Growth Inhibition of Enteric Bacteria by *Vibrio cholerae* in Nutrient Media Containing Lactate, Acetate, or Citrate¹

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In cross-streak tests on nutrient agar containing lactate, acetate, or citrate, the growth of certain enteric bacteria was inhibited by themselves or by *Vibrio cholerae*, or by both. This mimicked the activity of bacteriocins, but the phenomenon was attributable to the accumulation of carbonate and increased alkalinity of the media.

The cross-streaking procedure is routinely used for demonstrating the production of antibiotic agents like bacteriocins by bacteria (1). With the aid of this technique, bacteriocins have been identified in numerous species of bacteria (7). These bacteriocins have been defined as "bactericidal substances, apparently protein in nature, which are synthesised by certain strains of bacteria and are active against some other strains of the same or closely related species" (9).

In *Vibrio cholerae*, bacteriocins are not easily identified by such simple procedures (2, 10). Farkas-Himsley and Seyfried (5, 6) demonstrated that vibriocin (bacteriocin) production could be induced in a strain of *V. cholerae* by ultraviolet irradiation, anaerobiosis, and cold shock. Jayawardene and Farkas-Himsley (8) obtained good titers of this vibriocin through induction by mitomycin C, which also facilitated its purification. Wahba (10) showed that cold shock induced the production of vibriocin in numerous strains of *V. cholerae*. He also identified four strains of the same species that were susceptible to one or more of these vibriocins.

Chakrabarty et al. (3) applied the cross-streak technique for identifying the bacteriocins of *V. cholerae*. They observed that abundant production of bacteriocin took place on solid media containing a citrate-phosphate buffer (0.5 to 0.7% of each reagent) at pH 7.5 to 7.6. Eleven bacteriocin types of *V. cholerae* were identified. Each type was associated with the production of a bacteriocin that had a characteristic spectrum of activity on eight indicator strains. A noteworthy feature of this study was the demonstration that enteric bacteria belonging to diverse species such as *Escherichia coli*,

Shigella sonnei, and *Shigella flexneri* were susceptible to bacteriocins produced by *V. cholerae*. Consistent results were, in fact, obtained with these strains rather than with substitute susceptible strains of *V. cholerae*.

An unusual growth inhibitory phenomenon was observed in this laboratory when strains of *V. cholerae* were grown in the presence of lactate, acetate, or citrate-phosphate and then cross-streaked with indicator strains of enteric bacteria. This phenomenon was attributable to the accumulation of carbonate and alkalization of the test media.

Culture media. Nutrient broth contained, per liter of distilled water: peptone (Oxoid), 10 g; yeast extract (Oxoid), 2.5 g; and NaCl, 5 g. It was adjusted to pH 8.5 before sterilization.

Lactate broth contained, in addition, 3.6% sodium lactate (Lactolin, 60% sodium lactate; Boehringer, Ingelheim, Germany), acetate broth contained 2% sodium acetate (B.D.H.), and citrate-phosphate broth contained 0.6% each of trisodium citrate (B.D.H.) and K_2HPO_4 (B.D.H.). The pH of the citrate-phosphate broth was adjusted to 7.6 before sterilization. The media were solidified with 1% agar (Oxoid no. 3) when required. All media were sterilized at 15 lb/in² for 10 min.

Bacterial strains. The following 10 bacterial strains were used: *V. cholerae* (KB9 and KB92), *V. cholerae* El Tor (T50), *Escherichia coli* (58-161 = SR F⁻; 58-161 = SR F⁺, W677 = SR F⁻, and W677 = SR F⁺), *Salmonella typhi* (B-34-6 = SR), *Salmonella paratyphi* C (B-33-2 = SR), and *Shigella boydii* (B-37-3 = SR). All strains except *V. cholerae* (KB92) and *V. cholerae* El Tor (T50) were streptomycin-resistant mutants derived from streptomycin-susceptible parent strains.

Growth inhibition studies. On solid media, growth inhibition studies were done by the

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cross-streak procedure (1). In the case of bacterial strains, grown in liquid media, the cultures were centrifuged and the clear supernatants, after fortification with peptone, (1%; Oxoid) were solidified by the addition of 1% agar (Oxoid no. 3) and sterilized. The sterilized media were then poured into petri dishes and when set were streaked with indicator strains. Growth inhibition patterns were observed after overnight incubation at 37 C.

Other tests. The pH of supernatants was determined by means of a pH meter. Presence of bicarbonate or carbonate in the supernatants was identified by the effervescence caused by addition of dilute hydrochloric acid and bubbling the gas evolved through 0.15% solution of Ca(OH)_2 . Carbonates were identified by precipitation with a 10% solution of MgSO_4 at room temperature. Alkalinity was measured by adding an excess of 0.1 N HCl and back-titration with 0.1 N NaOH.

Growth inhibitory phenomenon in cross-streak tests on acetate, lactate, and citrate-phosphate nutrient agar. A checkerboard analysis of growth inhibition was carried out by the cross-streak procedure (1) with five representative bacterial species as test organisms. Four test media and six bacterial strains were used. Each strain was grown initially as a diametric streak for 48 h at 37 C before cross-streaking them with the same and other indicator strains. Growth inhibition patterns were recorded after overnight incubation (Table 1).

On lactate agar, *Salmonella typhi* (no. 3) and *Shigella boydii* (no. 5) were unable to inhibit the growth of the six indicator strains (no. 1 to 6), whereas *V. cholerae* (no. 1), *E. coli* (no. 2 and 6), and *Salmonella paratyphi* C (no. 4) showed inhibition of growth of five indicator strains (no.

2 to 6). On acetate agar, the growth of five indicator strains (no. 2 to 6) was inhibited in the vicinity of the diametrical streaks of the six strains (no. 1 to 6). On citrate-phosphate agar, five of the six strains (no. 2 to 6) were inhibited by *V. cholerae* (no. 1) and *Salmonella paratyphi* C (no. 4). The growth of *V. cholerae* (no. 1) was not inhibited in any of these tests. However, when the initial incubation period of the test strains (diametric streaks) was increased from 48 h to 72 to 96 h, inhibition of all the indicator strains was observed on lactate and acetate agar media. Such tests on plain nutrient agar gave uniformly negative results. The pattern of growth inhibition is illustrated in Fig. 1.

Inhibitory activity of citrate-phosphate nutrient broth culture supernatants of *V. cholerae* (KB9). To facilitate the identification of the growth inhibitory agent in these studies, tests were carried out with supernatants of *V. cholerae* (KB9) cultures in citrate-phosphate broth. The choice of the strain as well as the medium was based on the work of Chakrabarty et al. (3), who showed that citrate-phosphate buffer played an essential role in the synthesis of vibriocins. *V. cholerae* (KB9) was grown at 28 C in shake flasks in citrate-phosphate nutrient broth. Samples were withdrawn at 24-h intervals and centrifuged, and the supernatant fluids were converted to solid media as described earlier. These media were tested to see whether they would support the growth of the six test strains (Table 2). Five out of six indicator strains did not grow in media containing supernatants derived from citrate-phosphate broth cultures. No such inhibition was observed in control tests with nutrient broth culture supernatants. Since all the test media were sterilized at 15 lb/in² for 10 min before

TABLE 1. Growth inhibitory phenomenon observed in cross-streak tests on lactate, acetate, and citrate-phosphate agar.

Strain: diametrical streak ^a	Cross-streak in strains grown on:																							
	Nutrient agar						Lactate agar						Acetate agar						Citrate-phosphate agar					
	1 ^a	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
1	+	+	+	+	+	+	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-
2	+	+	+	+	+	+	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+
4	+	+	+	+	+	+	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-
5	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+
6	+	+	+	+	+	+	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+	+	+	+

^a 1, *Vibrio cholerae* (KB9); 2, *Escherichia coli* (58-161:SRF⁻); 3, *Salmonella typhi* (B-34-6:SR); 4, *S. paratyphi* C (B-33-2:SR); 5, *Shigella boydii* (B-37-3:SR); 6, *E. coli* (W677:SRF). Symbols: +, No growth inhibition; -, growth inhibition.

FIG. 1. Pattern of growth inhibition on lactate agar. Diametrical streak was done with *Vibrio cholerae* (KB92) incubated at 37 C for 48 h. Cross-streaks were done with: (left) top, *V. cholerae* El Tor T50; middle, *V. cholerae* KB92; bottom, *Escherichia coli* 58-161:SRF⁺; (right) top, *E. coli* 58-161:SRF⁻; middle, *E. coli* W677:SRF⁺; bottom, *E. coli* W677:SRF⁻.

TABLE 2. Growth inhibitory activity of culture supernatants of *Vibrio cholerae* (KB9)

Test strain	Medium ^a							
	A ₁	A ₂	A ₃	A ₄	B ₁	B ₂	B ₃	B ₄
<i>V. cholerae</i> (KB9)	+	+	+	+	+	+	+	+
<i>Escherichia coli</i> (58-161:SRF ⁻)	+	+	+	+	-	-	-	-
<i>Salmonella typhi</i> (B-34-6:SR)	+	+	+	+	-	-	-	-
<i>Salmonella paratyphi</i> C (B-33-2:SR)	+	+	+	+	-	-	-	-
<i>Shigella boydii</i> (B-37-3:SR)	+	+	+	+	-	-	-	-
<i>E. coli</i> (W677:SRF ⁻)	+	+	+	+	-	-	-	-

^a Media A₁ to A₄ were nutrient broth culture supernatants of *Vibrio cholerae* (KB9) derived after 24, 48, 72, and 96 h of incubation, respectively, at 28 C in a rotary shaker and solidified with agar. Media B₁ to B₄ were citrate-phosphate broth culture supernatants derived as above and solidified with agar. Symbols: +, Growth; -, no growth.

plating, it was clear that the inhibitory agent was heat stable. It was also observed that supernatants obtained after growing *V. cholerae* (KB9) and *E. coli* (W677:SRF⁻) in lactate broth inhibited the growth of all the test strains except *V. cholerae* when tested in this manner.

Detection of carbonate in supernatants of citrate-phosphate broth culture of *V. cholerae* (KB9). Supernatants of *V. cholerae* (KB9) grown in citrate-phosphate nutrient broth and in nutrient broth (control) were tested at 24-h intervals for pH and presence of carbonate and bicarbonate (Table 3). The cultures became progressively more alkaline dur-

ing continuous incubation. The pH of the supernatants increased progressively, from 8.84 to 9.12, in nutrient broth and from 9.14 to 9.50 in citrate-phosphate nutrient broth during 96 h of incubation. Although the pH variation was observed to be similar in the two media, supernatants of citrate-phosphate broth culture required more 0.1 N HCl for neutralization. The obvious reason for this was identified when the supernatant fluids were examined for the presence of carbonates. Effervescence occurred when dilute HCl was added to citrate-phosphate nutrient broth culture supernatants, but not with control samples of nutrient broth

TABLE 3. pH, alkalinity, and detection of carbonates in culture supernatants of *Vibrio cholerae* (KB9)

Test	Nutrient broth culture supernatant at:				Citrate-phosphate broth culture supernatant at:			
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
pH	8.84	9.03	9.20	9.12	9.14	9.34	9.48	9.50
Alkalinity ^a	219.7	229.2	214.9	210.1	558.6	577.7	577.7	572.9
Effervescence with dilute HCl	Nil	Nil	Nil	Nil	+ ^b	+	+	+
Solution of Ca(OH) ₂	Clear	Clear	Clear	Clear	Turbid	Turbid	Turbid	Turbid
Solution of MgSO ₄	Nil	Nil	Nil	Nil	PPT ^c	PPT	PPT	PPT

^a To 10 ml of each supernatant sample, 20 ml of 0.1 HCl was added, and back-titration of the excess acid was carried out by using standard alkali (0.1 N NaOH) and a solution of methyl red as indicator. Alkalinity of each supernatant sample was expressed arbitrarily as equivalent to milligrams of Na₂CO₃ per 100 ml of each sample.

^b +, Effervescence.

^c PPT, Precipitate.

culture supernatants (Table 3). The gas liberated was identified as CO₂. The presence of carbonates was further confirmed by precipitation with a solution of MgSO₄. Supernatants obtained by growing *V. cholerae* (KB9) and *E. coli* (W677:SRF⁻) in lactate broth media also showed the presence of carbonate by the tests described above (Table 3).

A growth inhibitory effect was observed in the vicinity of 48-h streak cultures of six different bacterial strains on nutrient agar media containing lactate, acetate, or citrate-phosphate buffer (Table 1). This was particularly well manifested on acetate agar as seen by growth inhibition of five test strains of enteric bacteria by themselves as well as by a strain of *V. cholerae*. On citrate-phosphate agar, these strains were inhibited by *V. cholerae* and *S. paratyphi* C. Only these two strains could utilize citrate, as shown by growth in Koser citrate medium (4). On lactate agar, these growth inhibitory effects were manifested by *V. cholerae* and three other strains.

The growth inhibiting agent was also present in culture supernatants of *V. cholerae* cultivated in citrate-phosphate broth. This facilitated its identification. It was seen that these supernatants were highly alkaline due to the accumulation of carbonate, which is the end product of citrate, lactate, and acetate metabolism. The growth inhibitory effects could therefore be due to either the presence of carbonate or the increased alkalinity of the media.

These experiments point to the unsuitability of nutrient media containing lactate, acetate, or citrate for prolonged cultivation of enteric bacteria or for the detection of specific antibiotic agents like bacteriocins.

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