

Bdellovibrio属細菌の生理生態学的研究 3

生菌培地ならびに死菌培地でのB.bacteriovorusの増殖

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Biological and Ecological Studies on *Bdellovibrio*

3. Growth of *B. bacteriovorus* in media composed of living and of autoclaved bacterial cells

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植松 勉・脇本 哲 : *Bdellovibrio* 属細菌の生理生態学的研究

3. 生菌培地ならびに死菌培地での *B. bacteriovorus* の増殖

Abstract

Five isolates of *Bdellovibrio bacteriovorus* obtained from paddy field soils of Japan were tested on their ability and mode of multiplication in media composed of autoclaved susceptible or resistant bacteria. The isolate Bd-N6801 successively transferred on *Xanthomonas oryzae* living cell medium was commonly used in this experiment.

Bd-N6801 multiplied in media composed of autoclaved cells of susceptible and also of some species of resistant bacteria. The multiplication was slightly retarded in the autoclaved cell (AC) medium than in the living cell (LC) medium. Autoclaved cytoplasm fraction of the bacteria also supported the multiplication of Bd-N6801. Bd-N6801 grew well on AC-agar plate, forming light yellowish colonies. Lytic zone appeared around each colony on AC-agar plate prepared from susceptible bacteria, in contrast to negative results with plate prepared from resistant bacteria.

Since the plaque forming units (PFU) on LC-agar medium closely corresponded with the colony forming units (CFU) on AC-agar medium, it is concluded that every cell of Bd-N6801 either in the plaque on LC-agar or in the colony on AC-agar possesses both parasitic and saprophytic propagation abilities. The morphological characteristics of the cells from the colony were identical with those from the plaque.

The other isolates of *B. bacteriovorus*, Bd-N6802, Bd-N6804, Bd-N6805, and Bd-N6806, also formed colonies on AC-agar medium. The ratio of CFU to PFU, however, varied much with different isolates.

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Introduction

It was formerly believed that *Bdellovibrio bacteriovorus* could multiply only in living host bacteria^{10,11}). Recently, attempts to grow the bacterium on media without living host cells have successfully been made by several workers. Burger *et al.*¹⁾ reported that a strain of *B. bacteriovorus* was able to grow on heat-killed or UV-inactivated cells of host bacteria. Shilo *et al.*⁷⁾ succeeded in culturing a host-independent mutant of *B. bacteriovorus* by using artificial medium, retaining their parasitic characteristics. Huang³⁾ confirmed the multiplication of *B. bacteriovorus* strain 6-5-S on resting, pasteurized, or autoclaved cells of *Spirillum sepeus* strain VHL in several kinds of suspending media

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supplemented with CaCl_2 and MgSO_4 . Another host-independent mutant of *B. bacteriovorus* UK was cultured on host-cell free artificial media by Dietrich *et al.*²⁾, retaining its parasitic capacities. It is evident from these results that some isolates of *B. bacteriovorus* possess both parasitic and saprophytic abilities.

The present work was carried out to make clear the fundamental problems concerning to multiplication of Japanese isolates of *B. bacteriovorus*.

Materials

Organisms *B. bacteriovorus* isolate Bd-N6801¹²⁾ successively transferred on *Xanthomonas oryzae* living cell medium was mostly used throughout the experiment. The original Bd-N6801 isolate and other isolates, Bd-N6802, Bd-N6804, Bd-N6805, and Bd-N6806 which had been kept in freeze dried condition were used occasionally. Susceptible bacteria, *Xanthomonas oryzae*, *X. pruni*, *X. phaseoli*, *Pseudomonas solanacearum*, *P. eriobotryae*, *P. mori*, *Escherichia coli* and *Aerobacter cloacae*, and resistant bacteria, *Corynebacterium michiganense*, *C. rathayi*, *X. campestris*, *Bacillus megaterium*, *B. subtilis*, *B. cereus*, *P. fluorescens* and *Agrobacterium tumefaciens* were used either in living culture or as autoclaved cell medium. *B. bacteriovorus* forms plaques on the susceptible bacteria, but does not on the resistant bacteria.

Media PSA medium¹⁵⁾ was used to grow the host bacteria. The composition was as follows: potato decoction 300 g, $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ 0.5 g, $\text{K}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 2.0 g, peptone 5 g, sucrose 20 g, agar 15 g, water 1 l, pH 6.8–7.0. "Living cell (LC) medium" means a concentrated suspension of bacterial cells in sterilized distilled water. To determine plaque forming units (PFU), LC-agar (LC plus agar 0.6%) medium was employed. "Autoclaved cell (AC) medium" was prepared by the following procedure. Bacterial cells cultured on PSA medium for 2 days were washed three times by centrifuging in sterilized distilled water. The final precipitant was resuspended in water to make concentration of 10^{10} to 10^{11} cells/ml, and autoclaved at 120°C for 20 min. To determine colony forming units (CFU), AC-agar medium (AC plus agar 0.6%) was used.

Methods and results

Multiplication of Bd-N6801 on LC and AC media of susceptible and resistant bacteria

The bacteria listed in Table 1 and 2 were cultured on PSA medium at 28°C for 2 days. Both LC

Table 1. Multiplication of Bd-N6801 in susceptible bacteria

Host	LC medium ^{a)}	Ratio ^{b)}	AC medium ^{a)}	Ratio ^{b)}
<i>X. oryzae</i>	2.6×10^7	$\times 3,800$	4.0×10^8	$\times 590$
<i>X. phaseoli</i>	3.0×10^7	$\times 4,400$	8.0×10^8	$\times 1,190$
<i>X. pruni</i>	2.2×10^7	$\times 3,200$	1.2×10^7	$\times 1,790$
<i>P. solanacearum</i>	1.5×10^7	$\times 2,200$	4.5×10^7	$\times 6,710$
<i>P. mori</i>	2.5×10^7	$\times 3,700$	7.6×10^7	$\times 11,260$
<i>P. eriobotryae</i>	5.5×10^6	$\times 820$	3.2×10^8	$\times 47,160$
<i>E. coli</i>	1.5×10^7	$\times 2,100$	4.4×10^7	$\times 6,570$
<i>Aer. cloacae</i>	1.0×10^8	$\times 15,300$	1.2×10^8	$\times 17,160$

a) PFU determined on 4th day after inoculation.

b) Initial concentration of Bd-N6801: 6.7×10^8 cells/ml.

Table 2. Multiplication of Bd-N6801 in resistant bacteria^{a)}

Host	LC medium ^{b)}	Ratio ^{c)}	AC medium ^{b)}	Ratio ^{c)}
<i>C. michiganense</i>	1.0×10^2	<1	2.0×10^6	$\times 2,240$
<i>C. rathayi</i>	$<1.0 \times 10^2$	"	$<1.0 \times 10^2$	<1
<i>B. subtilis</i>	"	"	2.4×10^5	$\times 250$
<i>B. cereus</i>	"	"	6.5×10^6	$\times 6,840$
<i>A. tumefaciens</i>	3.5×10^3	$\times 4$	3.3×10^5	$\times 350$
<i>B. megaterium</i>	9.5×10^4	$\times 100$	1.5×10^6	$\times 1,580$
<i>P. fluorescens</i>	$<1.0 \times 10^2$	<1	8.0×10^4	$\times 80$

a) Plaque did not appear on the lawn of these bacteria.

b) PFU determined on 4th day after inoculation.

c) Initial concentration of Bd-N6801: 9.5×10^3 cells/mL.

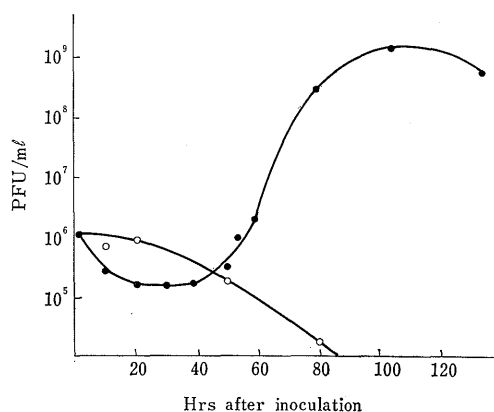


Fig. 1. Growth of Bd-N6801 in living (LC) and in autoclaved cells (AC) media prepared with *Bacillus subtilis*.

○ : LC medium. ● : AC medium.

and AC media prepared from these cultures were inoculated with 6.7×10^3 cells/mL of Bd-N6801 cells and were incubated at 30°C. The population of Bd-N6801 was determined in terms of PFU on the 4th day after inoculation. In AC media prepared with susceptible bacteria, Bd-N6801 multiplied well as in LC media (Table 1).

Bd-N6801 could not form plaques on the lawn of the resistant bacteria as listed in Table 2, although it multiplied to a slight degree on living *B. megaterium* and *A. tumefaciens* cells. On the other hand, Bd-N6801 multiplied rather well in AC media prepared from such resistant bacteria as *C. michiganense*, *B. subtilis*, *B. cereus*, *A. tumefaciens*, *B. megaterium*, and *P. fluorescens* (Table 2 and Fig. 1).

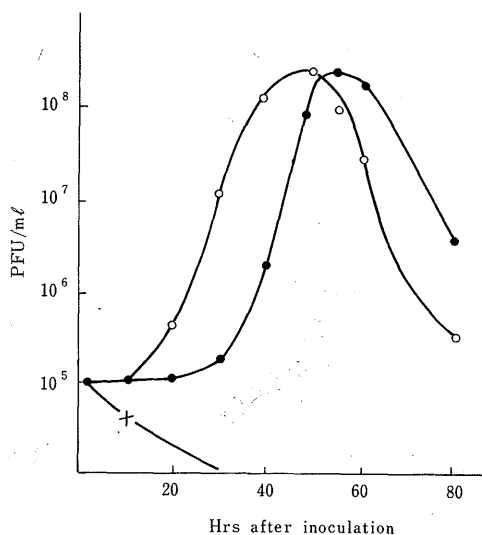
Growth of Bd-N6801 in LC and AC media of *X. oryzae* Both LC and AC media prepared from cultures of *X. oryzae* were inoculated with Bd-N6801 cells which had been prepared by passing through 0.45 μ Millipore filter, and were shaken at 31°C. Periodical plating with LC-agar medium composed of *X. oryzae* cells was carried out for PFU determination.

As shown in Fig. 2, the multiplication of Bd-N6801 in the AC medium was somewhat retarded in comparison with that in the LC medium. In both cases, PFU decreased rapidly after attaining maximum.

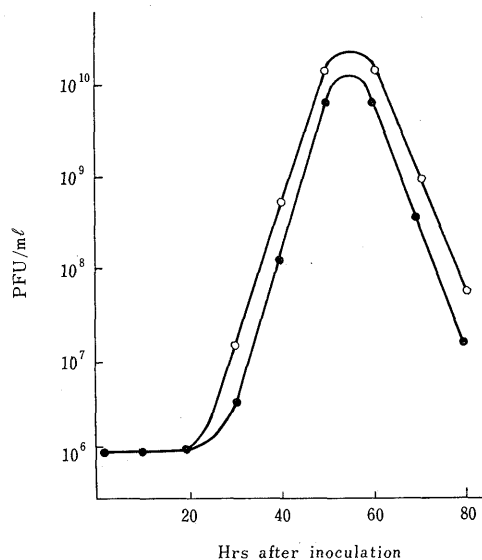
Table 3. Multiplication of Bd-N6801 in crude fraction of *Xanthomonas oryzae* cells

Medium	Hours after inoculation		Ratio
	0	100	
Washed intact cells	1.1×10^3	1.4×10^8	$\times 127,272$
Cytoplasm	//	2.0×10^7	$\times 18,181$
Cell wall ^{a)}	//	6.5×10^6	$\times 5,918$
Water (Control)	//	1.0×10^2	$\times <1$

a) Possibly contaminated with a small quantity of cytoplasm.

Fig. 2. Growth of Bd-N6801 in living (LC) and autoclaved cell (AC) media prepared with *X. oryzae*.

○ : LC medium. ● : AC medium.
+ : Control (PS medium).

Fig. 3. Growth of Bd-N6801 in fractions of *X. oryzae* cells.

○ : Cytoplasmic granule fraction.
● : Cytoplasmic soluble fraction.

Table 4. Multiplication of Bd-N6801 in cytoplasm of resistant bacteria

Medium	Hours after inoculation		Ratio
	0	100	
<i>A. tumefaciens</i>			
Broken cell ^{a)}	1.4×10^4	3.8×10^5	$\times 36$
Cytoplasm	//	8.5×10^5	$\times 80$
<i>B. subtilis</i>			
Broken cell	//	6.3×10^5	$\times 60$
Cytoplasm	//	2.0×10^6	$\times 160$
<i>C. michiganense</i>			
Broken cell	//	4.0×10^6	$\times 380$
Cytoplasm	//	5.0×10^6	$\times 476$

a) Ultrasonic treated and centrifuged at $3,000 \times g$ for 30 min to make the preparation free from intact cells.

Cell materials supporting multiplication of Bd-N6801 Fractionation of *X. oryzae* cells into components of cell wall, cytoplasmic granules, and soluble substances was carried out by the method of Kotani *et al.*⁴⁾ Each fraction was autoclaved and inoculated with Bd-N6801, followed by continuous shaking. A small aliquot of the suspension was plated periodically for PFU determination.

Bd-N6801 multiplied most abundantly in the cytoplasmic granule fraction, followed by the soluble fraction and cell wall fraction (Table 3). Both cytoplasmic granules and soluble substances in other experiment supported the multiplication to the same degree (Fig. 3). Bd-N6801 also multiplied in the cytoplasm fraction of such resistant bacteria as *A. tumefaciens*, *B. subtilis*, and *C. michiganense* (Table 4).

Colony formation of Bd-N6801 on AC-agar plate AC-agar medium inoculated with Bd-N6801 was plated by means of the double-layer technique, and incubated at 31°C. Bd-N6801 formed light yellowish colonies in the center of lytic zone by 4th to 5th day after plating (Fig. 4).

The morphology of the cells obtained from the colonies was almost identical with those from the plaques, except the existence of spiral form cell (Fig. 5).

Similarity in parasitism between Bd-N6801 cells from colonies and those from plaques A part of the colonies formed on the AC-agar medium was suspended in sterilized distilled water, and the titer was simultaneously estimated in PFU and in CFU by using LC-agar and AC-agar media, respectively. From the result shown in Table 5, it was confirmed that Bd-N6801 cells obtained from the colonies retained their parasitic ability to the host bacteria, as indicated by the close correspondence between CFU and PFU. Similar test was also conducted with Bd-N6801 cells from the plaques

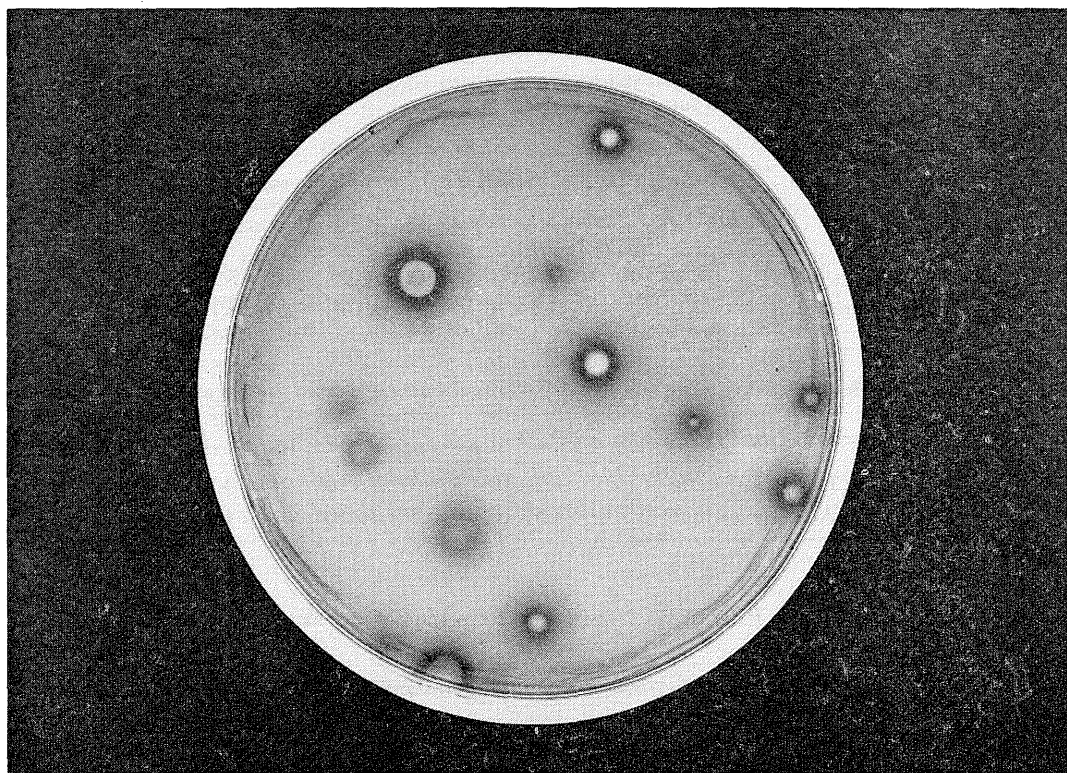


Fig. 4. Colonies of Bd-N6801 appeared on AC (*X. oryzae*)-agar medium (5 days after inoculation).

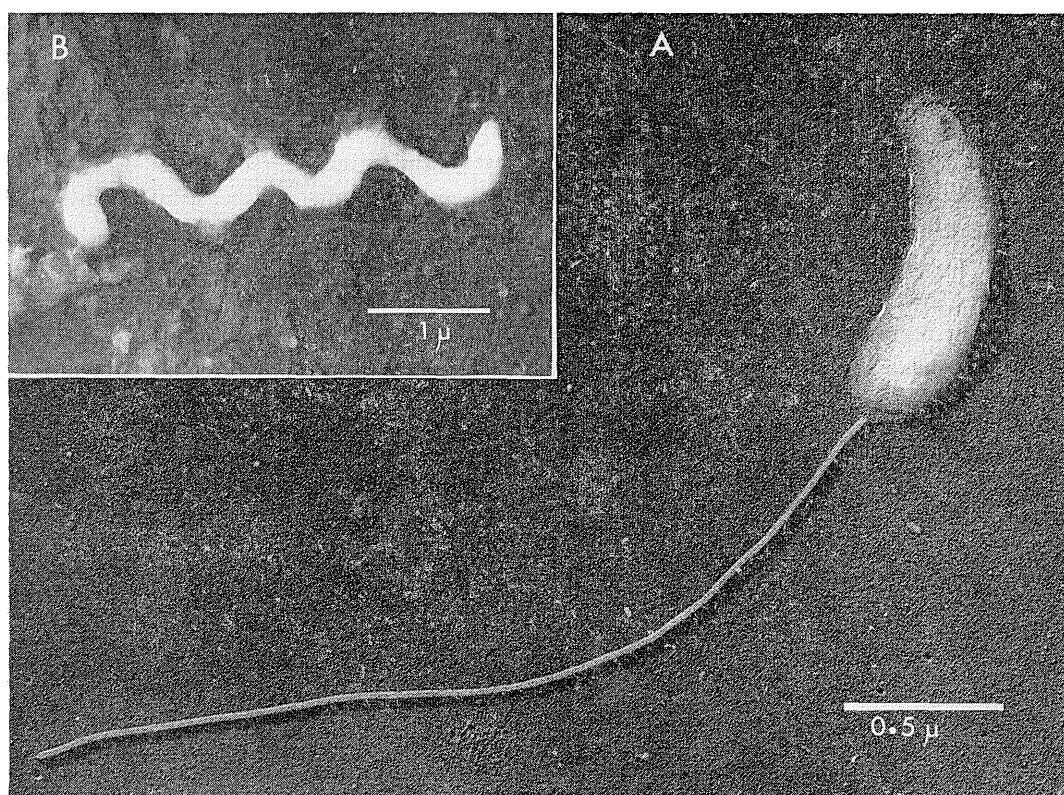


Fig. 5. Electronmicrograph of Bd-N6801 cells obtained from the colony.
 (A) Vibrio form cell.
 (B) Spiral form cell.

Table 5. Parasitism of Bd-N6801 cells obtained from the colonies

Exp. No.	1	2	3	4	5
PFU on LC-agar medium	8.2×10^5	1.2×10^6	1.5×10^6	7.4×10^5	1.3×10^6
CFU on AC-agar medium	1.1×10^6	1.5×10^6	1.5×10^6	6.2×10^5	1.5×10^6

Table 6. Colony formation of Bd-N6801 cells obtained from the plaques

Exp. No.	1	2	3
PFU on LC-agar medium	4.2×10^2	1.7×10^4	4.9×10^1
CFU on AC-agar medium	4.8×10^2	1.4×10^4	6.0×10^1

formed on LC-agar medium. PFU of Bd-N6801 cells from the plaques also closely corresponded with CFU (Table 6).

It is therefore concluded that every cell of Bd-N6801 in the plaques has an ability to form colony on AC-agar medium, and that individual cells in the colony retain their parasitic ability.

Colony formation of Bd-N6801 cells on various kinds of AC-agar medium AC-agar media listed in Table 7 were prepared by the above described method. The colony forming efficiency

(CFE) of Bd-N6801 on these media was tested by means of the usual plating technique.

Bd-N6801 cells formed their colonies on various kinds of AC-agar medium without much variation in CFE. Bd-N6801 cells formed lytic zone around each colony on media prepared with susceptible bacteria, but not on media prepared with resistant bacteria (Table 7).

Colony formation of other isolates of *B. bacteriovorus* on AC-agar medium Preparation of AC-agar medium and plating were done in the same way as in the case of Bd-N6801.

Table 7. Colony forming efficiency of Bd-N6801 cells on various kinds of AC-agar medium

Kinds of bacteria used for AC-agar medium preparation	CFU (per ml) ^{a)}
<i>P. solanacearum</i>	6.1×10^5
<i>E. coli</i>	3.5×10^5
<i>X. phaseoli</i>	2.7×10^5
<i>X. citri</i>	4.7×10^5
<i>X. oryzae</i>	7.9×10^5
<i>Aer. cloacae</i>	4.1×10^5
<i>A. tumefaciens</i> ^{b)}	8.1×10^5
<i>B. cereus</i> ^{b)}	$6.5 \times 10^{5c)}$
<i>S. sake</i> ^{b)}	$3.0 \times 10^{5c)}$
<i>B. subtilis</i> ^{b)}	$4.8 \times 10^{5c)}$
<i>C. michiganense</i> ^{b)}	$5.1 \times 10^{5c)}$

a) PFU on LC-agar medium prepared with *X. oryzae* was $8.4 \times 10^5/\text{ml}$.

b) Resistant bacteria.

c) No lytic zone appeared around the colony.

Table 8. Colony forming efficiency of the isolates of *B. bacteriovorus* on AC-agar medium prepared with *X. oryzae*

Isolates	PFU on LC-agar medium	CFU on AC-agar medium
Bd-N6802	9.7×10^5	8.1×10^5
Bd-N6804	5.4×10^5	4.0×10^2
Bd-N6805	2.6×10^6	8.0×10^2
Bd-N6806	4.4×10^6	1.1×10^4

It is concluded from the result shown in Table 8 that the relationship between PFU and CFU much varies according to the isolates. CFU of Bd-N6802 on AC-agar medium closely corresponded to PFU on LC-agar medium as in the case of Bd-N6801, whereas CFU of the other isolates was much lower than PFU.

Discussion

The parasitic bacterium *B. bacteriovorus* attacks many kinds of Gram-negative and also some kinds of Gram-positive bacteria. It attaches on the host bacterial cell surface, invades through cell wall, and reproduces within the bacterial cell^{1,5,9,10,11}.

Recently, Burger *et al.*¹⁾ reported that *B. bacteriovorus* can propagate on heat (80°C) treated host

bacteria. Varon *et al.*¹⁴⁾ also reported propagation of the organism on ultraviolet-irradiated host cells as well as cells treated at 70°C. Shilo *et al.*⁷⁾, succeeded in culturing *B. bacteriovorus* A3.12 isolate on a synthetic medium, reported that this strain retained their parasitic ability even after successive transfers. Huang³⁾ and Dietrich *et al.*²⁾ obtained similar results by using different isolates. These reports demonstrated that some strains of *B. bacteriovorus* do not necessarily require living host cells for their propagation.

The authors confirmed that *B. bacteriovorus* isolate Bd-N6801 successively transferred on living cell medium of *X. oryzae* grows well on a medium composed of autoclaved (120°C for 20 min) bacterial cells, and that this isolate retains parasitic ability even after successive transfers on this medium. Autoclaved cells of 8 species of susceptible and 6 species of resistant bacteria also supported the multiplication of Bd-N6801. Cytoplasmic granule fraction of the bacteria appeared to be essential to the growth of Bd-N6801. These results indicate that Bd-N6801 can grow saprophytically in the presence of some bacterial components without living host bacteria.

Bd-N6801 did not form lytic zone around the colony on AC-agar medium prepared with resistant bacteria. It appears that the lytic enzymes of Bd-N6801 can not destroy the cell wall and cytoplasmic components of the resistant bacteria.

Both isolates Bd-N6801 and Bd-N6802 showed close correspondence between PFU on LC-agar medium and CFU on AC-agar medium. It is suggested that almost all cells of these isolates have the ability of both saprophytic and parasitic multiplication. The majority of the cells of the other isolates, Bd-N6804, Bd-N6805, and Bd-N6806, however, may not be able to grow saprophytically.

Bd-N6801 did not form plaques on the lawn of *B. megaterium* and of *A. tumefaciens*, although it multiplied to some degree in the suspensions of these bacteria. This may be explained by extremely low plaque forming efficiency, but it is also possible that exudation of some nutrients from the cells supported the multiplication of Bd-N6801.

Acknowledgment

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和文摘要

Bdellovibrio 属細菌の生理生態学的研究

(3) 生菌培地ならびに死菌培地中での *B. bacteriovorus* の増殖

植 松 勉・脇 本 哲

水田土壌から分離した *Bdellovibrio bacteriovorus* の 5 株を用いて、各種の感受性細菌と非感受性細菌の生菌培地中、およびそれらを高圧滅菌して作った死菌培地中での増殖の有無および増殖様相について検討した。

B. bacteriovorus の 1 分離株 Bd-N6801 は、感受性細菌中でよく増殖するが、これらを用いて調製した死菌培地中でも増殖し、また数種の非感受性細菌の死菌培地中でも増殖することを確認した。死菌培地中での増殖様相は感受性細菌の生菌でのそれと類似しているが、増殖がやや遅れる傾向がある。

イネ白葉枯病菌の細胞構成成分を分画し、Bd-N6801 の増殖に関与する成分について検討し、細胞質画分で増殖することを確認した。

Bd-N6801 は死菌培地上に淡黄色のコロニーを形成し、感受性細菌の死菌培地上ではコロニーの周縁に透明な溶菌帯を形成する。非感受性細菌の死菌培地上でもコロニーは形成されるが、溶菌帯は認められない。

生菌培地上にできた溶菌斑と死菌培地上にできたコロニーから Bd-N6801 をとり、生菌培地と死菌培地を使って溶菌斑形成率とコロニー形成率とを比較した結果、その両者はほぼ一致した。しかも、コロニーから得た Bd-N6801 細胞の形態は溶菌斑から得たものの形態と同じであった。したがってすべての Bd-N6801 の細胞は寄生的能力とともに腐生的に増殖する能力も兼ね備えているものと思われる。

他の *B. bacteriovorus* の分離株、Bd-N6802, Bd-N6804, Bd-N6805, および Bd-N6806 も死菌培地上にコロニーの形成を認めたが、溶菌斑形成率とコロニー形成率との関係は菌株によっていちじるしく異なった。