ISOLATION AND CHARACTERIZATION OF *BDELLOVIBRIO* PARASITIC TO GRAM-NEGATIVE FISH PATHOGENIC BACTERIUM *AEROMONAS HYDROPHILA*

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Abstract: One strain of bacteria parasite-*Bdellovibrio* was isolated from cultured fish ponds in Taiwan. This strain is able to parasitize on *Aeromonas hydrophila*, designated as *Bdellovibrio* A-1. The morphology of host and parasite were examined by means of electron microscopy. The effect of medium composition, initial pH of medium, incubation temperature, addition of divalent cation and kinds and concentration of host bacteria on the growth of *Bdellovibrio* were investigated. The optimum temperature for the growth of *Bdellovibrio* in liquid medium was 25-29°C. The optimum initial pH of medium was pH 6.5. Growth of the parasite was dependent upon the addition of Ca^{2+} and Mg^{2+}. The highest lytic action of *Bdellovibrio* was obtained by using a higher concentration of fish pathogenic bacteria (4×10^8 cells/ml) as host.

INTRODUCTION

The methods of controlling fish diseases involved the addition of antibiotics or other chemical compounds, usually caused acute toxicity and chronic effect to the aquatic organisms. A more suitable alternative method would be the use of specific biological control agents to regulate fish diseases. The addition of bacteriophages has been used by several workers (Wu and Chao, 1982; Wu *et al.*, 1981.), however, the formation of resistant bacteria cause another problem. The uniquity and natural prediletion of *Bdellovibrio bacteriovorus*, described by Stolp and Petzold (1962) is to attack and destroy populations of several gram-negative bacteria. Miyamoto and Kuroda (1975), Miyamoto *et al.* (1976), Hanaoka (1981) as well as Horie and Kobayashi (1981) had isolated *Bdellovibrio* parasitic to *Vibrio parahaemolyticus* from fresh water and sea water. This led us to examine the feasibility of using these bacteria to parasitize population of gram-negative fish pathogens. Wang and Lin in 1983 had isolated a *Bdellovibrio* parasitic to methylotrophic bacteria from abnormal fermentation broth of local fermentation plan. Wang *et al.* (1983) also studied the occurrence of *Bdellovibrio* which might kill pathogenic bacteria of fishes in Taiwan aquaculture. The object of this study was to isolate a *Bdellovibrio* which can parasitize on fish pathogens *Aeromonas hydrophila* and physiological properties of the isolate were also investigated.

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MATERIALS AND METHODS

Bacterial strains and culture media

The fish pathogens used in these experiments were *Aeromonas hydrophila, Vibrio anguillarum* and *Edwardsiella tarda.* These bacteria usually caused fish diseases in Taiwan, were kindly supplied by Dr. H. Y. Chung, Department of Zoology, National Taiwan University. The other organisms used in studying the activity spectrum of the isolate were *Bacillus subtilis, Escherichia coli* and *Staphylococcus aureus* which were the stock collection of our laboratory. Tryptic soy broth (TSB, Difco) and nutrient broth (NB, Difco) were used for the growth of fish pathogens and other tested hosts, respectively. Low nutrient medium: yeast extract and peptone (YP) broth contained Difco yeast extract, 0.3%; Difco peptone, 0.06% and 0.05 M Tris-buffer at pH 6.5 was prepared as described by Stolp and Starr (1976). Dilute nutrient broth, NB/10; diluted tryptic soy broth, TSB/10 and diluted YP broth, YP/10 were prepared by adding 1 volume of NB or TSB or YP to 9 volumes of distilled-water. Double-layered agar plates were made by used of YP broth with the addition of 0.4 g/l of Ca^{2+}, Mg^{2+} and 0.06% agar for the top layer; 1.2% agar for the bottom layer.

Growth of host bacteria

Fish pathogens were grown in TSB at 28°C shaking water bath for 18 h. *Escherichia coli* and *Bacillus subtilis* were grown in NB at 37°C for 18 h. *Staphylococcus aureus* was grown in NB at 28°C for 18 h.

Isolation and cultivation of *Bdellovibrio* parasitic to fish pathogens

An aliquot of fish pond water from Tainan, Kao-Shung or Ping-Tung was collected and filtered through a membrane filter with 0.45 μm pore-size diameter. Suitable amounts of filtrates were added to fish pathogen *Aeromonas hydrophila* in YP/10 broth and incubated at 28°C. After 1 to 2 days of incubation, 0.1 ml of appropriate dilutions of the culture was plated with fish pathogens by using Adams' double-layer plating procedures (1959). After incubation at 28°C for two to four days, the plaques were appeared. The plaques were cut out of the top-layer were suspended in YP and purified three times by double layer plating procedure. The organism isolated was cultivated either in agar plate by double-layer plating procedure or in YP broth with host cells by shaking culture. It was confirmed by phase contrast microscopy (Zeiss Photomicroscope III) directly and by transmission electron microscopy (TEM).

Electron microscopy

Cells for TEM were dropped on specimen grid (300 mesh), negatively stained with 2% PTA (potassium phosphotungstate) and examined with a Hitachi H-600 electron microscope.

Lytic activity

Lytic activity of *Bdellovibrio* was determined by the double layer plating technique. Bacteriolytic activity was measured by following the decrease in turbidity (Shimazu spectrophotometer UV 160).

RESULTS

Isolation

Filtrate of fish pond water was plated with fish pathogen, *Aeromonas hydrophila*, clear plaques were appeared after two days of incubation at 28°C (Fig. 1) and enlarged progressively. Phasecontrast microscopic observation revealed the clear region of plaques consisted of highly motile microbes which are very small in size. The organism isolated from the clear region was purified, characterized and named *Bdellovibrio* A-1.

Electron microscopic observation

By TEM, cells of uninfected *Aeromonas hydrophila* were rods, 0.83-1 μm × 2.3-2.8 μm in size (Fig. 2). The isolate was vibrio shape, 0.24 μm × 1.1 μm in size with single polar flagellum (Fig. 3). The sequences in the interaction between *Bdellovibrio* A-1 and *Aeromonas hydrophila* were showed in Fig. 4 observed by TEM. The infectious organisms became rounded when aged and exhibited surface projections when negatively stained with 2% PTA (Fig. 4). Besides the vibriod and rounded shape cell of *Bdellovibrio*, they also showed long straight and spiral forms (Fig. 5). The ghosted remnants of host bacteria were left after progeny swarmers swam away (Fig. 6).

Effect of medium composition on the growth of *Bdellovibrio*

Different media were used to prepare bottom agars for the cultivation of *Bdellovibrio*. The results showed that *Bdellovibrio* can grow on YP, 1/10 NB and 1/10 TSB, but not on NB or TSB (Table 1).

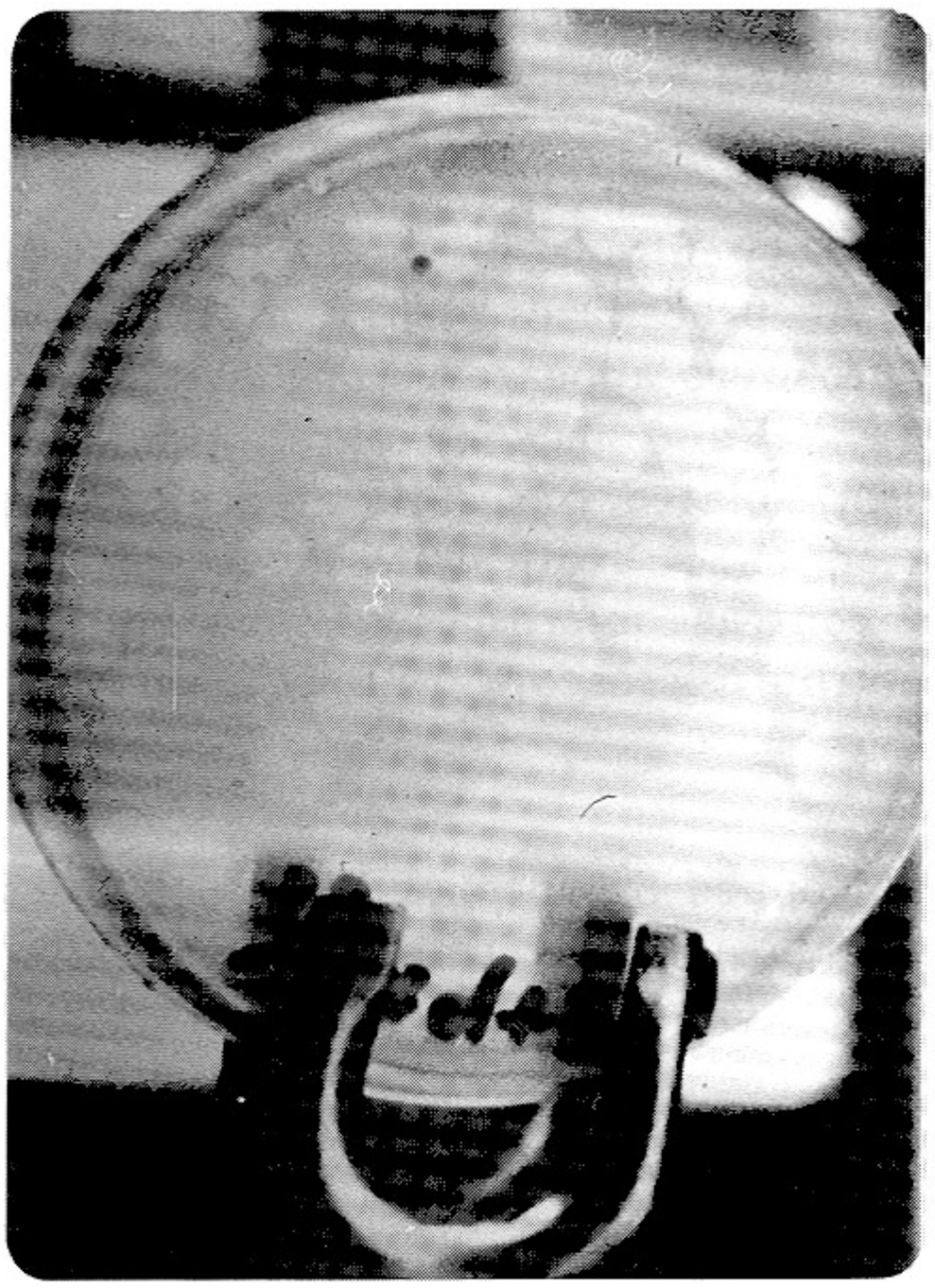


Fig. 1. Plaques formed by isolate A-1 on lawn of *Aeromonas hydrophila*.

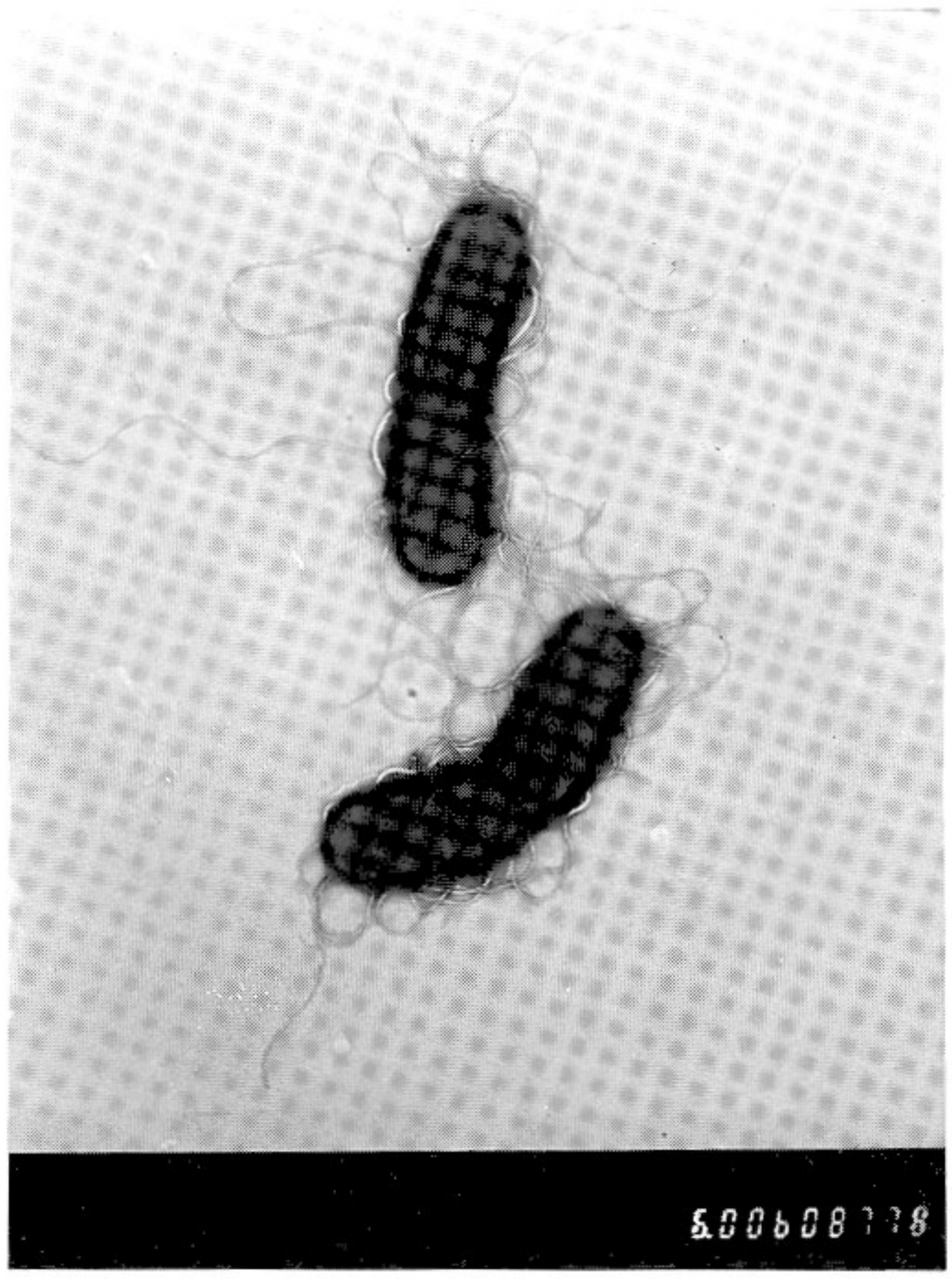


Fig. 2. Morphology of uninfected *Aeromonas hydrophila*, which was negatively stained with 2% PTA and observed by TEM. ×5,400.

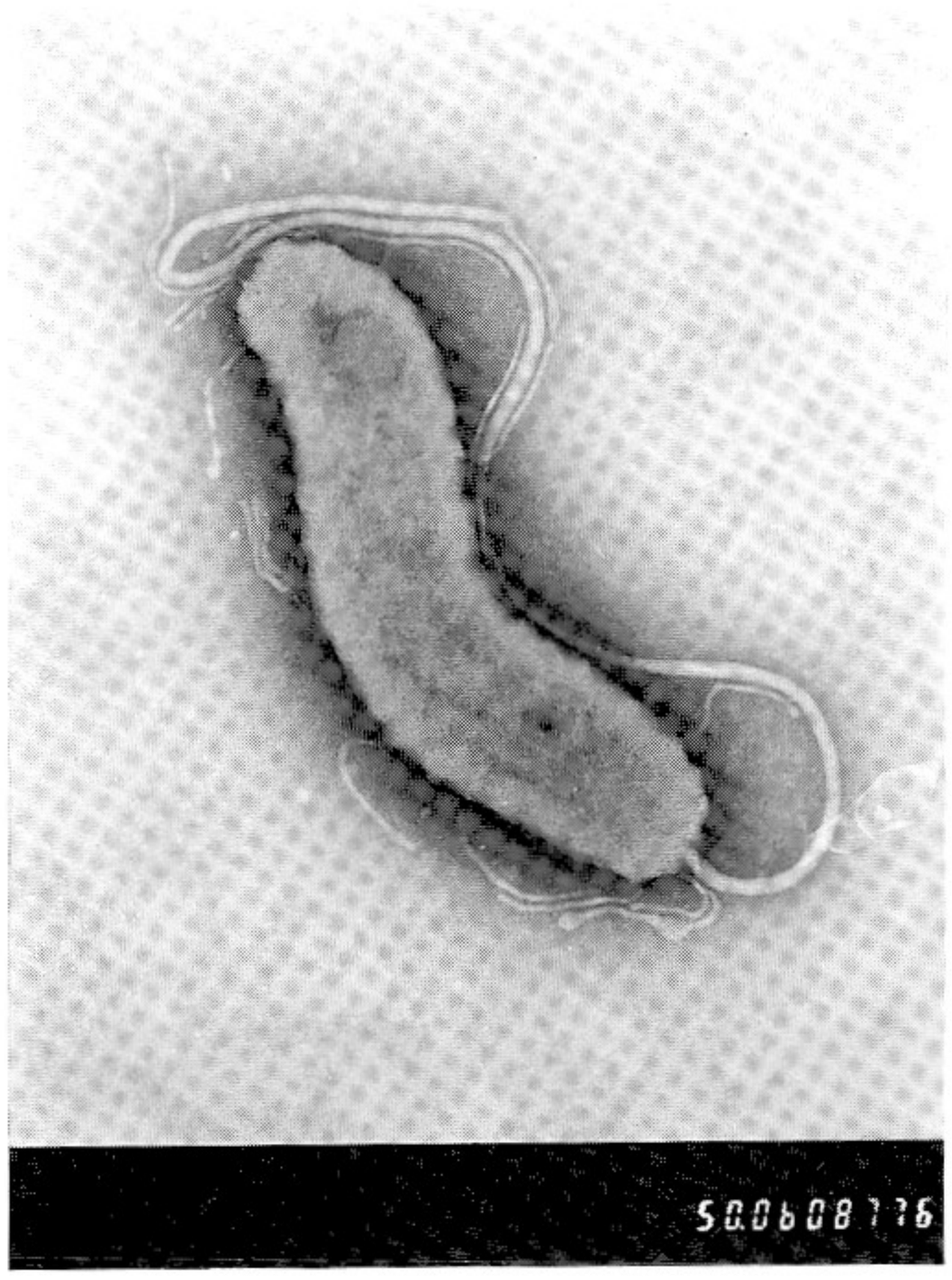
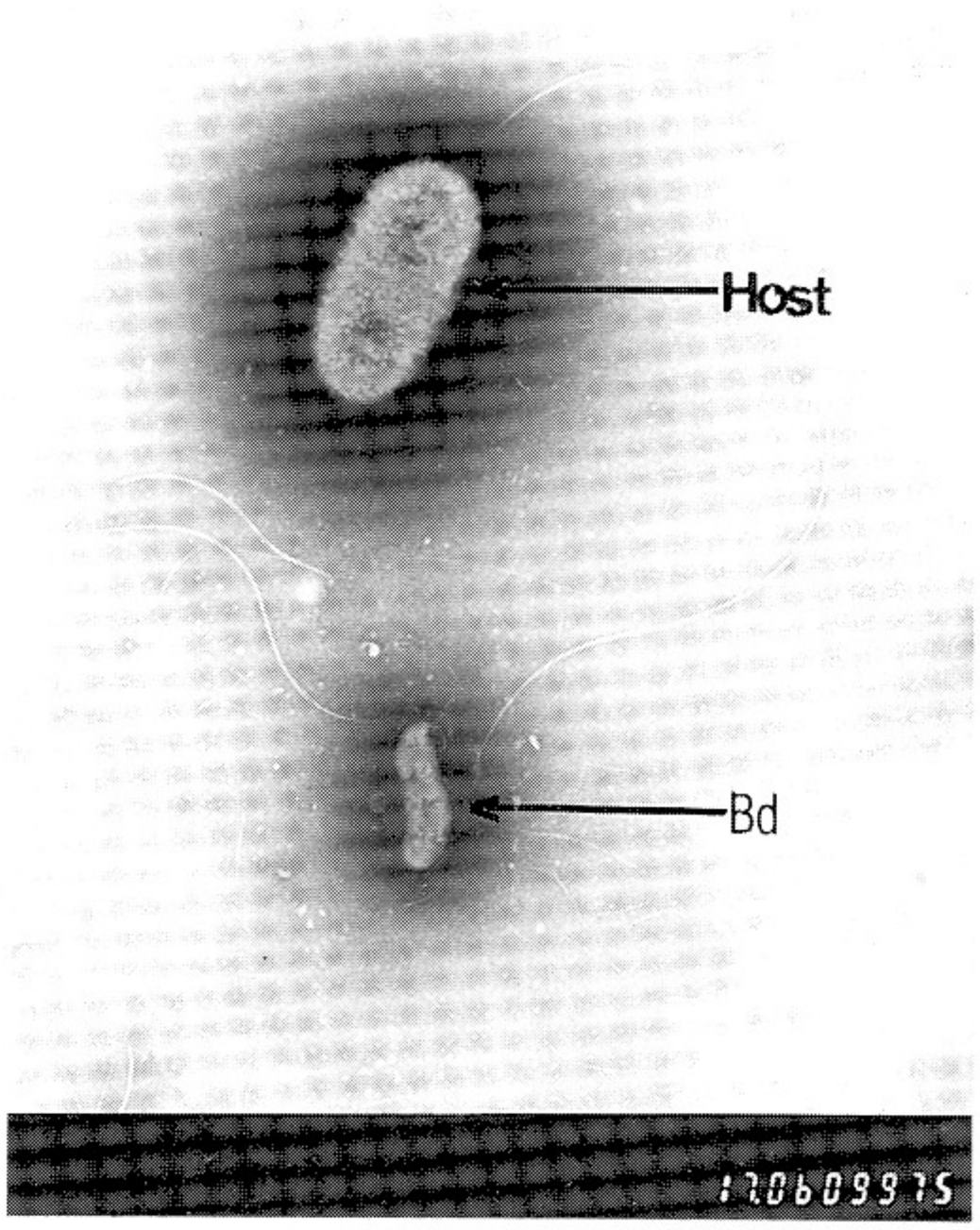


Fig. 3. Morphology of isolate A-1, which was negatively stained with 2% PTA and observed by TEM. ×45,000.



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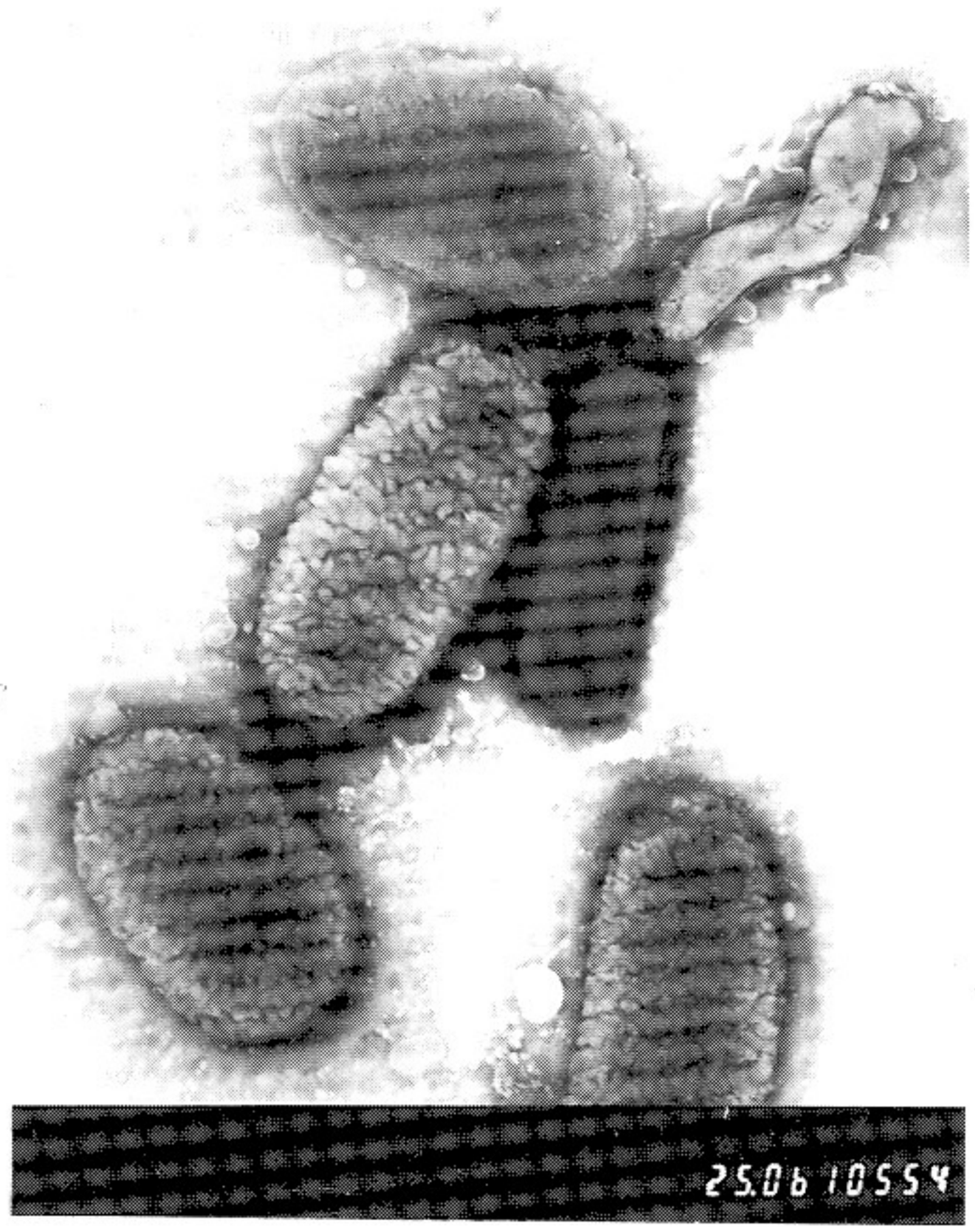

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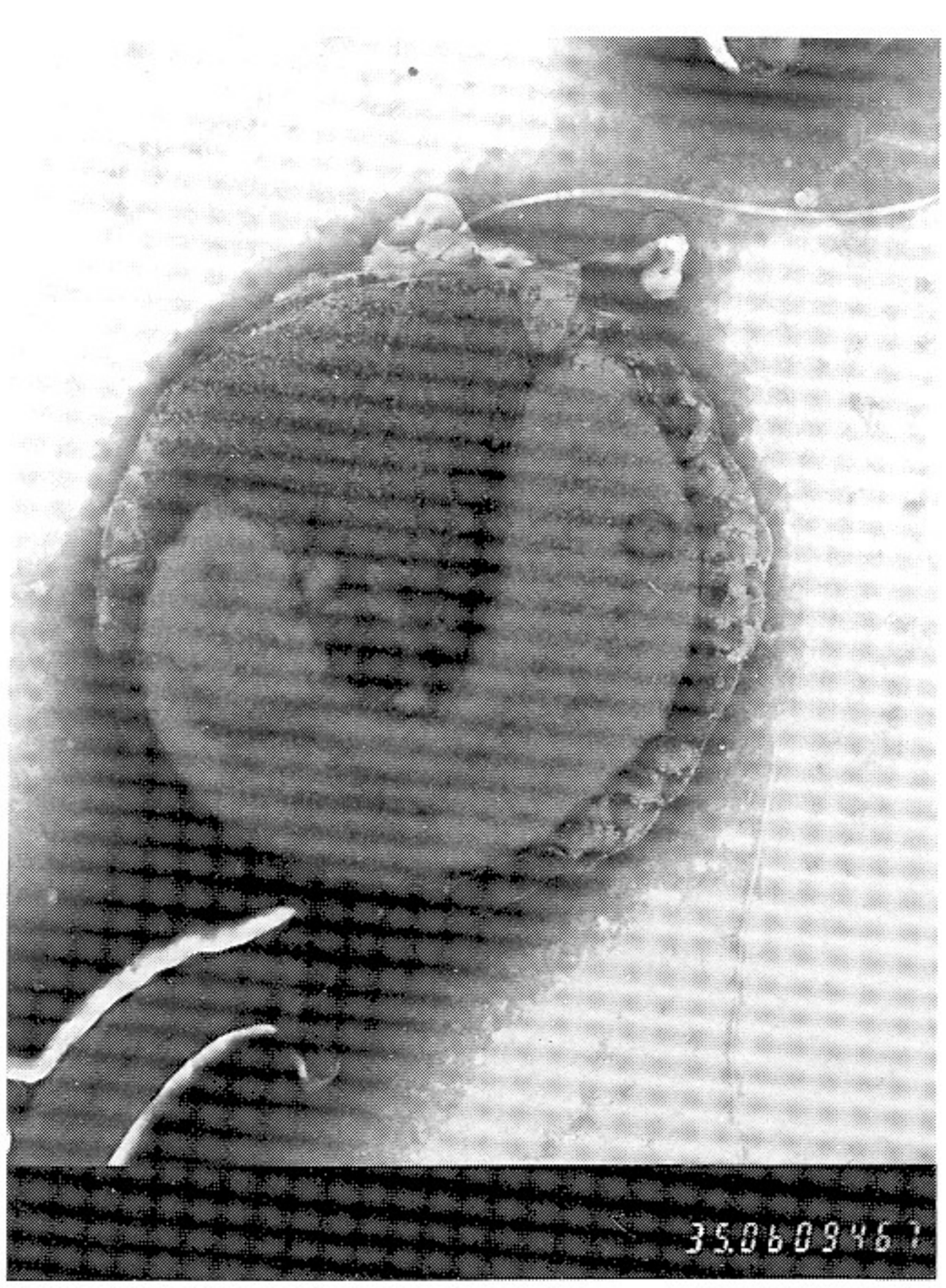

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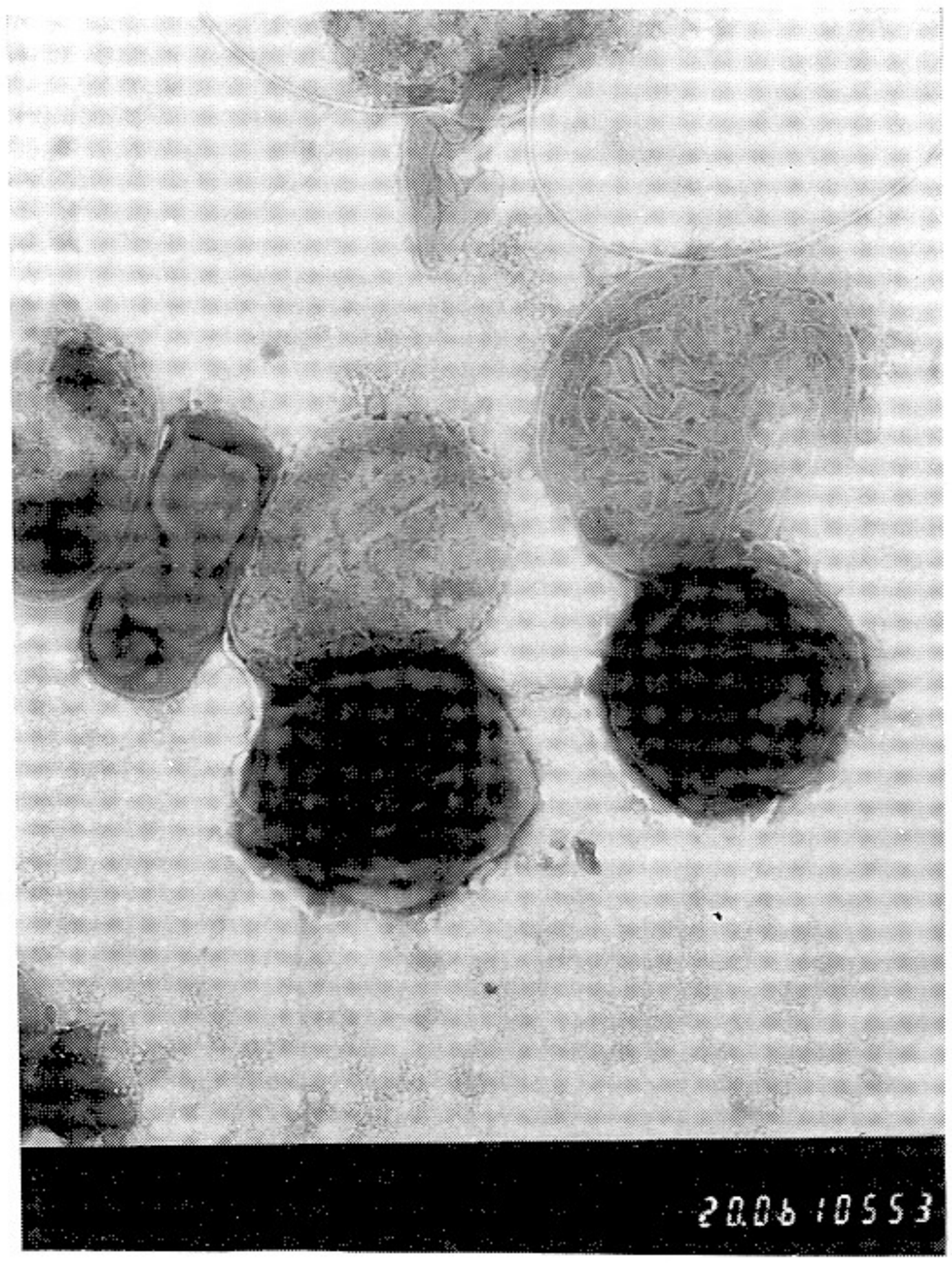
Fig. 4. Sequences in the interaction between *Bdellovibrio* A-1 and *Aeromonas hydrophila*. (A) ×15,300, and (B) ×22,500, initial contact of *Bdellovibrio* with *Aeromonas hydrophila*; (C) ×31,500, complete invasion and spheroplast formation.



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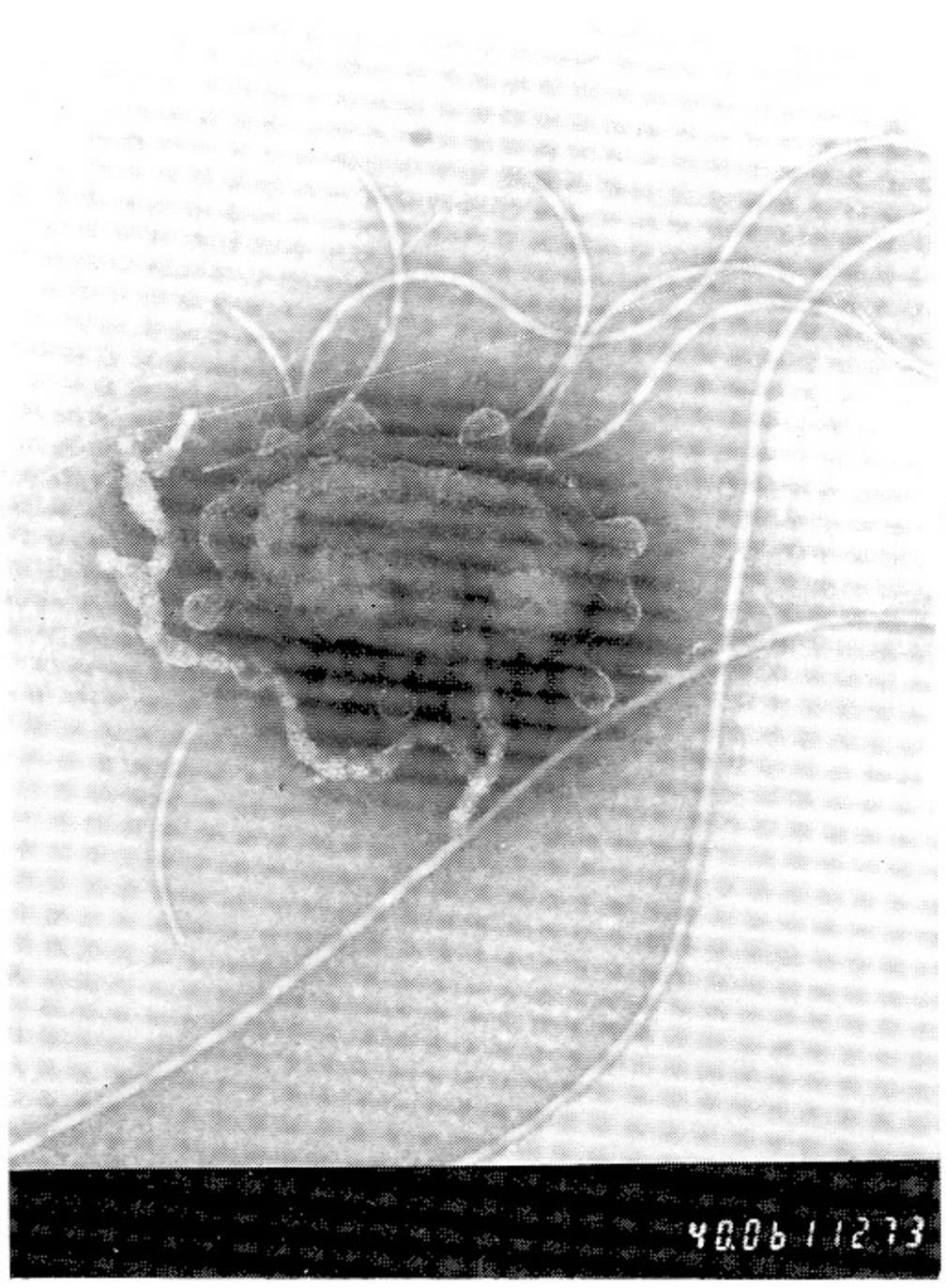

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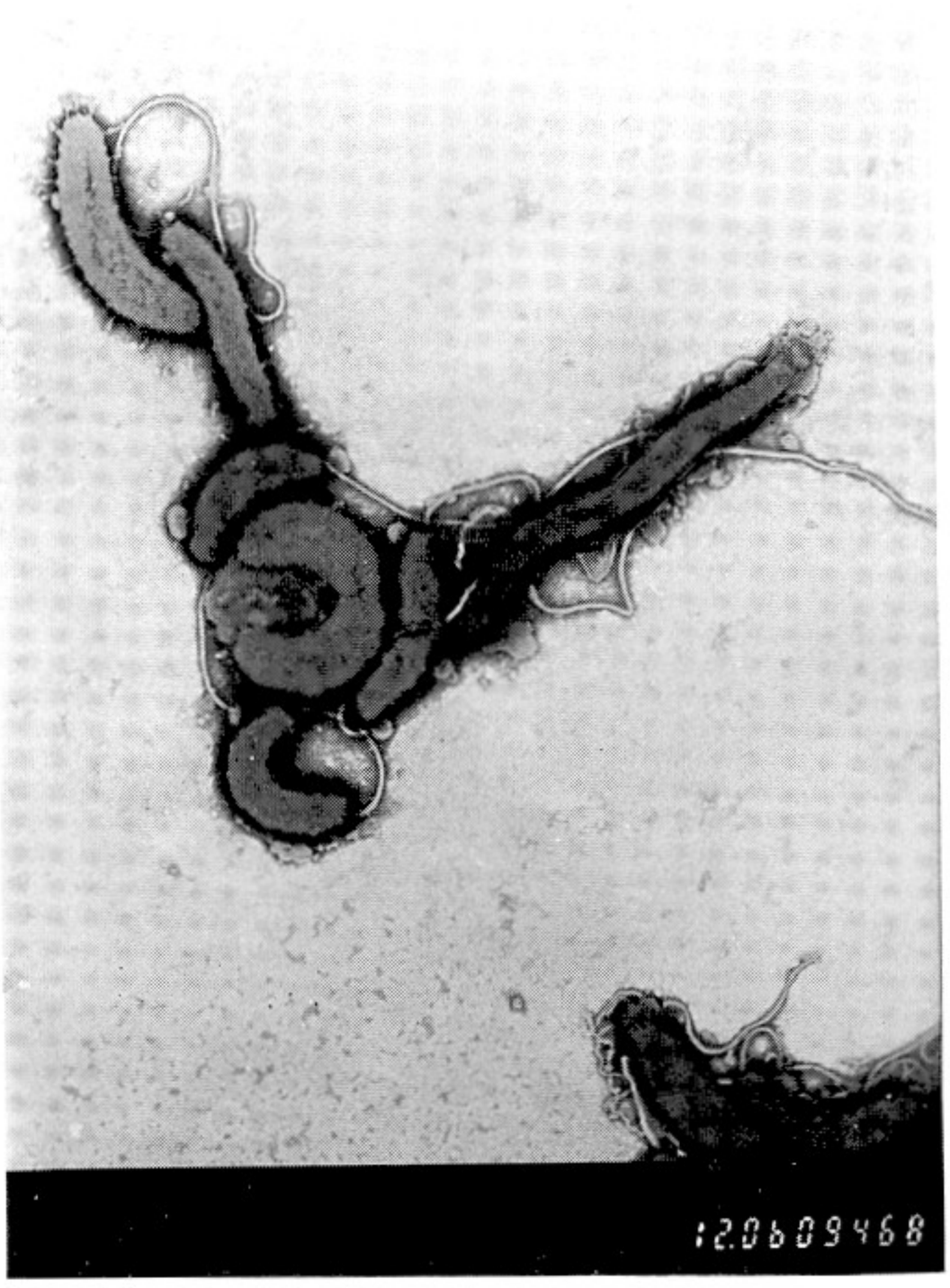
Fig. 4. Sequences in the interaction between *Bdellovibrio* A-1 and *Aeromonas hydrophila*. (D) ×18,000, (E) ×18,000, Bdelloplast formation; (F) ×36,000, surface projection formed on rounded cell of *Bdellovibrio*.



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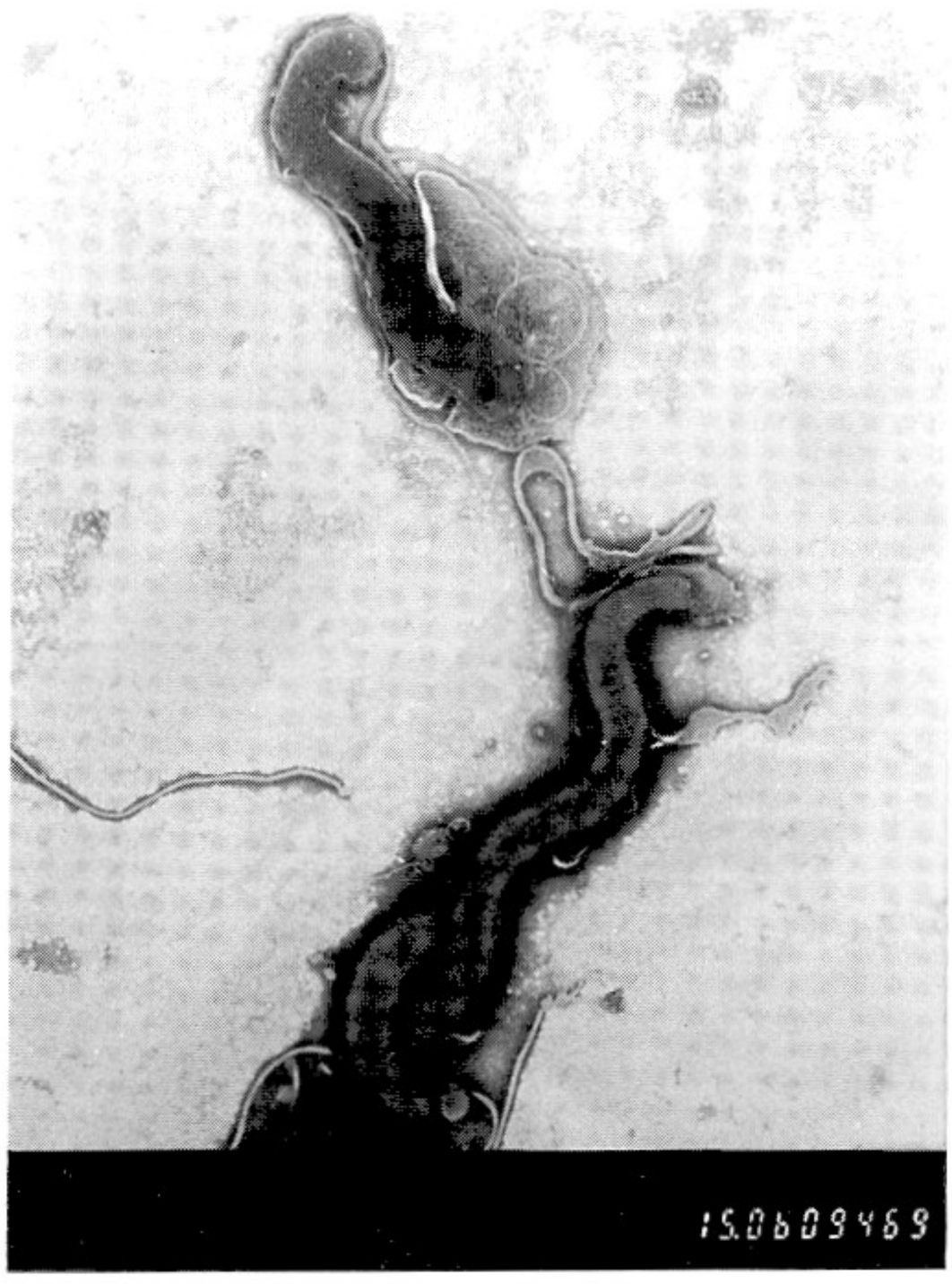

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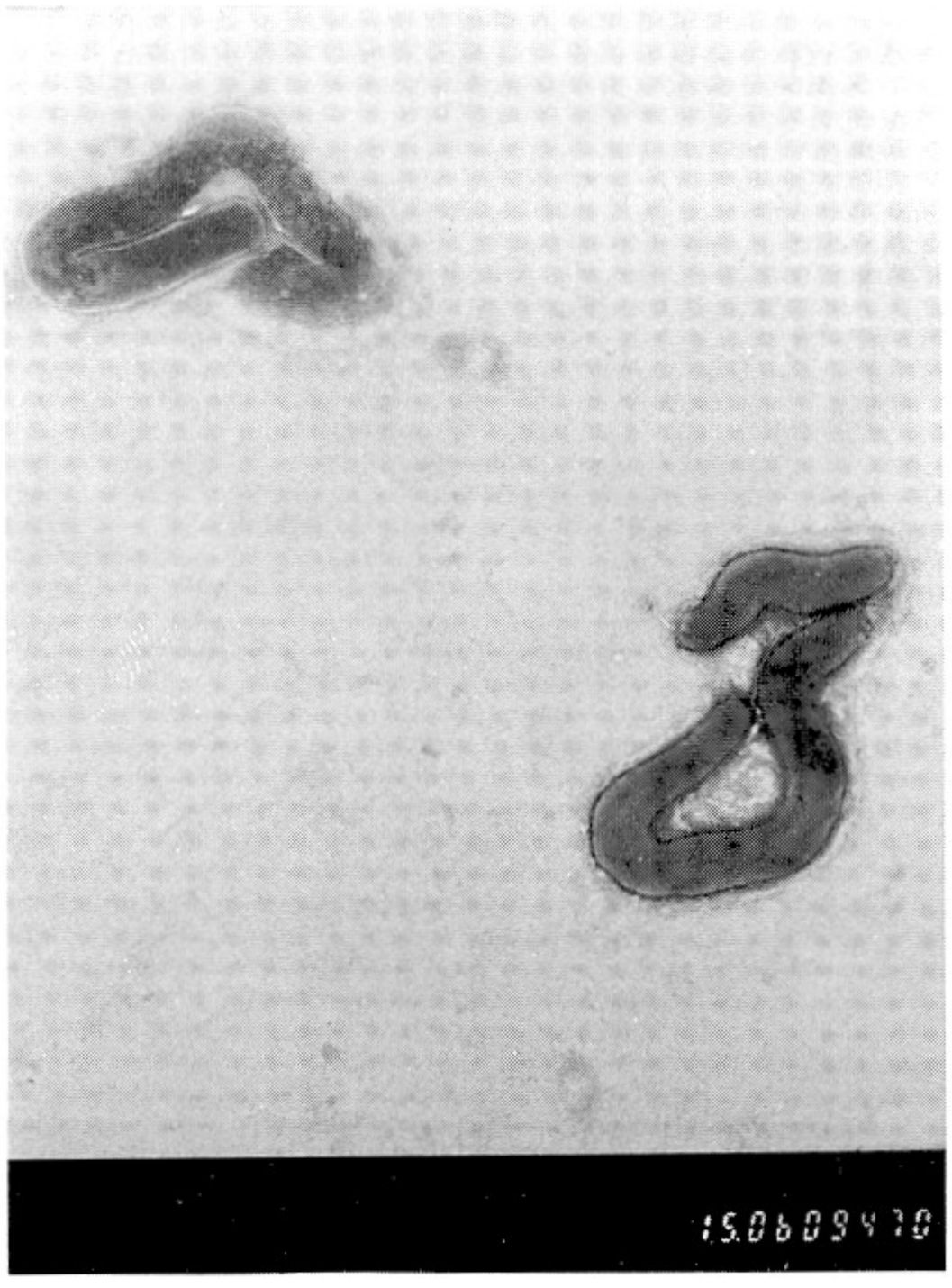

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Fig. 5. Morphologically heterogeneous cells of lysates. (A). ×10,800; (B) and (C) ×13,5000.

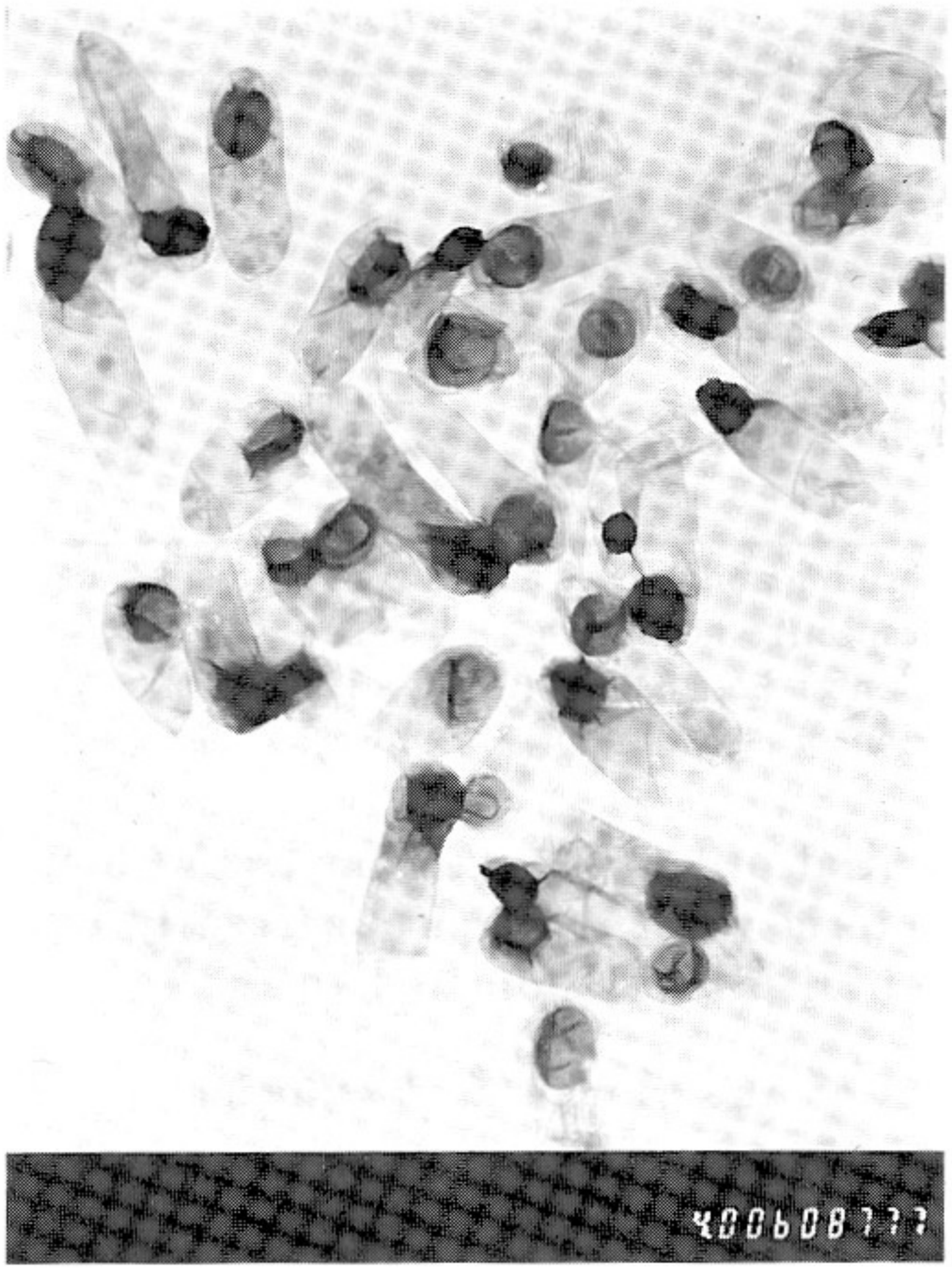


Fig. 6. The ghosted remnants of host bacteria formed after progeny swarmers swim away.

Table 1. Effect of various kinds of media on the plaque formation of *Bdellovibrio*

Medium	Dilution	Plaque formation*
NB	no dil.	−
	1/10	+
TSB	no di.	−
	1/10	+
YP	no dil.	+

* Plaque-forming ability was determined on the 3rd day after inoculation on each medium.
Concentration of host bacteria: 4.0×10^7 cells/ml.
Concentration of *Bdellovibrio*: 2.0×10^6 cells/ml.

Spectrum of lytic activity

The host range of A-1 was investigated on the basis of plaque formation by double-layer agars with the bacteria listed (Table 2). Isolate A-1 formed plaques on three Gram-negative fish pathogens. On the other hand, the isolate did not form plaques on the plates of *Escherichia coli* and gram-positive bacteria tested, such as *S. aureus* and *B. subtilis*.

Effect of initial pH value of media on the growth of *Bdellovibrio*

The initial pH value of the medium is critical for the growth of *Bdellovibrio*.

Table 2. Host spectra of *Bdellovibrio* A-1 lytic activity

Host bacteria	Plaque formation*
Edwardsiella tarda	+
Vibrio anguillarum	+
Aeromonas hydrophila	+
Staphylococcus aureus	−
Bacillus subtilis	−
Escherichia coli	−

* Plaque-forming ability was determined on the 3rd day after inoculation on the respective host lawns.
Concentration of host bacteria: 4.0×10^7 cells/ml.
Concentration of *Bdellovibrio*: 2.0×10^6 cells/ml.

When changing the initial pH of the medium from 6.5 to 8.2, Fig. 7 showed that the growth of host bacteria was inhibited apparrently due to the development of *Bdellovibrio* when the initial pH was adjusted to 6.5.

Effect of divalent cations on the growth of *Bdellovibrio*

Isolate A-1 was investigated to determined whether it required cation for the growth of *Bdellovibrio*. The reduction of turbidity were shown, when 0.2 g/l to 0.8 g/l Ca^{2+} and Mg^{2+} were added (Fig. 8) to the growth medium of *Bdellovibrio* and their host.

Effect of temperature on the growth of *Bdellovibrio*

Aeromonas hydrophila inoculated with isolate in YP liquid medium, were incubated at temperature ranging from 25° to 37°C. The maximum inhibition of growth on host bacteria was obtained at 25° to 29°C (Fig. 9).

Effect of the concentration of host bacterium on the growth of *Bdellovibrio*

Aeromonas hydrophila cultured on 5 ml TSB broth at 28°C for one day was washed with distilled water. The final precipitate was resuspended in 0.5, 5 or 50 ml of YP/10. Point five ml of these bacterial suspension was added with 0.5 ml of *Bdellovibrio* to YP broth to determine the growth inhibition of host bacteria due to the presence of *Bdellovibrio*. The result (Fig. 10) showed that the highest reduce of turbidity was obtained by using a highly concentrated suspension (4.0×10^8 cells per ml) of *Aeromonas hydrophila*.

DISCUSSION

Bdellovibrio had been known to infect and lyse Gram-negative bacteria. The distinct characteristics used for the identification of *Bdellovibrio* are: (1) plaques were formed on lawns of suitable bacteria and increased in size over several days (Starr and Stolp, 1976) (Fig. 1), (2) the small, highly motile, vibriod (Fig. 3) *Bdellovibrio* could enter into the periplasmic spaces of other bacterial cells; they lived and developed there-in (Fig. 4-Fig. 5); they grew into non-motile, large, helical or serpentine forms (Fig. 5) and after multiple fission of the helical element, they generate one flagellum per progeny cell (Fig. 3), the swarmers swam away

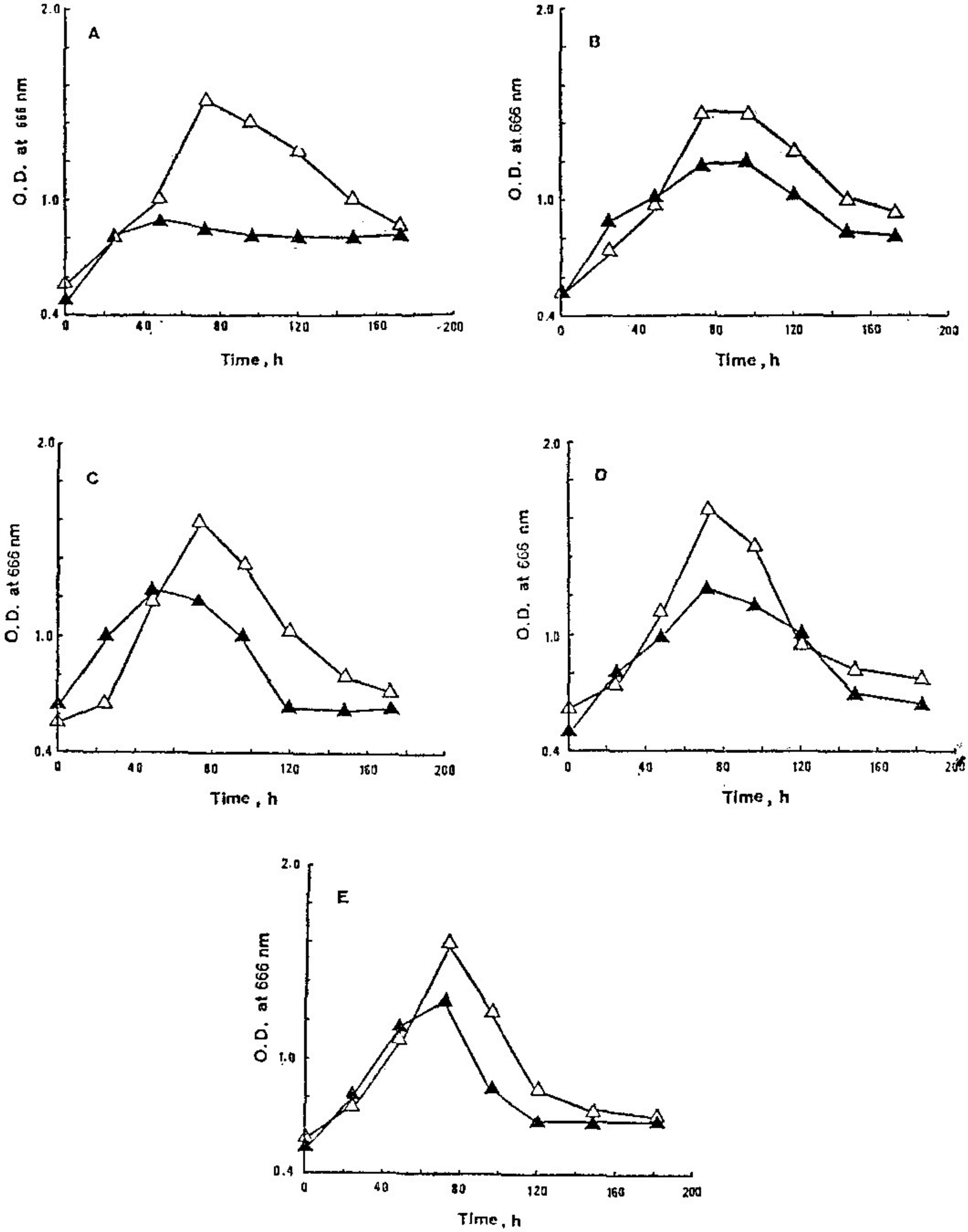
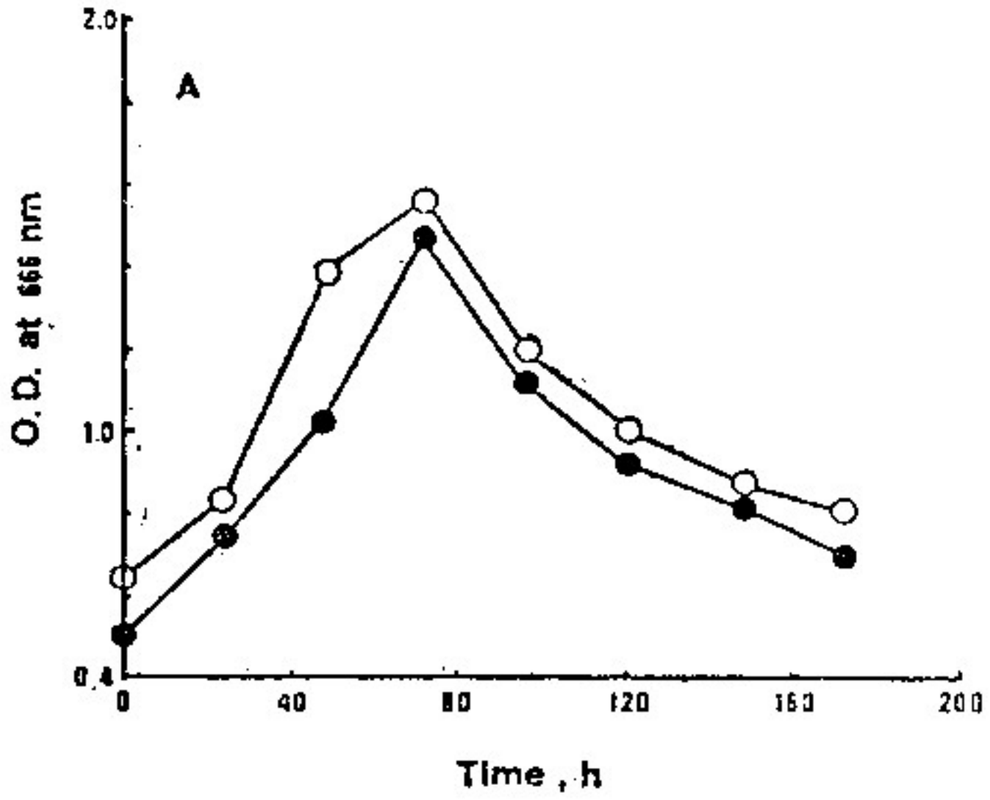
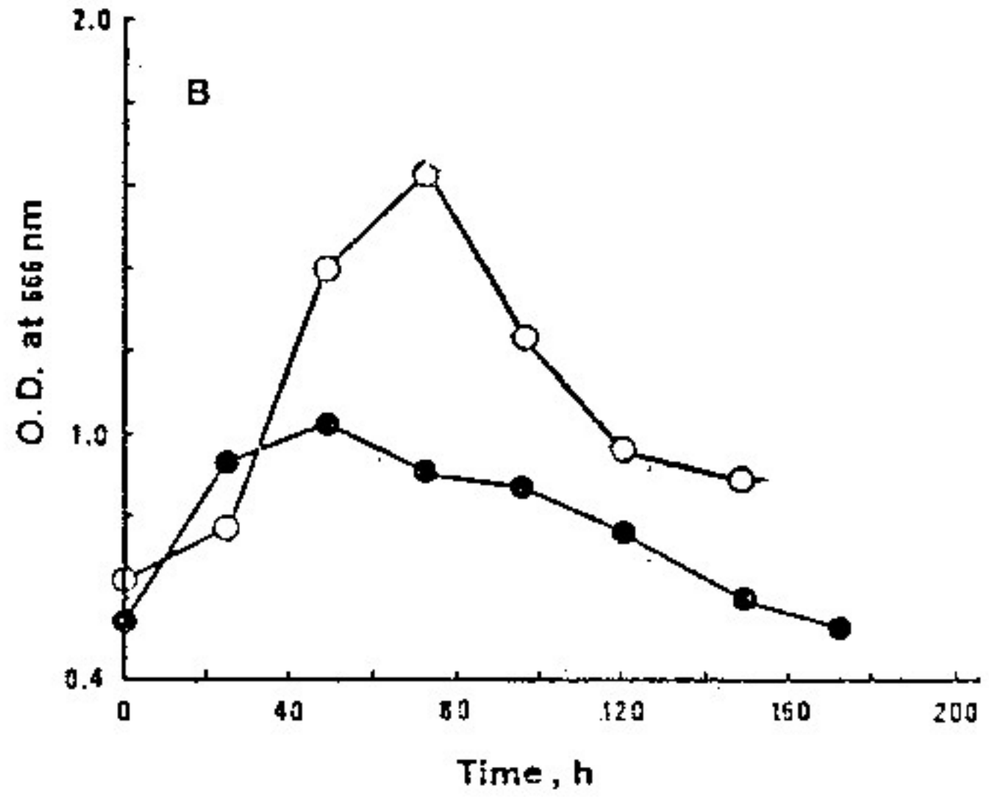
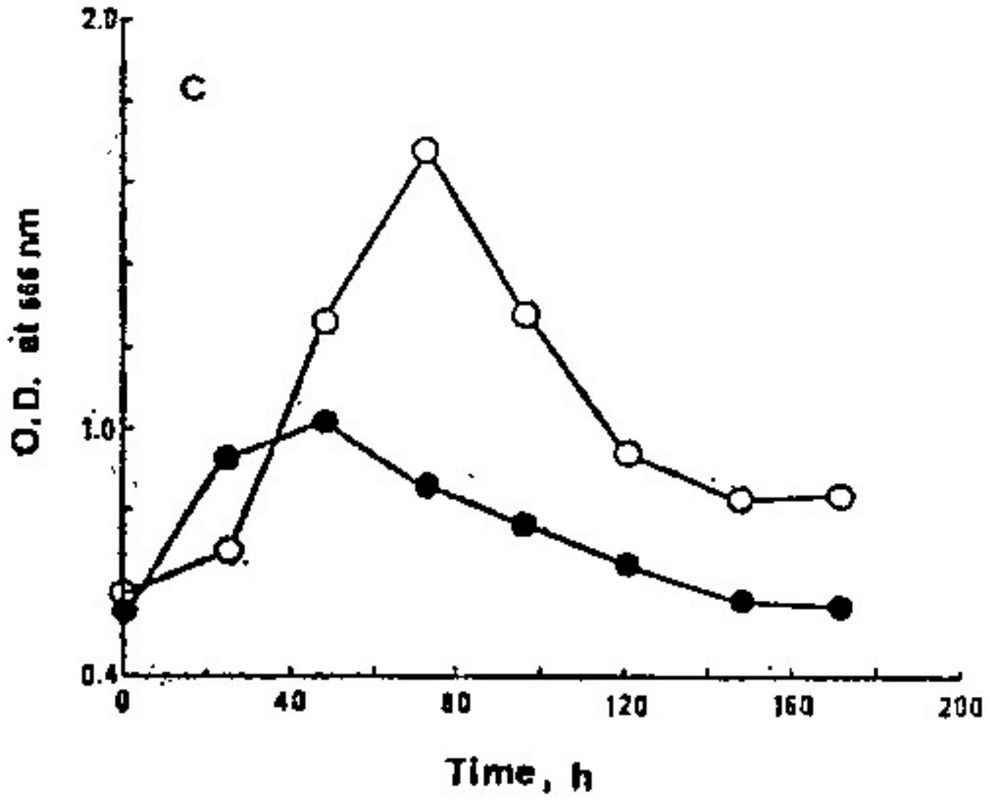
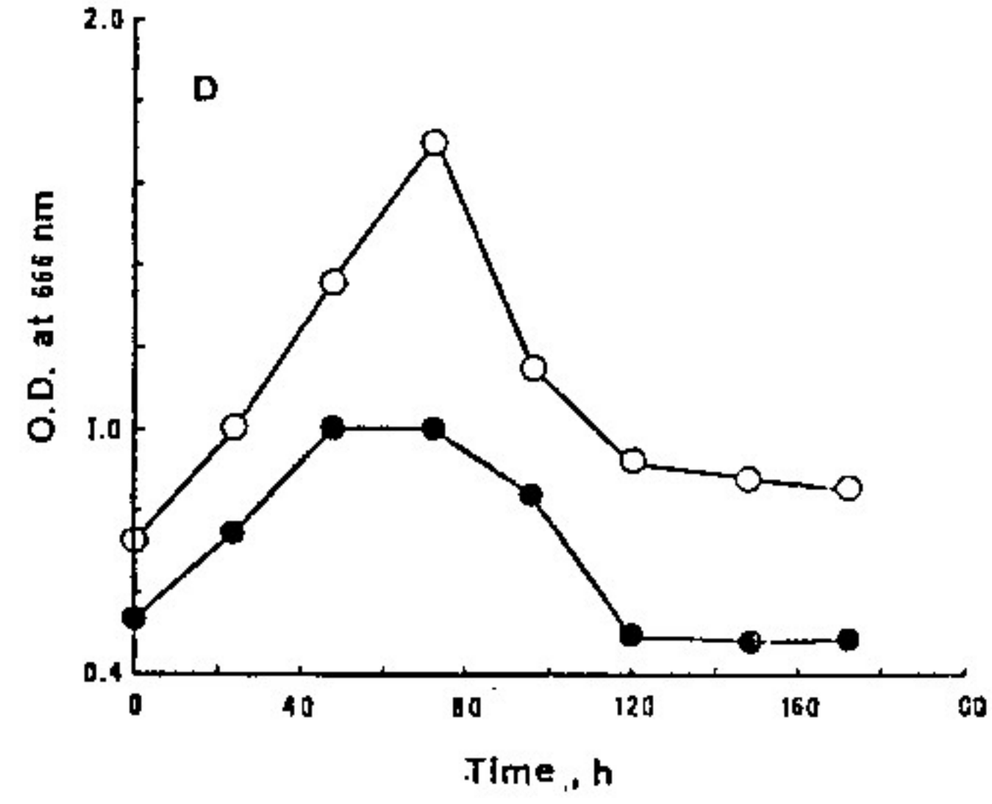


Fig. 7. Effect of initial pH value of medium on the inhibition of growth of host bacteria. (A) pH 6.5; (B) pH 7.2; (C) pH 7.5; (D) pH 7.8; (E) pH 8.2. (△--△): *host cell alone*; (▲--▲): *host plus Bdellovibrio*. Concentration of host bacteria: 4.0×10^7 cells/ml. Concentration of *Bdellovibrio*: 2.0×10^6 cells/ml.









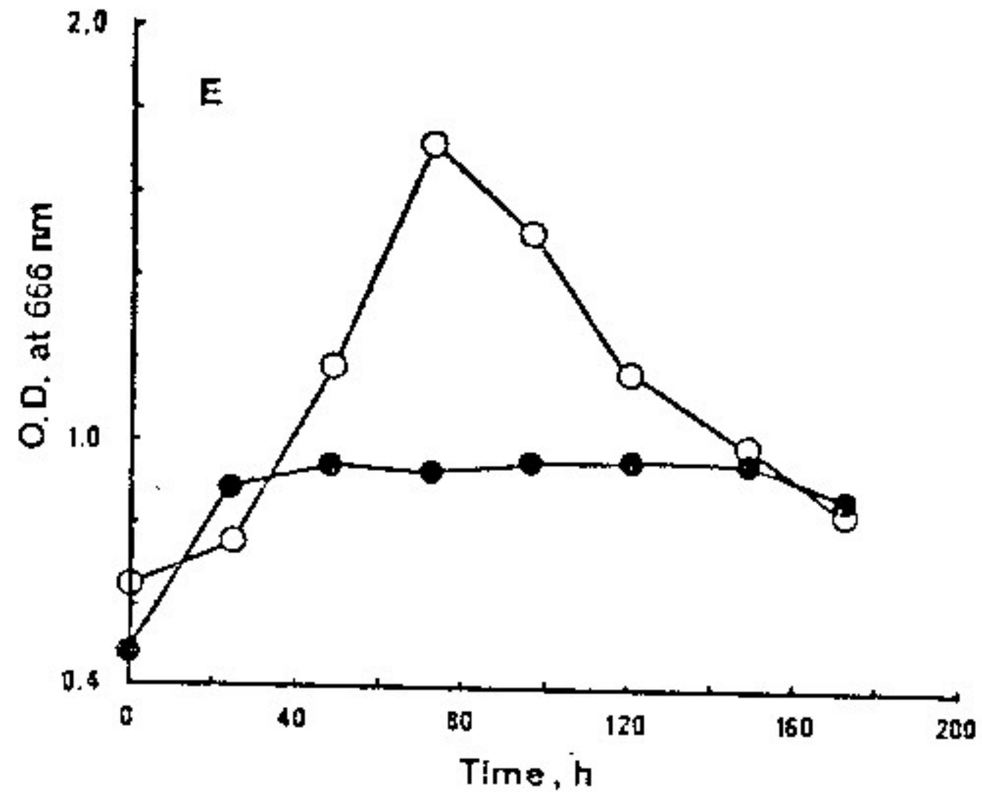


Fig. 8. Effect of concentration of cacium and magnesium ion on the inhibition of growth of host bacteria. (A) not added; (B) 0.2 g/l; (C) 0.4 g/l; (D) 0.6 g/l; (E) 0.8 g/l of Ca^{2+} and Mg^{2+} was added. (○--○): host cell alone; (●--●): host plus *Bdellovibrio*. Concentration of host bacteria: 4.0×10^7 cells/ml. Concentration of *Bdellovibrio*: 2.0×10^6 cells/ml.

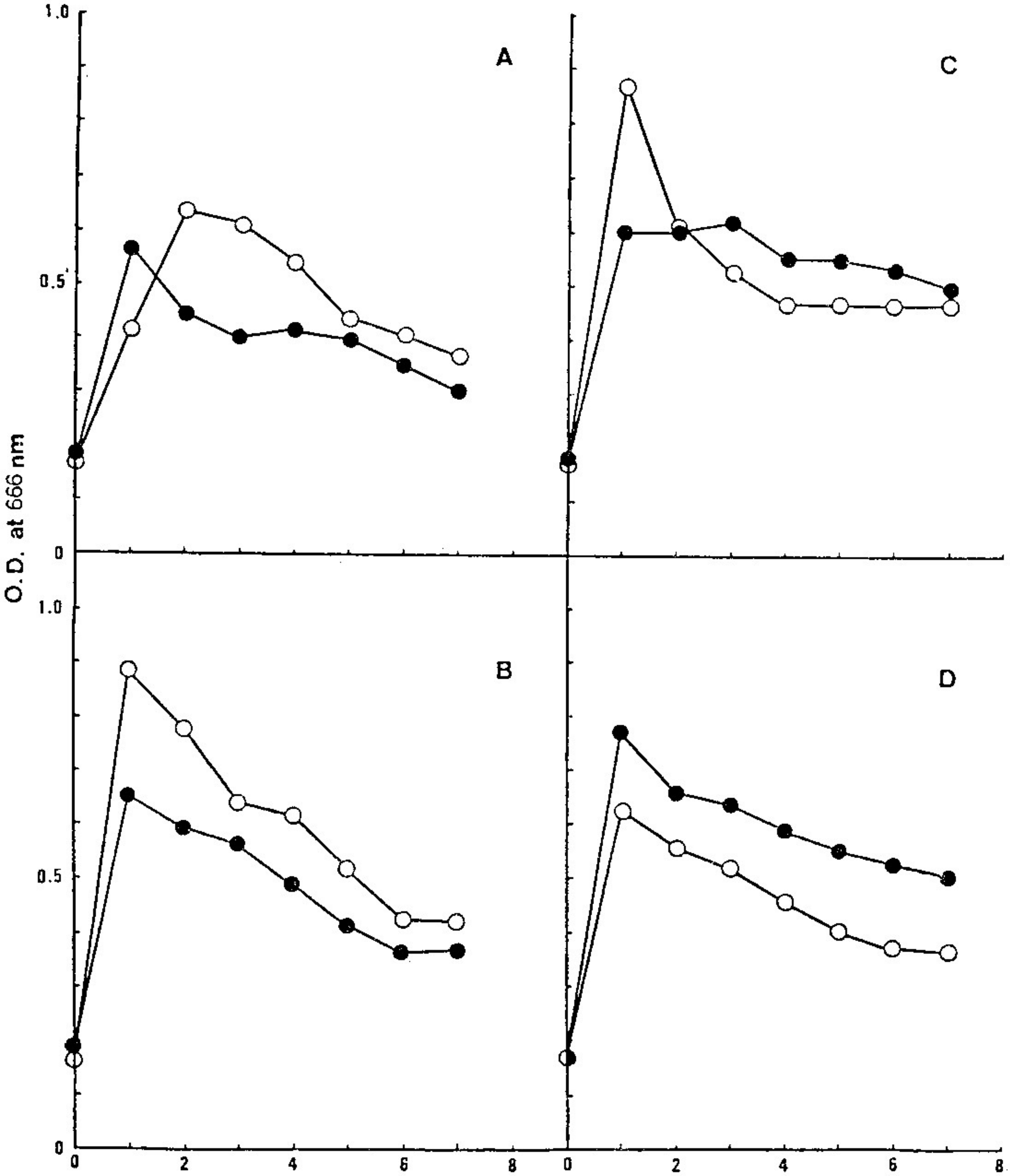


Fig. 9. Effect of temperature on the inhibition of growth of host bacteria. (A) 25°C; (B) 29°C; (C) 33°C; (D) 37°C. (○--○): host cell alone; (●--●): host plus *Bdellovibrio*. Concentration of host bacteria: 4.0×10^7 cells/ml. Concentration of *Bdellovibrio*: 2.0×10^6 cells/ml.

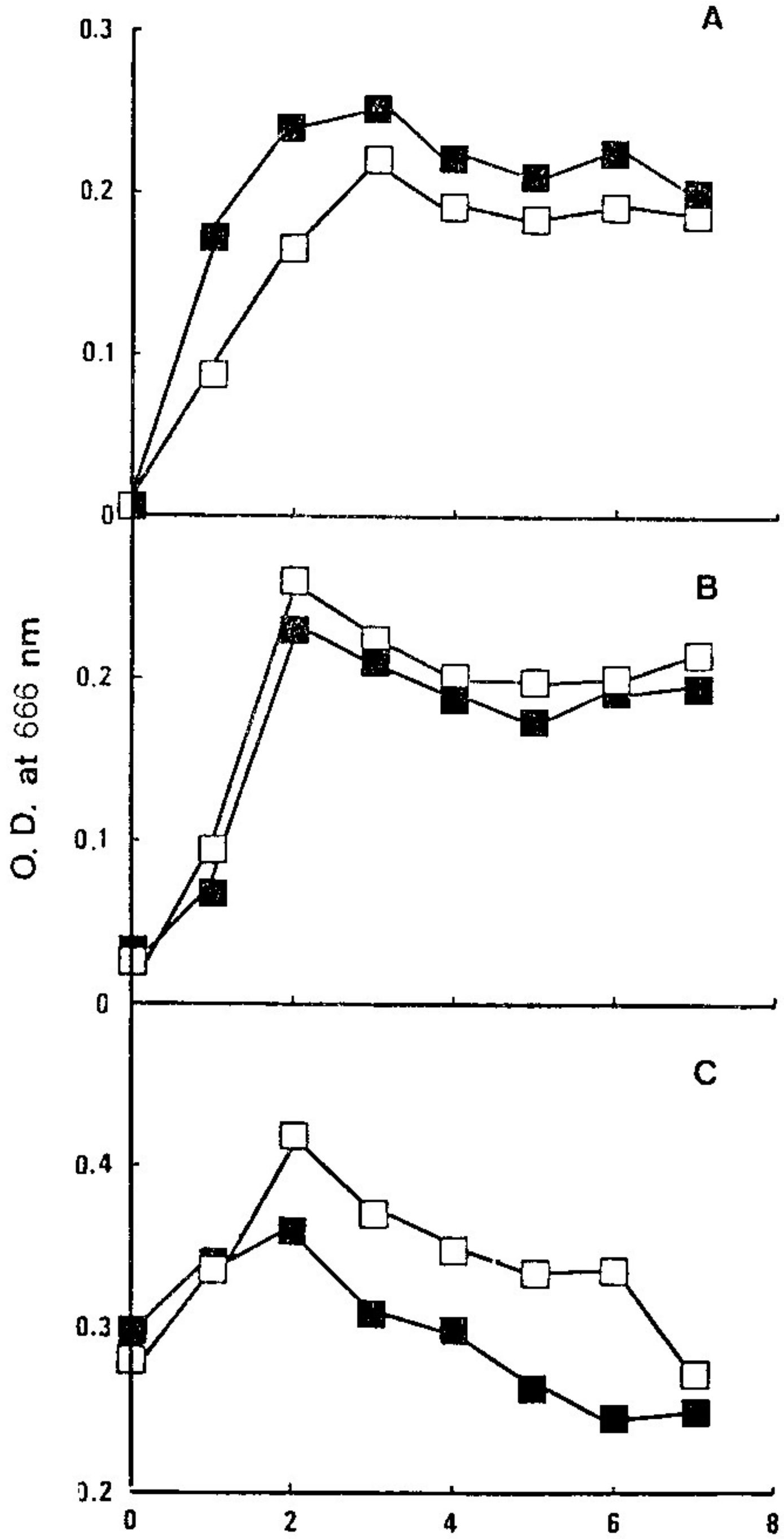


Fig. 10. Effect of concentration of host cells on the growth of *Bdellovibrio*. (A) 1/10×original suspension; (B) original suspension *; (C) 10× original suspension.
(□--□): host cell alone; (■--■): host plus *Bdellovibrio*.
*: concentration of original suspension: 4.0×10^7 cells/ml.
Concentration of *Bdellovibrio*: 2.0×10^6 cells/ml.

from the ghosted remnants of host bacteria (Fig. 6). This dimorphic life-cycle may well be a general (and quit unusual) feature of all *Bdellovibrio* (Starr and Stolp, 1976). (3) Liquid cell suspensions of suitable associant bacteria, when acted upon by *Bdellovibrio* swarmers, were reduced in turbidity. (Fig. 10-Fig. 13) (Starr and Stolp, 1976). (4) *Bdellovibrio* cell surface sensitive to some electron dense negative staining reagent, surface projections were revealed on intact *Bdellovibrio* cells after negative staining with certain compound (Fig. 4). The small rounded forms of *Bdellovibrio* (Fig. 4) were always present in old lysates and the number increases as the lysates aged (Abram and Davis, 1970). (5) Parasitism on bacteris of different genera (Table 2) are common characteristics of all *Bdellovibrio* (Starr and Stolp, 1976). (6) Gram-negative.

Starr and Stolp (1976) pointed out that development of *Bdellovibrio* plaques is favored by use media of low nutrient. In this study, we found that the isolate could only grow on YP as well as diluted NB and TSB, but not on undiluted NB and TSB (Table 1). *Bdellovibrio* A-1 showed a wide host range when tested for plaque forming on Gram negative fish pathogens, but not on *Bacillus subtilis*, and *Staphylococcus aureus* (Table 2). According to the studies of Starr and Seidler (1971), parasitic capacity of all *Bdellovibrio* strains is limited to Gram-negative host species; but not to Gram-positive bacteria.

In geneneral, the addition of divalent cation has been found to support the growth of *Bdellovibrio* (Fig. 8). Crothers and Robison (1971) found that *Bdellovibrio.* cultivated in the presence of viable host cells, exibits an absolute requirements for Ca^{2+} or Mg^{2+}. Starr and Seidler (1971) also pointed out that the addition of cation ion is necessary for *Bdellovibrio* to start infectious cycle.

Uematsu *et al.*[7] has mentioned that the plaque formation was considered to be a result of competitive multiplication between the host and parasite and the maximum multiplication of *Bdellovibrio* parasitic to *Xanthomonas oryzae* was obtained at 30°C. In this study, we found that the maximun growth of *Bdellovibrio* was obtained at 25 to 29°C (Fig. 9).

These results suggested that the use of *Bdellovibrio* as a biological control agent is feasible. Experiments are in progress which involved the survival of *Bdellovibrio* in the culture fish pond and the stability of *Bdellovibrio* during preservation.

ACKNOWLEDGEMENT

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寄生於格蘭氏陰性魚病細菌 *Aeromonas hydrophila* 的噬菌弧菌之篩選及其特性

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摘　要

由臺北近郊分離出一株能寄生於 *Aeromonas hydrophila* 的噬菌弧菌，經多次重覆單離，並做電子顯微鏡觀察鑑定後將其定名爲 *Bdellovibrio* A-1，並探討影響寄主關係的因子。噬菌弧菌產生的最適溫度爲 25～29°C，最適酸鹼質爲 pH 6.5，噬菌弧菌的生長需要添加二價陽離子 Ca^{2+} 和 Mg^{2+}。噬菌弧菌對三種魚病細菌均會造成感染，而宿主細菌的添加濃度以 4×10^8 cells 較適合於噬菌弧菌的生長。