF13. Bac DF13-r indicates a strain isolated as resistant to the bacteriocin DF13 from strain S458.

<sup>b</sup> Rept. no. MBL/1965/8 of the Medical Biological Laboratory of the National Defence Research Organisation TNO, 1965.

of bacteriocin were tested by a modification of the method described by Fredericq (10, 11). A 3-ml amount of soft agar at 50 C, seeded with 0.1 ml of a fresh culture of the indicator strain S 15 (diluted to  $E = 0.200$  in BHI medium), was layered upon 20 ml of Brain Heart Agar. Drops (0.03 ml) of suitably diluted sample were spotted on the surface of the plates. After incubation overnight at 25 C, the bacteriocin titer was estimated. The bacteriocin titer (called "killing units" in this paper) was arbitrarily defined as the highest dilution which gave a clear inhibition zone. The specific activity of a bacteriocin solution is defined as "killing units" per milligram of protein.

For determination of the activity spectrum of the bacteriocin, we used the same double-layer method with various indicator strains as described by Stouthamer and Tieze (31).

*Preparation and purification of bacteriocin.* A 300-ml amount of an overnight culture of *E. cloacae* strain DF13 (S 458) was inoculated into 5 liters of fresh BHI medium in a Micro Ferm Laboratory Fermentor, model MF 114 (New Brunswick Scientific Co., New Brunswick, N.J.). The culture was incubated at 37 C with constant stirring (200 rev/min). The aeration rate was adjusted to 2 liters of air per min. When the turbidity of the culture had increased to extinction 0.5, mitomycin C (1.0  $\mu\text{g}/\text{ml}$ ) was added and incubation was continued for 10 min. The cells were then harvested by centrifugation and resuspended in fresh prewarmed BHI medium; incubation was continued for 3 hr. After this period, chloroform (7 ml/liter) was added, and the mixture was stirred for 5 min. Cells were removed by centrifugation at  $23,000 \times g$ , and ammonium sulfate was added to the supernatant fluid to obtain 40% saturation. After centrifugation of the precipitate, more ammonium sulfate was added to 60% saturation. The precipitate was allowed to stand overnight, and then was collected by centrifugation and dissolved in Tris buffer (pH 7.0). The concentrated bacteriocin solution was then dialyzed against the same buffer for 24 hr in cellophane tubing. This solution will be referred to as "crude bacteriocin." For further purification, 10 ml of "crude bacteriocin" was applied on a column of Sephadex G-150 (3 by 40 cm), which had previously been equilibrated with 0.05 M Tris buffer (pH 7.0). The column was then washed with the same buffer. Fractions were collected on an LKB 3400 B RadiRac fraction collector, and ultraviolet absorption at a wavelength of 280 m $\mu$  was measured with an Uvicord II LKB 8300, equipped with a LKB 6520 A Chopper Bar Recorder (LKB-Produkter AB, Stockholm, Sweden). All experiments were carried out at 4 C.

*Electron microscopy.* The supernatant fraction of a mitomycin C-induced culture was centrifuged for 1 hr at  $57,000 \times g$ . The pellet was suspended in 0.1 M ammonium acetate. Negatively stained specimens were prepared by depositing a drop of the sample solution on a carbon-coated grid. After 1 min, the excess of the solution was withdrawn by touching the edge of the grid with filter paper. Then a drop of a 2% solution of phosphotungstic acid adjusted to pH 7.0 with potassium hydroxide was deposited on the grid and

withdrawn after 1 min. When dry, the specimens were ready for electron microscopy. A Philips E.M. 200 electron microscope was used.

*Estimation of molecular weight.* The molecular weight of bacteriocin was estimated by the method of Martin and Ames (22). Catalase ( $S_{20,w} = 11.3S$ ) was used as standard enzyme (22). Samples to be tested were centrifuged until clear and mixed with catalase (10,000 units/ml, final concentration); 0.10 ml of this mixture was layered on 4.6 ml of a linear sucrose-gradient (5 to 20% sucrose in 0.05 M Tris buffer, pH 7.0). After centrifugation for 16 hr at  $135,000 \times g$  at 4 C, the centrifuge tube was punctured, and fractions of equal volume were collected. Each fraction was diluted with 2 ml of a 0.9% NaCl solution and analyzed for bacteriocin and catalase activity. Catalase was assayed by following the decrease in absorbancy at a wavelength of 240 m $\mu$  of a 2.5-ml reaction mixture containing 1.0 ml of potassium phosphate buffer, pH 7.5 (30  $\mu\text{M}$ ), 1 ml of H<sub>2</sub>O<sub>2</sub> solution (36  $\mu\text{M}$ ), and 0.5 ml of the diluted fraction, during 100 sec. Reactions were carried out in a Unicam SP 800 ultraviolet spectrophotometer (Unicam Instruments Ltd., Cambridge, England). The sedimentation coefficient (or approximate molecular weight) of the bacteriocin was determined by a simple ratio of mobilities of bacteriocin and catalase.

*Transfer of bacteriocinogeny.* Transfer experiments were carried out by means of the mixed culture method of Ozeki, Stocker, and Smith (25), modified in accordance with the results of Mulczyk and Duguid (24). The donor and acceptor bacteria could be distinguished by nutritional deficiencies of the donor or of both of them. All experiments were done at 37 C. Bacteria were grown in BHI medium for 18 or 48 hr, and then 6 ml of the donor culture was mixed with 2 ml of the acceptor culture. The mixture was incubated for 24 hr. After that, the culture was centrifuged, the sediment was resuspended in 0.9% NaCl solution, and 0.1-ml amounts of serial 10-fold dilutions were spread on plates of minimal salts-agar, supplemented, if necessary, with appropriate nutrients to allow growth of the acceptor bacteria only. The selective plates were incubated overnight, after which all colonies from a number of plates were reinoculated on plates of the same medium. Fifteen inocula at regular distances were used on each plate. After overnight incubation, the bacteria were killed by chloroform vapor. The plates were overlaid with 3.5 ml of soft agar, seeded with about  $10^7$  indicator bacteria. For isolation of acceptor cells which had also acquired resistance against antibiotics, a similar method was followed, except that before being killed colonies were replicated on selective plates appropriately supplemented with antibiotics and incubated overnight.

For rapid isolation of recipient cells which had received antibiotic resistance, the method of Datta, Lawn, and Meynell (7) was used. Donor and acceptor cells were grown for 6 hr in BHI, and then 1 ml of each was added to 8 ml of fresh broth and incubated for 24 hr. After that time, 0.1-ml amounts of serial 10-fold dilutions of the mixture were spread on a set of plates of minimal salts-agar with nutrients and

antibiotics to allow selection of acceptor bacteria which had acquired resistance against antibiotics. Then, individual colonies were isolated and tested for bacteriocin production.

### RESULTS

**Production of bacteriocin.** In BHI medium, cells of *E. cloacae* strain DF13 produced bacteriocin spontaneously. The amount of bacteriocin produced was relatively small; the supernatant fluid contained about 20 killing units/ml. Later, we found that mitomycin C could induce bacteriocin production, as has been described in a number of other cases (8, 20, 29). About 1  $\mu\text{g/ml}$  was most efficient for induction of bacteriocin formation in actively growing cultures. The time course of the production is shown in Fig. 1. After removal of mitomycin C, the turbidity of the culture increased steadily for about 45 min, and then suddenly fell, after which growth started again. During the first period in which the turbidity increased, there was no rise in the number of colony-forming cells. In this period, the formation of filamentous cells, as recently described by Suzuki, Pangborn, and Kilgore (32), was observed. The amount of bacteriocin liberated quickly rose after removal of the mitomycin C and then remained constant for several hours, after which the bacteriocin concentration decreased. Usually, about 10,000 killing units per

ml of supernatant fluid were attained. Induction with 0.1  $\mu\text{g}$  of mitomycin C per ml gave a bacteriocin production which was about one-tenth as great; with concentrations higher than 1.0  $\mu\text{g}$  of mitomycin C per ml, no increase in bacteriocin production could be attained.

**Partial purification of the bacteriocin.** Purification of the bacteriocin was performed as described in Materials and Methods. During gel filtration of "crude bacteriocin" on Sephadex G-150, the sample was separated into three fractions having ultraviolet absorption at 280  $m\mu$  (Fig. 2). The first fraction shows a small bacteriocin activity in addition to a high ultraviolet absorption. The major part of the bacteriocin activity is eluted in the second fraction with low ultraviolet absorption. The third fraction has no biological activity. Table 2 shows the specific activity at several steps of the purification procedure. As can be seen from this table, the specific activity increases 10-fold after ammonium sulfate precipitation and once again seven times after gel filtration. The identity of the bacteriocin during the purification is controlled by the activity spectrum. This spectrum is quite the same for the supernatant fluid, the "crude bacteriocin," and the purified bacteriocin eluted as the second fraction by gel filtration.

Upon investigation of the three fractions under an electron microscope, phagelike particles were found in the first fraction. In the second and third fractions, no phagelike particles nor other structural components could be detected.

**Chemical nature of the bacteriocin.** Biological activity of partially purified bacteriocin preparations is completely destroyed by incubation with trypsin. These preparations contain large amounts of protein as estimated by the Lowry test (21).

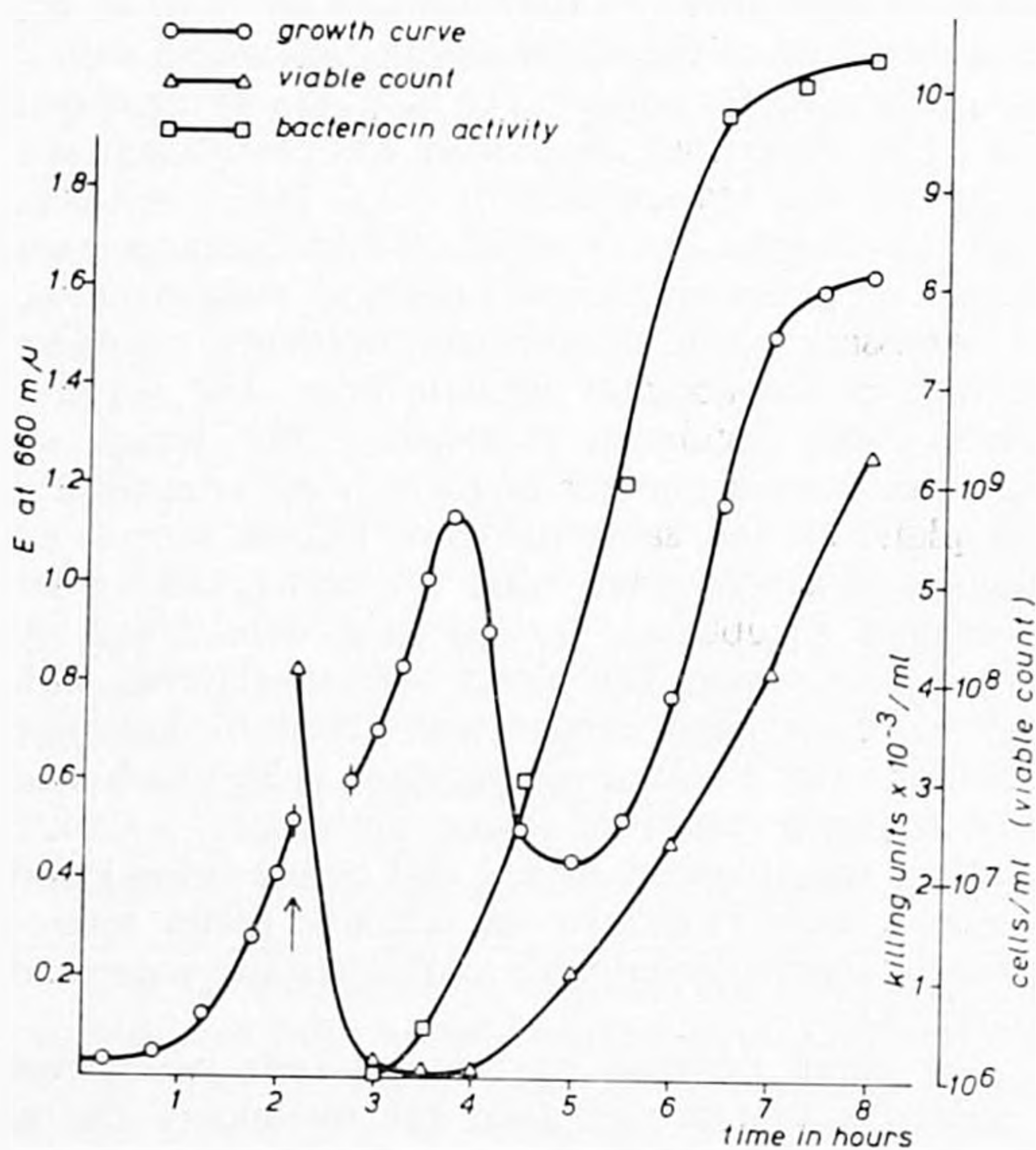


FIG. 1. Bacteriocin production by *Enterobacter cloacae* strain DF13 after induction with 1.0  $\mu\text{g}$  of mitomycin C/ml, added as indicated by the arrow. (The interruption in the growth curve is caused by centrifugation.)

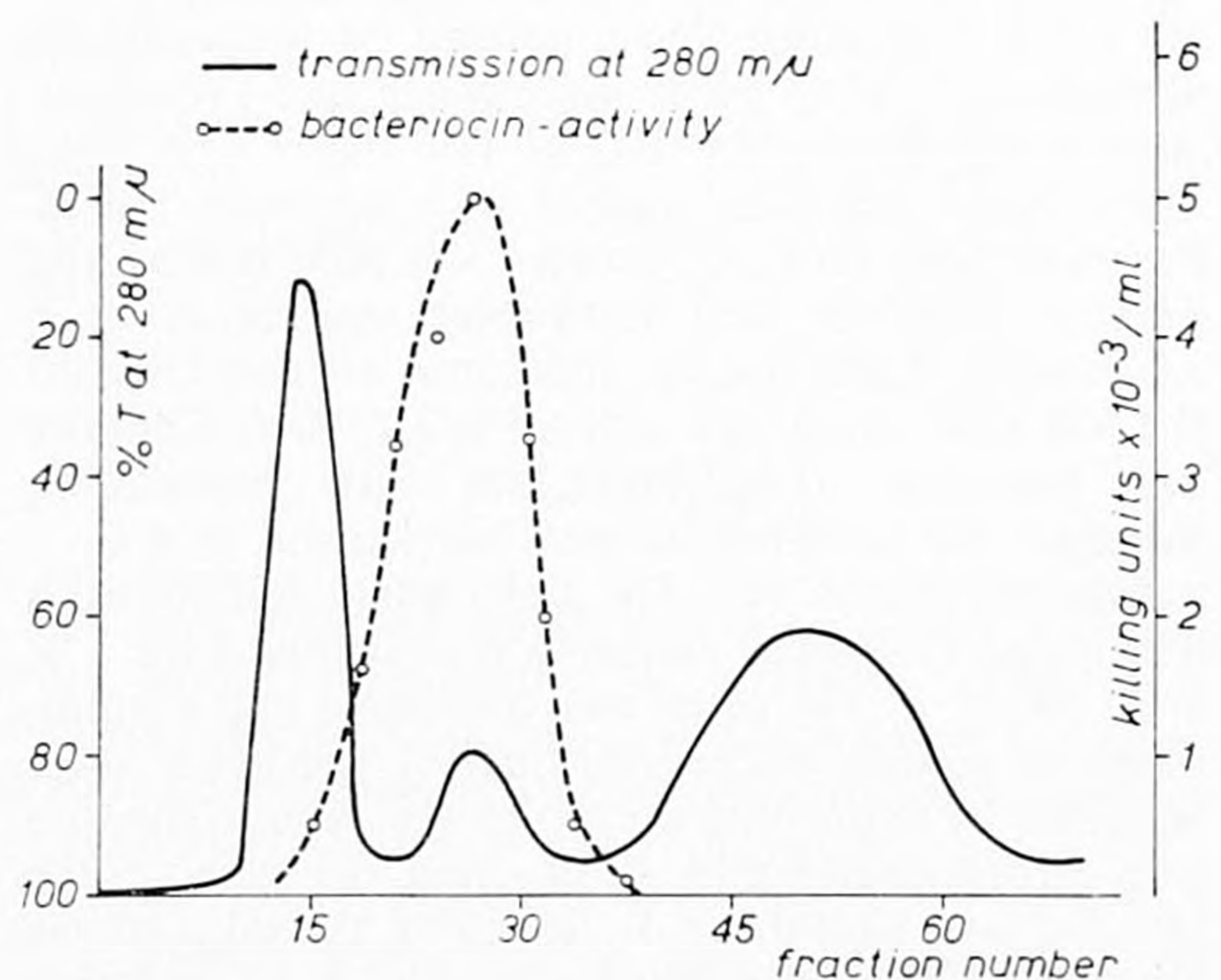


FIG. 2. Gel filtration of "crude bacteriocin" on Sephadex G-150.

TABLE 2. Purification of bacteriocin

Fraction	Specific activity $\times 10^{-3}$ (killing units/mg of protein)
Supernatant fluid.....	3.2
Crude bacteriocin.....	31
After gel-filtration (Fig. 2)	
Top of the first fraction.....	4
Top of the second fraction....	200
Second fraction (average).....	62.5
Third fraction.....	0

RNA, measured with the orcinol reagent (23), DNA, measured with the diphenylamine reagent (5), and sugars, measured with the anathrone reagent (28) are either completely absent or are present in negligible amounts (less than 2.5% of the amount of protein). We may conclude that the bacteriocin is a protein.

*Estimation of molecular weight.* To estimate the molecular weight of the bacteriocin, the sedimentation behavior of bacteriocin and catalase was studied in a sucrose gradient. The distribution of bacteriocin and catalase activity after centrifugation is shown in Fig. 3. From eight experiments, the sedimentation constant  $S_{20,w}$  of the bacteriocin could be calculated as  $4.4 \pm 0.2S$  at 3 C. Assuming a partial specific volume of  $0.73 \text{ cm}^3/\text{g}$ , the molecular weight of the bacteriocin can be calculated as  $61,000 \pm 4,000$ .

*Detection of phagelike particles by electron microscopy.* As can be concluded from the literature (4, 9, 17, 19, 27, 29, 30), cultures of some bacteriocinogenic bacteria contain detectable numbers of phagelike particles. After induction with mitomycin C ( $1.0 \mu\text{g}/\text{ml}$ ), the culture lysate of *E. cloacae* strain DF13 was therefore investigated under an electron microscope. After negative staining, we found phagelike particles (Fig. 4C). On the sheath, we could distinguish annuli, coarse helices, a base plate, and very delicate tail fibers. As can be seen (Fig. 4A), the tail is contractile. After contraction the core is exposed. Thus, this phage fits into the first class of morphological phage types of Bradley (3). The head exhibits a six-sided outline, which is best preserved (Fig. 4B) by addition of  $5 \times 10^{-3} \text{ M MgCl}_2$  to the culture medium, as described by Seaman et al. (29). If the head is empty, we can see within it a basal knob on the proximal end of the core. Two variants of this phage exist with respect to the dimensions of the head. Between 85 and 90% of the measured particles have a head with an axial diameter of 350 A (Fig. 4A, C), and 10 to 15% have a head with an axial diameter of about 540 A (Fig. 4B). Figure 4E shows both variants. With respect to the

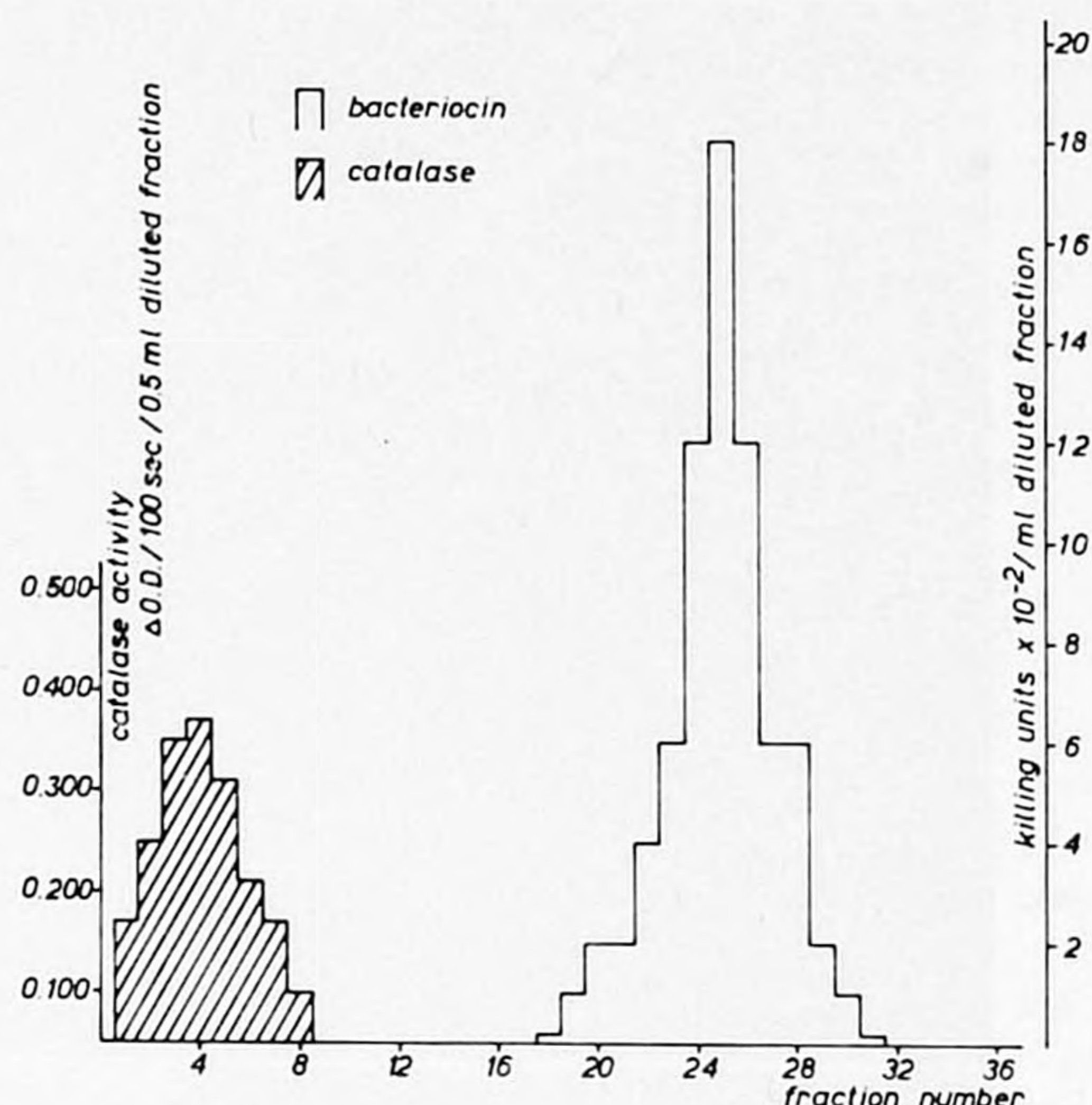


FIG. 3. Centrifugation of a mixture of partially purified bacteriocin (fraction 2 after gel filtration) and catalase on a linear sucrose-gradient (5 to 20% sucrose in  $0.05 \text{ M}$  Tris buffer, pH 7.0).

head morphology, the tail morphology, and the dimensions of the tail, there is no apparent difference between the two variants. We will refer to both variants as phage type DF13.

Apparently intact phages were found relatively rarely. Most of the particles were incomplete, so we found phages with full or empty head and contracted sheath (Fig. 4A), empty heads without tail (Fig. 4B), and tails with extended or contracted sheath without a head (Fig. 4A, E). All dimensions are given in Table 3.

*E. cloacae* strain O2, which was used as acceptor strain in transfer experiments, has also been investigated in the same manner. In this strain, too, we found phagelike particles with contractile tails. We never found an intact phage, but found mostly empty heads and very rarely (2% of all observed phagelike particles) a phage with empty head and contracted sheath (Fig. 4D). The dimensions are given in Table 3. We will refer to this phage as phage type O2.

The differences between phage type DF13 and phage type O2 are found mainly in the dimensions of their tails (Table 3). Furthermore, the surface of the head of phage type O2 shows a triangular annulus, and the core does not have a basal knob at its proximal end (Fig. 4D).

It must be emphasized that although intact phagelike particles are observed in culture lysates of *E. cloacae* strain DF13, we have been unable, until now, to find a suitable host for the multiplication of these phagelike particles. Thirty-five

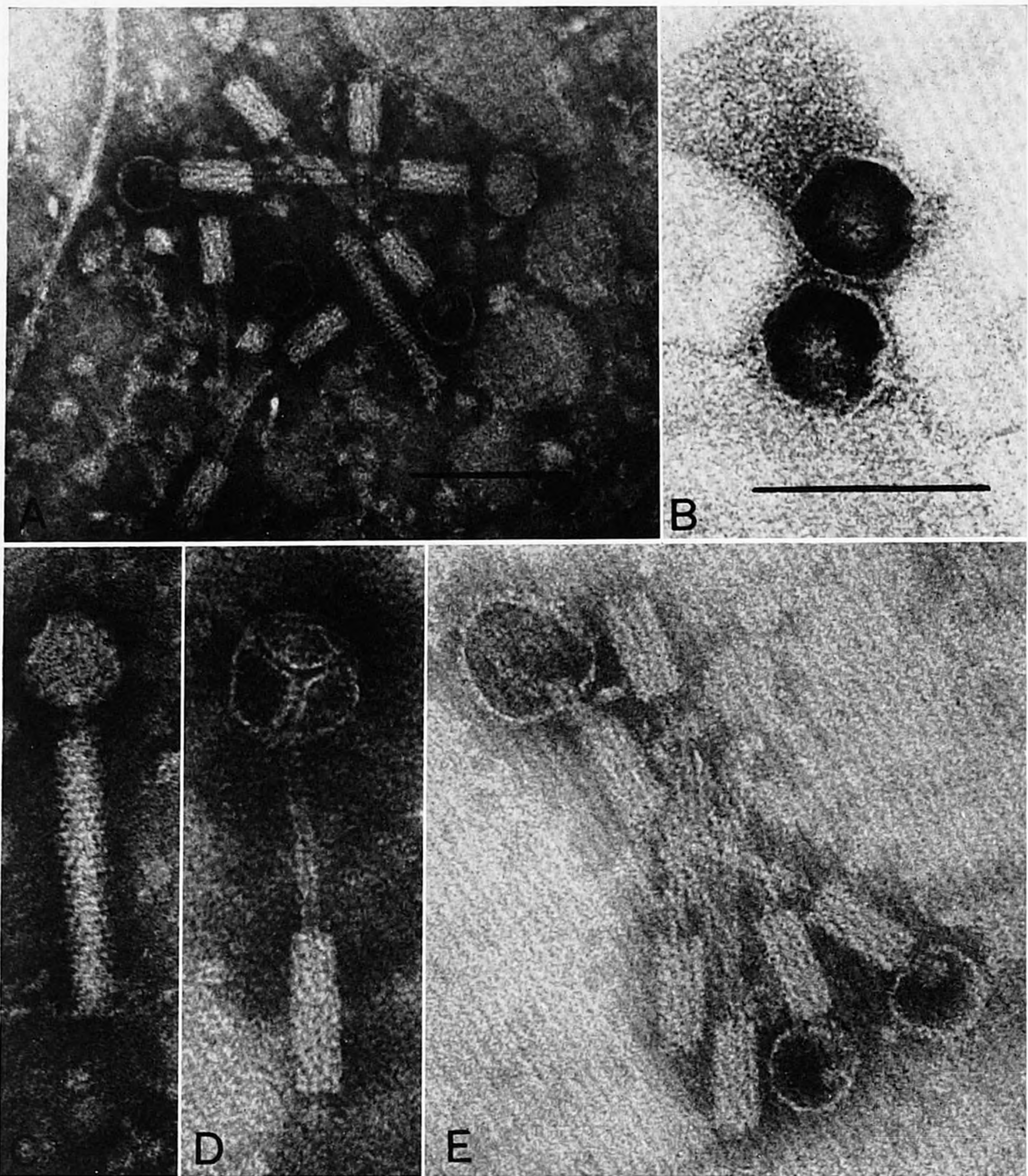


FIG. 4. Electron micrograph of phagelike particles from *Enterobacter cloacae* strains DF13 and O2 stained with phosphotungstic acid. (A and E) Phagelike particles type DF13. (B) Large empty heads of type DF13. (C) Intact phage particle type DF13. (D) Phage particle type O2 with contracted sheath and empty head. Magnification of A =  $\times 200,000$ ; B, C, D, and E =  $\times 300,000$ . Marker represents  $0.1 \mu$ .

strains of *Klebsiella* species, five strains of *E. cloacae*, and four strains of *Escherichia coli* have been tested by the double-layer method for detection of plaque formation without success. All these strains have been used previously (31) for determination of the activity spectrum of bacteriocin.

*Transfer experiments.* As shown in Table 4, transfer of bacteriocinogeny is possible from *E. cloacae* strain DF13 to *E. cloacae* strain O2 with

frequencies varying between 65 and 90% of the total final acceptor population. The results of different transfer experiments indicate that the highest transfer is reached if the precultures are in the postlogarithmic phase before mixing. Overnight incubation of the mixture for 18 hr is then sufficient to obtain a high transfer (Table 4).

Transfer of the bacteriocinogenic factor occurs frequently in combination with transfer of resistance against streptomycin, sulfanilamide, and

TABLE 3. Dimensions of phagelike particles produced by strains of *Enterobacter cloacae*

Dimension	Avg dimensions (A)	
	Phage type DF13	Phage type O2
Diameter of head...	350, 540 <sup>a</sup>	590
Length of contracted sheath....	450	700
Length of extended sheath.....	1,190	Never observed
Diameter of sheath..	190	220
Length of core.....	1,190	1,630
Diameter of core...	80	90

<sup>a</sup> In phage type DF13, two types of heads exist with different diameters.

TABLE 4. Transfer of bacteriocinogeny in *Enterobacter cloacae* strain DF13 × *E. cloacae* strain O2 crosses

Strain no.		No. of colonies tested	(Bac DF13) transfer (%)	Incubation time of pre-culture (hr)
Donor	Acceptor			
S 478	H 478	405	78	18
S 165	H 478	399	65	48
S 478	H 456	135	90	48

penicillin. The results of an analysis of all the tested colonies from a transfer experiment are shown in Table 5. It is evident that the frequency of transfer of resistance against these three antibiotics is also very high.

Transfer of bacteriocinogeny or resistance against antibiotics by means of a culture filtrate, induced or not induced, was not possible, indicating that transfer cannot be due to transduction by the DF13 phage. It may be concluded that, in all these cases, transfer is due to conjugation.

As mentioned above, in our transfer experiments the donor and acceptor cells could be distinguished by nutritional deficiencies. In most experiments, the acceptor strain needed methionine or histidine for growth. In none of these experiments was transfer of bacteriocinogeny and streptomycin resistance found to be accompanied by chromosome transfer. A chromosome transfer with frequencies lower than 10<sup>-8</sup> could not be detected because our mutants revert with this frequency. Also, in many other experiments with strains with other nutritional deficiencies no chromosome transfer was observed (*unpublished data*). Thus, we may conclude that transfer of

TABLE 5. Analysis for bacteriocinogeny and resistance against antibiotics of the colonies of the transfer experiment S 478 × H 456 (Table 4)

Factors	No. of colonies which obtained the indicated factors	Percentage of total no. of colonies
None.....	2	1.5
(Bac DF13)(str sul)		
(pen).....	109	80.8
(Bac DF13)(pen).....	12	8.8
(Bac DF13).....	1	0.7
(pen).....	11	8.1

chromosome material does not occur in this conjugation system.

From the high frequency of transfer of bacteriocinogeny and the absence of transfer of chromosome material, we may conclude that bacteriocinogeny is determined by a plasmid, as had been demonstrated before for colicin production in *E. coli* (6, 25). Similarly, resistance against these three antibiotics must be explained by the presence of a resistance-transfer factor. Separate transfer of resistance against streptomycin and sulfanilamide was never observed. These two properties are most probably located on one single factor. Transfer of penicillin resistance is sometimes observed without transfer of the bacteriocinogenic factor and without transfer of the resistance against streptomycin and sulfanilamide (Table 5). Thus, penicillin resistance seems to be located on a separate plasmid. Transfer of the factor determining resistance against streptomycin and sulfanilamide without transfer of the bacteriocinogenic factor was not observed in the experiment of Table 5.

The separate transfer of these factors was observed by the rapid selection method for transfer of streptomycin resistance. This method lowers the transfer frequency to about 5 × 10<sup>-6</sup>. Therefore, this method is not suitable for detection of transfer of bacteriocinogeny alone, as very high numbers of individual colonies would have to be tested. However, it is satisfactory as a rapid test for transfer of resistance against streptomycin, whether or not it is accompanied by transfer of the bacteriocinogenic factor.

Most of the resulting streptomycin-resistant colonies were found to have also received the bacteriocinogenic factor (Table 6). In the same way, transfer of the bacteriocinogenic factor in addition to the factor determining streptomycin resistance was observed in *Enterobacter cloacae* strain DF13 × *E. coli* K-12S crosses. Furthermore, it was observed that *E. cloacae* strain O2



TABLE 6. *Transfer of bacteriocinogeny associated with transfer of resistance against streptomycin*

Cross, donor × acceptor <sup>a</sup>	Frequency of str-r of total final acceptor population	Percentage of resistant acceptor cells which obtained (Bac DF13)	Isolate
(1) S 165 × S 89 . . . .	$1.3 \times 10^{-5}$	100	S 231
(2) S 231 × H 475 . . .	$1.2 \times 10^{-7}$	43	S 237
(3) S 237 × S 89 . . . .	$6.0 \times 10^{-6}$	89	
(4) S 165 × H 447 . . .	$1.0 \times 10^{-5}$	4	

<sup>a</sup> (1) = *Enterobacter cloacae* strain DF13 × *E. cloacae* strain O2; (2 and 3) = *E. cloacae* strain O2 × *E. cloacae* strain O2; (4) = *E. cloacae* strain DF13 × *Escherichia coli* K-12S.

stocks which had received both bacteriocinogeny and streptomycin resistance could transfer their factors to other mutants of *E. cloacae* strain O2 (Table 6).

*Investigation of isolated recipient cells.* From a number of crosses, acceptor cells were isolated which had received one or another of the factors studied. From some of these strains (S 231, S 232, S 237, S 239), the bacteriocin production was induced and the bacteriocin was purified. The elution profile of gel-filtration of "crude bacteriocin" preparations of these strains on Sephadex G-150 was exactly identical with that of the wild type (Fig. 2). Furthermore, the activity spectrum of the purified bacteriocin and its molecular weight were also exactly the same as of the wild type. The purification procedure was also applied to strain S 238, which acquired resistance against streptomycin and sulfanilamide but did not produce bacteriocin. In the elution profile of gel filtration of the ammonium sulfate-precipitated supernatant fluid of this strain, the second protein peak is absent.

By means of electron microscopy, the isolated strains were investigated on reception of the ability to produce phage type DF13. This was possible as the dimensions of the phages produced by *E. cloacae* strains DF13 and O2 are different (Table 3). The results of the various *Enterobacter* crosses for ability to produce phage type DF13 are shown in Table 7. A number of these cells have indeed received the ability to produce this phage, but there is no correlation between this ability and bacteriocinogeny. Neither has the resistance against antibiotics any correlation with these two factors.

If *E. coli* K-12 S, which is completely free from any prophage, is used as acceptor, this

TABLE 7. *Investigation of various categories of recipient cells from Enterobacter cloacae strain DF13 × E. cloacae strain O2 crosses for ability to produce phage type DF13*

No. of strains tested	Isolated from crosses <sup>a</sup>	Factors obtained	No. of strains which had phage type DF 13	
			Obtained	Not obtained
2	A, C	(Bac DF13) ( <i>str sul</i> ) ( <i>pen</i> )	2	0
1	B	(Bac DF13) ( <i>str sul</i> )	0	1
3	A, B	(Bac DF13) ( <i>pen</i> )	1	2
4	A, C, D,	(Bac DF13)	3	1
3	B, E	( <i>str sul</i> )	1	2
3	B	( <i>pen</i> )	1	2
5	A	None	0	5

<sup>a</sup> Code of crosses, donor × acceptor: A = S 478 × H 456; B = S 165 × H 478; C = S 165 × S 89; D = S 165 × H 475; E = S 237 × S 89.

method is easier. Four cells which had received the ability to produce bacteriocin were investigated for the production of phage type DF13. The supernatant fluid of two of them contained empty heads only; in the supernatant fluid of the other two, we could not observe phagelike particles.

#### DISCUSSION

*E. cloacae* strain DF13 has been found to produce a bacteriocin inducible by mitomycin C. The culture lysate of an induced culture contains a considerable number of phagelike particles and intact phages. For purification of the bacteriocin, we made use of gel filtration to separate these phagelike particles from substances of lower molecular weight. The bacteriocin was eluted separately from the structural components and has a molecular weight of about 61,000, showing similarity to the molecular weights of megacine and colicin A (2, 15). These results suggest that we must distinguish between real bacteriocins with relatively low molecular weight and defect lysogenic phages with intact killing capacity. Thus, we may conclude that at least three types of bacteriocinogenic bacteria exist: (i) bacteria producing phagelike particles which represent the bactericidal principle, (ii) bacteria producing bacteriocins of relatively low molecular weight; (iii) bacteria producing bacteriocins in addition to phagelike particles without killing activity.

The transfer experiments show that bacteriocinogeny in *E. cloacae* strain DF13 is determined

by a plasmid, which can be transferred by conjugation. As transfer was most successful if post-logarithmic donor and acceptor cultures were used, the conjugation system is somewhat similar to that described by Mulczyk and Duguid (24), who studied the kinetics of transfer of the colicinogenic factor col I-CT2 in crosses between a colicinogenic strain of *Shigella flexneri* and a noncolicinogenic strain of the same species. Some of the recipient cells which have received the bacteriocinogenic factor or resistance factors also have received the ability to produce the defective phage of the donor. However, not all of the strains which have received bacteriocinogeny produce the defective phage of the donor, and some strains, which have received the ability to produce defective phage, have not received bacteriocinogeny. Thus, bacteriocin formation and production of the defective phage are determined by separate genetic entities. Our results exclude the possibility that the bacteriocin is a product formed during the multiplication of the defective phage. If the bacteriocin was a product formed during this multiplication, it might be expected that both bacteriocin and phagelike particles would be determined by one single genetic entity. However, this result does not invalidate the hypothesis of Fredericq (12) and Ivànovics (16), that bacteriocins are the products of defective lysogeny. The possibility cannot be excluded that the bacteriocinogenic factor is another defective prophage, which is only capable of producing that part of a phage that is responsible for the killing of sensitive bacteria.

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## LITERATURE CITED

1. ADELBERG, E. A., M. MANDEL, AND G. C. C. CHEN. 1965. Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in *Escherichia coli* K 12. *Biochem. Biophys. Res. Commun.* **18**:788-795.
2. BARRY, G. T., D. L. EVERHART, AND M. GRAHAM. 1963. Colicine A. *Nature* **198**:211-213.
3. BRADLEY, D. E. 1965. The morphology and physiology of bacteriophages as revealed by the electron microscope. *J. Roy. Microscop. Soc.* **84**:257-316.
4. BRADLEY, D. E., AND C. A. DEWAR. 1966. The structure of phage-like objects associated with non-induced bacteriocinogenic bacteria. *J. Gen. Microbiol.* **45**: 399-408.
5. BURTON, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**: 315-323.
6. CLOWES, R. C. 1964. Transfert génétique des facteurs colicinogènes. *Ann. Inst. Pasteur* **107**: 74-92.
7. DATTA, N., A. M. LAWN, AND E. MEYNELL. 1966. The relationship of F type piliation and F phage sensitivity to drug resistance transfer in R<sup>+</sup>F<sup>-</sup> *Escherichia coli* K12. *J. Gen. Microbiol.* **45**:365-376.
8. DE WITT, W., AND D. R. HELINSKI. 1965. Characterization of colicinogenic factor E<sub>1</sub> from a non-induced and mitomycin C induced *Proteus* strain. *J. Mol. Biol.* **13**:692-703.
9. ENDO, H., K. AYABE, K. AMAKO, AND K. TAKEYA. 1965. Inducible phage of *Escherichia coli* 15. *Virology* **25**:469-471.
10. FREDERICQ, P. 1957. Colicins. *Ann. Rev. Microbiol.* **11**:7-22.
11. FREDERICQ, P. 1963. Colicines et autres bactériocines. *Ergeb. Microbiol. Immunol. Exptl. Therapy.* (Berlin) **37**:114-161.
12. FREDERICQ, P. 1963. On the nature of colicinogenic factors: a review. *J. Theoret. Biol.* **4**:159-165.
13. HAAN, P. G. DE, A. H. STOUTHAMER, H. S. FELIX, AND A. K. MOL. 1963. Transfer of F' from *Escherichia coli* K12 to *Escherichia coli* B and to strains of *Paracolobacter* and *Klebsiella*. *Antonie van Leeuwenhoek. J. Microbiol. Serol.* **29**:407-420.
14. HADJIPETROU, L. P., J. P. GERRITS, F. A. G. TEULINGS, AND A. H. STOUTHAMER. 1964. Relation between energy production and growth of *Aerobacter aerogenes*. *J. Gen. Microbiol.* **36**: 139-150.
15. HOLLAND, I. B. 1961. The purification and properties of megacin, a bacteriocin from *Bacillus megaterium*. *Biochem. J.* **78**:641-648.
16. IVÀNOVICS, G. 1962. Bacteriocins and bacteriocin-like substances. *Bacteriol. Rev.* **26**:108-118.
17. ISHII, S., Y. NISHI, AND F. EGAMI. 1965. The fine structure of a pyocin. *J. Mol. Biol.* **13**:428-431.
18. KAGEYAMA, M., AND F. EGAMI. 1962. On the purification and some properties of a pyocin, a bacteriocin produced by *Pseudomonas aeruginosa*. *Life Sci.* no. 9, p. 471-476.
19. KELLENBERGER, G., AND E. KELLENBERGER. 1956. Etude de souches colicinogènes au microscope électronique. *Schweiz. Z. Pathol. Bakteriologie.* **19**:582-597.
20. KINGSBURY, D. T. 1966. Bacteriocin production by strains of *Neisseria meningitidis*. *J. Bacteriol.* **91**:1696-1699.
21. LOWRY, O. H., A. L. FARR, N. J. ROSEBROUGH, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
22. MARTIN, R. G., AND B. N. AMES. 1961. A method for determining the sedimentation behaviour of enzymes: application to protein mixtures. *J. Biol. Chem.* **236**:1372-1379.

23. MEJBAUM, W. 1939. Über die Bestimmung kleiner Pentosemengen, insbesondere in Derivaten der Adenylsäure. *Z. Physiol. Chem.* **258**:117-120.
24. MULCZYK, M., AND J. P. DUGUID. 1966. Influence of the state of fimbriation on transmission of the colicinogenic factor col I between strains of *Shigella flexneri*. *J. Gen. Microbiol.* **45**:459-477.
25. OZEKI, H., B. A. D. STOCKER, AND S. M. SMITH. 1962. Transmission of colicinogeny between strains of *Salmonella typhimurium* grown together. *J. Gen. Microbiol.* **28**:671-687.
26. REEVES, P. 1965. The bacteriocins. *Bacteriol. Rev.* **29**:25-45.
27. SANDOVAL, H. K., H. C. REILLY, AND B. TANDLER. 1965. Colicin 15: possibly a defective bacteriophage. *Nature* **205**:522-523.
28. SCOTT, T. A., AND E. H. MELVIN. 1953. Determination of dextran with anthrone. *Anal. Chem.* **25**:1656-1661.
29. SEAMAN, E., E. TARMY, AND J. MARMUR. 1964. Inducible phages of *Bacillus subtilis*. *Biochemistry* **3**:607-612.
30. STICKLER, D. J., R. G. TUCKER, AND D. KAY. 1965. Bacteriophage-like particles released from *Bacillus subtilis* after induction with hydrogen peroxyde. *Virology* **26**:142-145.
31. STOUTHAMER, A. H., AND G. A. TIEZE. 1966. Bacteriocin production by members of the genus *Klebsiella*. *Antonie van Leeuwenhoek. J. Microbiol. Serol.* **32**:171-182.
32. SUZUKI, H., J. PANGBORN, AND W. D. KILGORE. 1967. Filamentous cells of *Escherichia coli* formed in the presence of mitomycin C. *J. Bacteriol.* **93**:683-688.