JOURNAL OF BACTERIOLOGY, July 1969, p. 339-340

Vol. 99, No. 1
Printed in U.S.A.

New Method for the Detection of a Lethal Factor in Vibrios

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Received for publication 6 February 1969

When vibriocinogenic vibrios were mixed with an indicator strain, "lacunas" (plaquelike clearings) were observed, thus providing a new method for detection of vibriocins.

There have been controversies as to the existence of vibriocin. Although Wahba's cold-shock method (8) seems a promising technique for demonstration of vibriocins, we could not obtain constant positive results by the cold-shock method. However, particles associated with vibriocin activity were reported by Lang et al. (5) and by Jayawardene and Farkas-Himsley (3).

Recently, we succeeded in detecting a vibriocin by a new method. This vibriocin could not be demonstrated by the usual bacteriocin detection techniques, including streak culture (7) and stab culture (2) methods, nor by the cold-shock method, but it was demonstrated by the "lacuna" formation method. When approximately 50 to 100 vibriocinogenic vibrios and the proper concentration of indicator vibrios were mixed and cultured in semisolid (0.3%) Brain Heart Infusion (BHI)-agar media overlayered on solid BHI-agar media, "lacunas" (plaquelike clearings) were observed (Fig. 1). Incubation at 37 C for 6 hr and then at room temperature overnight gave consistent results. The VC154 strain of Asiatic cholera vibrio, isolated in India in 1957, was routinely used as the indicator strain. The "lacuna" consists of a circular growth inhibition zone with a microcolony of vibriocin-producing vibrios at the center, just like the centered plaque formed by lysogenic bacteria. The number of "lacunas" produced approximated that of the vibriocinogenic vibrios with colony-forming ability. The possibility that the "lacuna" had been produced by lysogenic vibrios was negated by inseparability of vibrio-killing activity from living cells as described below. During a recombination experiment with *Vibrio cholerae*, Bhaskaran (1) also found that strains with fertility factor (P + strains) produced plaque-like clearings when tested on other strains devoid of fertility factor (*P*− strains). P+ strains (V58P+ and V63 SRP+), kindly supplied by K. Bhaskaran, were proved by our method to produce "lacunas"

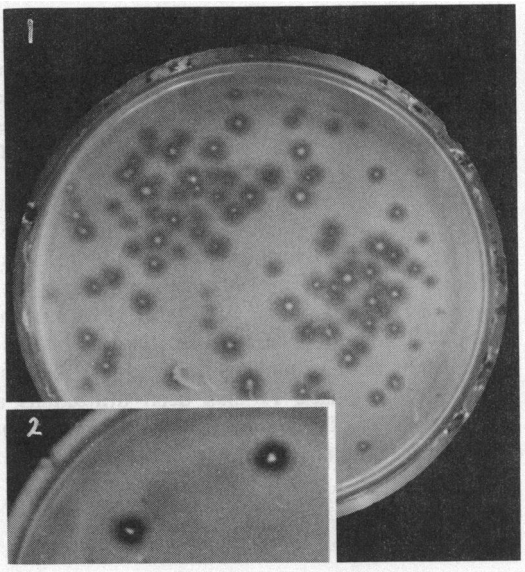

FIG. 1. *"Lacunas" (plaquelike clearings) formed on indicator cells by plating samples of cultures of a vibriocinogenic strain.*

FIG. 2. *Part of a plate showing inhibition zones produced by stab cultures of a vibriocinogenic strain (HP47 strain of El Tor cholera vibrio) against indicator cells (VC154 strain of Asiatic cholera vibrio).*

against P− strains (V58P− and V63SRP−) and our indicator strain. This fact may indicate that zygotic induction is involved in the formation of the "lacunas."

All attempts by various methods to separate vibriocins from living vibriocinogenic vibrios were unsuccessful: the supernatant fluid of the broth culture of vibriocinogenic vibrios had no inhibitory effect; killing of vibriocinogenic vibrios by treatment with chloroform, heat, or freeze-thawing also reduced vibriocin activity; treatment with proteolytic enzymes, including Nagarse (a bacterial protease) and trypsin, had no effect on

either viability of vibrios or vibriocin activity. Treatment with mitomycin C, an effective inducer of bacteriocins and temperate phages, could not induce the production of vibriocin separable from vibrio cells. By means of electron microscopy, particles resembling phages or phage tails could not be detected in cultures of vibriocinogenic strains, with or without mitomycin C, nor in mixed culture of vibriocinogenic strains and the indicator strains. Growth inhibition zones of indicator vibrios could be found by the stab culture method only when vibriocinogenic vibrios had not been killed by exposure to chloroform vapor (Fig. 2). Bacteriocin inseparable from living bacteria has also been recently reported for group A streptococci (4). Five strains of El Tor cholera vibrios (HP47, 36, 23, 18-1, and 18-2) among 410 strains of vibrio examined were found to produce this type of vibriocin. All five strains were isolated in Thailand in 1966. Most of the Asiatic and El Tor cholera vibrio strains were sensitive to the vibriocin, but all NAG vibrio strains were resistant.

Vibriocin-resistant colonies were isolated from vibriocin-sensitive Asiatic cholera vibrios after contact with the vibriocin. All of the resistant colonies acquired not only vibriocinogeny but also resistance to phage IV. Phage IV is known to be lytic for all Asiatic cholera vibrios but not for El Tor cholera vibrios, and the test for susceptibility to phage IV has been utilized as a reliable differentiating tool for both types of vibrio (6). It is interesting that vibriocinogeny was apparently accompanied by acquisition of resistance to phage IV.

The mode of transfer of vibriocinogeny was further analyzed by using a mixed broth culture of vibriocinogenic El Tor cholera vibrios and streptomycin (SM)-resistant indicator Asiatic cholera vibrios. Transferred vibrios could be selected by SM-containing agar and treatment with phage IV, since vibriocinogenic El Tor cholera vibrios were killed by SM and phage IV eliminated nontransferred indicator Asiatic cholera vibrios. According to the results of a preliminary experiment, transfer of vibriocinogeny occurred as early as 1 hr after contact, and the frequency of transfer was of the order of 10^{-4} to 10^{-5}.

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