Biological Properties of Protoplasts Produced by Sucrose-induced Autolysis of
Clostridium saccharoperbutylacetonicum

by

K. H. Choi

Laboratory of Applied Microbiology, Department of Agricultural Chemistry,
Kyushu University, Fukuoka, 812, Japan.
(Received January 27, 1978)

Sucrose溶液中에서 誘起되는 *Clostridium saccharoperbutylacetonicum*의 自己溶解現象에 依하여 形成된 Protoplast의 性狀에 關한 研究

崔　　慶　　浩
日本國　福岡市　九州大學　農學部　農藝化學科　應用微生物學敎室
(1978년 1월 27일 수리)

Summary

The young cells of *Clostridium saccharoperbutylacetonicum* were rapidly autolysed by exposing them to the hypertonic solution of sucrose(0.3-0.6M) without any other supplement to decompose the rigid cell wall. The cells were converted into the spherical cells by lysis.

The spherical cells had following properties: (1) they were absent in the cell wall and osmotically fragile. (2) they were stabilized in the existence of 0.4M sucrose and 5mM $MgSO_4$. (3) they were resistant against adsorption of phage particles. (4) they allowed infection of the isolated phage DNA and produced progeny phage particles. (5) they were able to biosynthesize their macromolecules for a few hours according to a balanced manner of biosynthesis. (6) they were able to produce the bacteriocin particles by mitomycin C treatment. (7) they were unable to multiply.

These results were all in the level of typical properties of bacterial protoplasts. It was apparent that the spherical cells formed by lysis occcurring by treatment with hypertonic sucrose were protoplasts.

Introduction

Many kinds of gram positive bacteria were converted into protoplasts by using the cell wall-lytic enzymes[1-3] or antibiotics[4,5] Sometimes, they were produced autolytically[6,7] Throughout these works, sucrose was used to prevent the formed protoplasts from further lysis. Sucrose, in this report, was used not ont only for the produced spherical cells to prevent from lysis, but also used for the clo-

stridial cells to be rapidly lysed.

By many authors, the biological properties of protoplasts were investigated[8,9]. The principal property of protoplasts was suggested firstly as the posses of many of physiological capabilities of the parent cells and secondly as the lack of ability to multiply (10). In spite of this clear suggestion on the properties of protoplasts, there are a few bacterial forms, spheroplasts and L-form cells, so resembled to the protoplasts that confusions are made among them. In fact, questions were made from early against the confused use of the word "protoplasts"[5,9].

The technique, used in this report to form the spherical cells, is unique in the role of sucrose. Therefore, it was desired to clarify the biological properties of the cells to understand whether they were protoplasts or not. From this point of view, some biological properties of the spherical cells were investigated.

From here, the spherical cells shall be abbreviated into PLC(protoplast-like cells), while intact cells shall be abbreviated into Ic.

Materials and Methods

Bacterial strains. *C. saccharoperbutylacetonicum* Nl-4(ATCC 13564) (11) was used without otherwise mention. Two mutant strains of Nl-611 and Nl-504 were used for the transfection of phage DNA. They are resistant mutants against the adsorption of phage HM 2(11). Strain Nl-8 was also used as the indicator for clostocin O (a kind of bacteriocin)[12,13].

Media and cultural conditions. Cells were grown for 3 to 4 hr at 30°C until they reached early exponential growth phase. Cultural broth contained glucose (40g), yeast extract (2g), ammonium acetate (3g), Bacto-tryptone (6g), KH_2PO_4 (0.5g), $MgSO_4 \cdot 7H_2O$ (0.4g) and $FeSO_4 \cdot 7H_2O$ (0·01g) per liter (pH 6.5). Soft agar medium containing 0.6% agar was used for the assay of phage and bacteriocin activity.

Preparation of PLC. Cells were harvested by centrifugation(10,000×g for 10 min at 25 to 30°C) during their early exponential growth phase. They were resuspended in buffered sucrose solution(0.4 M) which dissolved 5mM of $MgSo_4 \cdot$ Sodium phosphate buffer(1/60M, pH 6.5) was used as the suspending fluid for

the cells. The cells were incubated for 60 min at 30 °C.

Adsorption of phage particles on PLC. Phage HM 2 and HM 3(14) were added to PLC wiith various multiplicity of infection(m. o. i). After incubation of the cells for 15 min at 30°C, the cells were removed by centrifugation(10,000×g for 10 min at 2°C). The number of phage particles remaining in the supernatant fluid was assayed by double layer plate technique.

Fractionation and analysis of macromolecular content. Macromolecules(DNA, RNA and protein) of PLC were fractionated according to the modified STS (Schmidt-Thannhauser-Schneider's) method[15]. The content of DNA, RNA and protein was analysed respectively according to Burton's diphenylamine method (15), Mejbaum's orcinol method[16] and Lowry's phenol-reagent-method[17].

Analysis of incorporation of radio-labelled precursor. PLC were cultivated in the stabilized fresh medium containing ^{32}P-orthophosphoric acid(2.5 μCi/ml) or methyl-^{3}H-thymidine (1.0μCi/ml). After fractionation of DNA, RNA and lipid, radioactivity of ^{32}P incorporated in each macromolecule was measured with a gas flow counterAloka, TDC-10). Radioactivity of ^{3}H in PLC was measured with a scintillation counter (Beckman, LS-250) after alkaline decomposition of the cell. Alkaline decomposition was carried out for 15 hr at 37°C with N KOH. The scintillation fluid was consisted of 0.6% diphenyloxazole (Beckman) and toluene-methanol. The radioisotopes were purchased from Daiichi Radioisotope Lab., Ltd..

Induction of bacteriocin. PLC were cultivated for 3 hr in the presence of mitomycin C(Kyowa Hakko), and then, the cells were removed by centrifugation (3,000×g for 20 min at 2°C). The activity of clostocin O liberated into the supernatant fluid was assayed. The activity of clostocin O was represented as an arbitrary unit of activity(unit/ml) which was defined as the reciprocal of the highest dilution cleary showing inhibition zone against strain Nl-8 by spot test. One unit of bacteriocin was approximately equal to 1×10^8 particles/ml of clostocin O.

Preparation of infectious phage DNA. DNA was isolated from purified phage HM 2, which had a final

titer of 1×10^{18} p. f.u./ml, according to phenol techn-
ique[18]. Isolated DNA was dissolved in saline-citrate
buffer(pH 7.0), and dialysed against the same buffer
for 48 hr at 4°C. This preparation contained 2-3 mg/
ml of DNA.

Transfection. DNA preparation(0.2ml) was added
on PLC culture(2ml) which contained about 2×10^8
cells/ml. The infected cells were cultivated for 3 hr,
and then, the number of active phage particles liber-
ated into the supernatant fluid was assayed.

Osmotic shock. PLC were harvested from 50ml of
culture, and then, they were rapidly resuspended in
the same volume of distilled water. Turbidity was
measured at 660nm. For control system, the same
volume of 0.4M sucrose solution was used. After
centrifugation, the absorptiom of supernatant fluid
was measured at 260 and 280nm.

Results

Sucrose-induced autolysis. As shown in Fig. 1,
cells were rapidly lysed as soon as they were exposed
to the hypertonic sucrose. Lysis occurred between
the sucrose concentration of 0.3 to 0.6M. Maximum
lysis was apparent at the concentration of 0.35M
sucrose showing a 50% loss of initial turbidity. Lysis
progressed rapidly for 30 min, and then it was leve-
lled off. After lysis for 40 min, more than 99% of
the rod-shaped clostridia were converted into the
spherical cells (PLC). The cells were apparently
induced to be autolysed by the concomitant sucrose
(sucrose-induced autolysis[19]).

Osmotic sensitivity of PLC. As shown in Table

**Table 1. Changes of optical density of the
suspension of PLC by osmo tic shock***

Wave	Shocked	Control
660nm	0.03	0.38
260nm	2.76	0.88
280nm	1.44	0.50

* Osmotic shock was given to the PLC by suspending
  them in the distilled water. For the control system,
  0.4M sucrose solution was used instead of distilled
  water. OD_{260} and OD_{280} were measured after elimin-
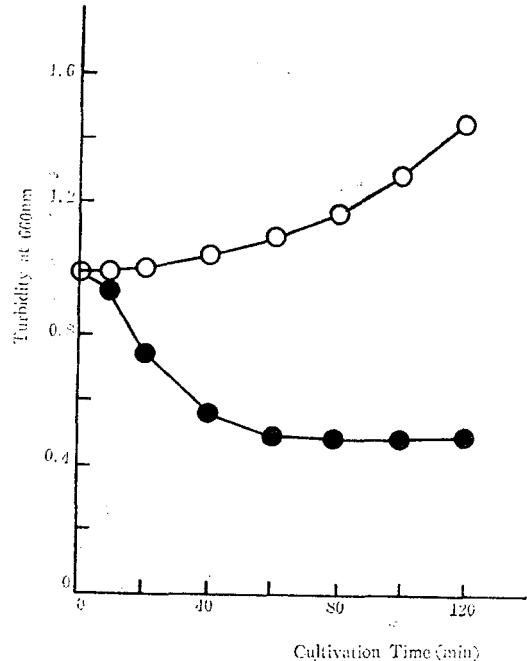 ation of the cell debris.

Fig. 1. Properties of protoplasts
Progress of sucrose-induced autolysis of clostrid-
ia Cells. Cells were inoculated in the fresh medium
containing 0.35M sucrose, and then, incubated anaer-
obically at 30°C. Initial OD_{660} of the culture was
adjusted to 0.6. Turbidity was expressed as the OD
$_{660}$ of measured time against that of initial. ●——●
sucrose, ○——○ : control

1, PLC immediately lost more than 90% of their
turbidity by osmotic shock, while they liberated their
cellular components. which showed high absorption of
ultraviolet ray. Shocked PLC showed bilayer plasma
membrane only by electron microscopy, while intact
PLC contained cellular components inside of the me-
mbrane. These results indicate that PLC are fragile
to osmotic shock.

**Effect of sucrose concentration on the stability
of PLC.** Experiment was performed to understand the
concentration of sucrose to stabilize PLC. The cells
were incubated for 30 min in sucrose solution of
various concentrations, and then they were havested
and subjected to osmotic shock. The supernatant
fluid was recovered before and after osmotic shock,
and the absorption of ultra violet ray was measured
respectively. In the process, it was expected that the
supernatant fluid before osmotic shock showed the

lowest adsorption at the concentration of sucrose app-ropriate to stabilize the PLC, while it showed the highest absorption after osmotic shock.

As shown in Fig. 2, the absorption was lowest at the concentration of sucrose near 0.4M before osmotic shock. At the same concentration of sucrose, the su-pernatant fluid after osmotic shock of PLC showed the highest absorption. This result indicates that PLC are most stable in 0.4M sucrose solution.
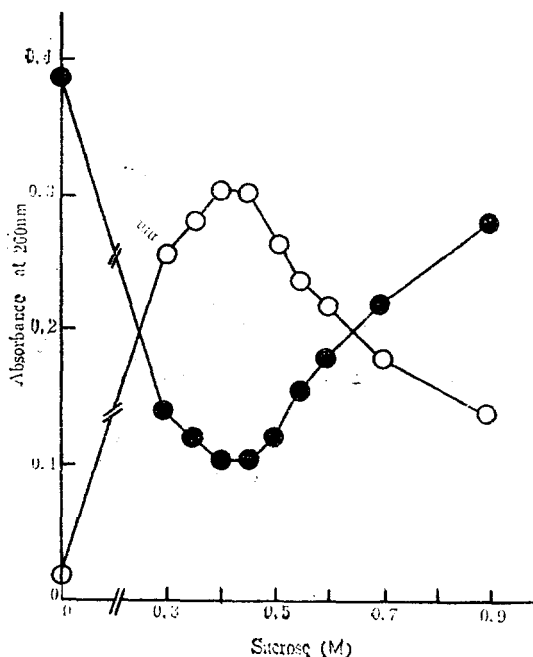

Fig. 2. Properties of protoplasts(2)

Effect of sucrose concentration on the stabilty of PLC. The lower absorption before osmotic shock ind-icates the better effect on the stability of PLC. On the contrary, the higher absorption after osmotic shock indicates the better effect on it. ●——● : before osmotic shock, ○——○ : after osmotic shock.

Resembled experiment was carried out to underst-and the effffect of Mg^{2+} to stabilize the PLC. Increased concentration of Mg^{2+} upto 5mM was effective.

Resistance of PLC against adsorption of bacte-riophage. Experiment was carried out to understand whether PLC were absent in the cell wall or not. Phage HM 2 and HM 3 are virulent phages which have their receptors on the surface of wall. As showin in Table 2, PLC showed always much lower adsoption of phage particles than intact cells. More-over, the adsorption rate of PLC decreased rapidly as

Table 2. Adsorption rate of phage particles*

m.o.i.[1]	HM 2		HM 3	
	PLC[2]	IC[3]	PLC	IC
0.1	18.1	99.9	14.9	96.5
0.5	9.7	99.9	8.9	92.3
1.0	4.8	99.7	2.1	86.8
5.0	1.2	99.5	0.7	95.7

* PLC were infected with phage HM 2 and 8Hm 3, and then, they were incubated for 15min at 30°C. The number of phage particls remaining in the supernatant fluid was assayed. The number of phage particles disappeared from the supernatant fluid was estimated and represented as the per cent against the number of infected phage particles.
1) Multiplicity of infection
2) Protoplast-like cells
3) Intact cells

the multiplicity of phage particles increased. This result indicates that PLC are completely absent in the cell wall.

Growth of PLC. PLC were able to grow volumet-rically, and showed a 50% doubling of turbidity during succeeding cultivation for 3 hr. PLC were plated on the soft agar medium to form colonies. Despite of efforts, it was unsuccessful to form colonies. This result may indicate that PLC are unable to multiply.

Macromolecular biosynthetic ability of PLC. Ability of PLC to biosynthesize their macromolecules was investigated through analysis of the macromola-cular content and incorporation of radiolabelled precursors. As shown in Fig. 3, PLC showed a bala-nced increase in the content of DNA, RNA and protein. Simiar result was exhibited by the further investigation carried out by using the radio-labelled orthophosphoric acid. As shown in Fig. 4, each mac-romolecular fraction(DNA, RNA and lipid) showed a balanced incorporation of ^{32}P during early period of cultivation. On this experiment, it was noticed that there was little further incorporation of radiois-otope into the DNA fraction after 2 hr from the start of cultivation, while there was continuous incorpation of it into RNA and lipid fraction. To certify the weakened ability of PLC to biosynthesize DNA at the rate period of cultivation,
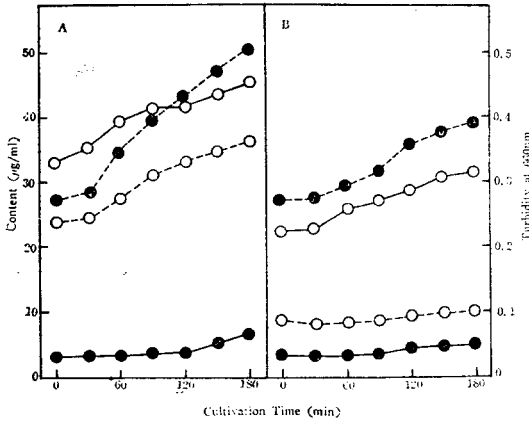
Fig. 3. Properties of protoplasts (3)
Macromolecular content in the PLC. Macromolecules were fractionated according to the STS method, and the content of each macromolecule was analysed according to the method described in materials and methods. ○······○ : turbidity, ●——● : DNA, ○—○ : RNA ●······● : protein. A: Intact cell B: PLC
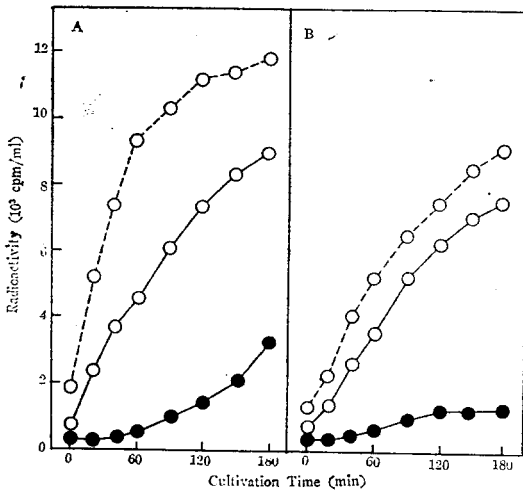

Fig. 4. Properties of protoplasts
Incorporation of ^{32}P into the macromolecules. macromolecules were fractionated according to the same method used in Fig. 3. Radio-activity was measured with a gas flow counter. ●——● : DNA, ○——○ : RNA ○······○ : lipid. A: intact cell B: PLC

metyl-^{3}H-thymidine was added to the PLC which were cultivated already for 3 hr. As shown in Fig. 5, PLC incorporated little radioisotope.

These results indicate that PLC biosynthesize their macromolecules accoding to a balanced manner of biosynthesis during a limited time of cultivation which may concern with the doubling time. The unb-
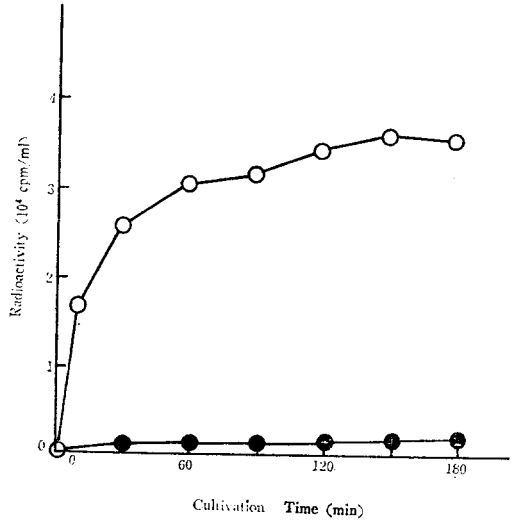

Fig. 5. Properties of protoplasts
Incorporation of ^{3}H-thymidine into PLC. PLC were cultivated for 3 hr, and then, cultivation was continued in the presence of ^{3}H-thymidine. After decomposition of PLC with N KOH for 15 hr, radio-activity was measured with a scintillation counter. ●——● : PLC, ○——○ : intact cells

alanced biosynthesis at the late period of cultivation indicates that the ability of PLC to biosynthesize DNA is damaged priory to that of other macromolecules such as RNA and protein.

Induction of bacteriocin. Strain Nl-4 is able to produce a phage tail-like bacteriocin "clostocin O" by treatment of low concentration of mitomycin C. As shown in Table 3, PLC produced clostocin O as well as the parent cells. They showed a liberation of clostocin which was approximately equel to a 50%

Table 3. Production of bacteriocin

Mitomycin C (µg/ml)	PLC	IC
0	—	—[1]
0.1	3—5[2]	—
0.5	0—2	5—10
1.0	—	0—2
5.0	—	—

* PLC were cultivated for 3 hr in the prsence of mitomycin. The activity of clostocin O liberated into the supernatant fluid was assayed.
1) unit/ml
2) No bacteriocin was detected.

of liberation from intact cells as the maximum. PLC liberated the bacteriocin at lower concentration of mitomycin C than intact cells.

This result indicates that PLC success the capability to produce bacteriocin from the parents cells. It is considered that concentration of the drug for PLC to produce clostocin O becomes low by the lack of cell wall.

Infection of isolated phage DNA. It was firmed that there was no contamination of infectious phage particles in the preparation of phage DNA. PLC were prepared from the strain Nl-611, Nl-504 and Nl-4, and they were respectively infected with the isolated DNA. The former two strains were resistant mutants against HM 2 phage adsorption. They were used in this experiment to prevent the produced progeny phage particles from readsorption on the contaminated debris of wall or intact cell.

PLC of each strain produced a 0.8 to 1.2×10^9 p.f. u./ml of infectious progeny phage particles. Their burst size was approximately 5 to 8% of intact cells. No phage particles were detected when DNA decomposed by vg/ml of DNase (Sigma, type I) was infected to the PLC. This result indicates that PLC can produce phage particles as well as the parent cells.

Discussion

Osmotic fragility, releasing cellular component which absorb the ultraviolet ray by osmotic shock, has been known as a basic property of bacterial protoplasts, although it can not be an evidence for the complete absence of cell wall[4,20]. It was clear that PLC had no cell wall on their surface from the electron microscopic observation[19] and the resistance against phage adsorption.

On the manner of macromolecular biosynthesis in protoplasts, different results were reported. The autoplasts(autolytically prepared protoplasts) of *Streptococcus faecalis* showed a balanced incorporation of radio-labelled precursors into DNA, RNA and protein [21]. On the other hand, protoplasts of *Bacillus megaterium*[22] and yeast spheroplasts[23] showed only a weak ability of DNA biosynthesis. It is difficult to judge which manner of macromolecular biosynthesis

the PLC are belong to, because they show a combination of the manners; balanced biosynthesis at early period of cultivation and weak biosynthesis of DNA.

Generally, DNA biosynthesis in a bacterium is closly linked with the formation of septum. It is necessary to synthesize the septum for a bacterium to initiate biosynthesis of new DNA[24] It is considered that the lack of ability to built cell wall including the septum of the PLC gives a limit to DNA biosynthesis.

Many reports demonstrated that protoplasts success many of the biological properties of parent cells such as spore formation in some spore forming bacteria[25] and the production of phage particles[8,26]. PLC also produced bacteriocin and phage particles as well as the parent cells.

The properties of PLC can be summarized as firstly the lack of cell wall, secondly the impossibility of multiplication and thirdly the success of some biological properties of parent cells. These properties are all in the level of typical protoplasts. The spherical cells produced by sucrose-induced autolysis of clostridial cells were protoplasts.

要　　約

嫌氣性細菌인 *C. saccharoperbutylacetonicum*이 同調濃度의 sucrose溶液中에서 他 溶菌酵素 또는 抗生物質을 添加함이 없이 細胞壁이 急激히 溶解되어 球狀의 菌으로 變化하는 特異한 現象이 確認되었다. 形成된 球狀의 菌은 細胞壁이 完全히 除去된 原形質體로서 삼투壓 shock에 弱하고 bacteriophage吸着能을 상실하였으며 分裂增殖이 不可能하였다. 그러나 生體高分子의 生合成이 可能하며 mitomycin C處理에 依하여 bacteriocin을 生産하였으며 分離한 phage DNA의 感染에 依하여 子孫 phage를 生産하였다. 即 同調濃度의 sucrose 溶液中에서 誘起되는 溶菌에 依하여 生成된 球狀의 菌은 細胞壁을 가지지 아니한 原形質體로서 母細胞의 菌學的 特性을 계승한 狀態에 있었으며 典型的인 protoplast로 判斷되었다.

References

1. Weibull, C., *J. Bacteriol.*, **66**, 688 (1953)
2. Joseph, R. and G.D. Shockman, *J. Bacteriol.*, **118,** 735 (1974).

3. Calandra, G.B., K.M. Nugent and M.R. Cole, *Appl. Microbiol.*, **29**, 90 (1975).

4. Kaback, H.R., Methods in Enzymology, vol. 22, Ed. by W.B. Jacoby, p. 99, Academic press, New York and London, (1971).

5. Chandler, H.M. and R.C. Hamilton, *J. Gen. Microbiol.*, **88**, 179 (1975).

6. Joseph, R. and G.D. Shockman, *J. Bacteriol.*, **118**, 735 (1974).

7. Mohan, P.R., P.K. Donald, R.S. Pianotti, R.L. Epstein and B.S. Schwarz, *J. Bacteriol*, **90**, 1355 (1964).

8. Weibull, C., *Ann. Rev. Microbiol.*, **12**, 1 (1958).

9. Martin, H.H., *J. Threot. Biol.*, **5**, 1 (1963).

10. Mitchell, P. and J, Moyle, *J. Gen. Microbiol.*, **16**, 184 (1957).

11. Hongo, M. and A. Murata, *Agr. Biol. Chem.*, **29**, 1135 (1965)

12. Ogata, S., F. Kato and M. Hongo, *Agr. Biol. Chem.*, **40**, 1093 (1976).

13. Ogata, S., O. Mihara, Y. Ikeda and M. Hongo, *Agr. Biol. Chem.*, **26**, 1413 (1972).

14. Ogata, S., N. Nagao, Z. Hagao, Z. Hdaka and M. Hongo, *Agr. Biol. Chem.*, **33**, 1541 (1969).

15. Mijuno, S., Methods for Isolation and Deter min-ation of Nucleic acids, Tokyo University Press, Tokyo, p. 19, (1974).

16. Mejbaum, W., Z. *Physiol. Chem.*, **258**, 117(1939).

17. Soejima, M. and K. Sugawara, Methods for Protein Analysis, Tokyo University Press, Tokyo, p. 14, (1974).

18. Kaiser, A.D. and D.S. Hogness, *J. Mol Biol.*, **2**, 392 (1960).

19. Ogata, S., K.H. Choiand M. Hongo, *Agr. Biol. Chem.*, **39**, 1247, (1975).

20. Weibull, C., Microbial Protoplasts, Spheroplasts and L-Forms, Ed. by L.B. Guze, The Williams and Wilkins Co., New York, (1968).

21. Roth, G.S., G.D. Shockman and L. Daneo-Moore, *J. Bacteriol.*, **105**, 710 (1971).

22. Weibull, C and H. Beckman, *J. Bacteriol.*, **79**, 638 (1960).

23. Hutchson, H.T. and L.H. Hartwell, *J. Bacteriol.*, **94**, 1697 (1967).

24. Paradee, A.B., P.C. Wu and D.R. Zusman, Bacterial Membranes and walls, Ed. by L. Leive, Marcel Dekker Inc., Amsterdam p. 357, (1973).

25. Salton, J.M.R., *J. Gen. Microbiol.*, **13**, IV(1955).

26. Benzinger, R. and D.Z. McCorquodable, *J. Virology*, **16**, 1(1975).