INDUCTION OF PROTOPLAST-LIKE CELLS IN *BIFIDOBACTERIUM BIFIDUM* ES5

I. INDUCTION OF PROTOPLASTS AND L-FORMS BY PENICILLIN

REIKO NAKAI AND ATSUSHI TAKAGI

Department of Bacteriology, Tottori University School of Medicine,
Yonago 683, Japan

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A method for protoplast induction with penicillin G (PCG) in *Bifidobacterium bifidum* Es5 was examined. Growing cells were converted into protoplasts in a nutrient broth containing PCG without addition of any osmotic stabilizers. The maximal rate of protoplast induction was approximately 50% after 6 hr incubation with 1 unit PCG per ml. Supplementing the PCG-treatment culture with 0.1 to 0.2 M sucrose raised the induction rate to 80% or more. It is suggested that sucrose makes the bacteria more sensitive to PCG, and subsequently stabilizes the protoplasts. In addition, sucrose by itself caused a decrease in the number of viable cells but not in the turbidity of the culture without PCG. L-colonies of strain Es5 developed from bacterial suspensions and also from protoplast suspensions inoculated on nutrient agar plates containing PCG alone or PCG and sucrose.

Bifidobacteria are one of major constituents of enteric flora in humans from nurslings to adults, and have been assumed for a long time to be closely related to human health. However, many are still in doubt about the microbes: about the mechanism of adaptability to various environment of nutrition and of atmospheric oxygen level (1-4) and about the genetic background of their characteristic properties of morphogenesis, cell division and metabolism (5). To study those problems, protoplasts are useful tools. They are available for many purposes such as gene transfer (6, 7), preparation of membranes or intracellular components (8-10), and for studies of the structure and function of the cell surface (11-13). Protoplast induction in *Bifidobacterium* has been reported only for *B. bifidum* var. *pennsylvanicus*, by treatment with lysozyme (14). The cells of *B. bifidum* Es5, on the other hand, are less sensitive to digestion with lysozyme and are not converted into protoplasts by that treatment.
There are several other reagents for protoplast induction (15), for example, a wall lytic enzyme, an inhibitor of cell wall synthesis, and an autolysin or a non-specific substance such as glycine or MgSO₄ (16). It is well known that penicillin G (PCG) interferes in the cross-linking of peptidoglycan, a final stage in cell wall biosynthesis, and that it kills or lyses sensitive bacteria. In such events, bacterial autolysin(s) is thought to play certain important and essential roles (17). It is also suggested that the enzyme prevents reversion of protoplasts and L-forms to walled state (18).

In this report, we describe a method for protoplast and L-form induction in *B. bifidum* Es5 by treatment with PCG. L-forms which are able to multiply in the wall-less state are very interesting. Investigation of the mechanism, including the reversion steps, may offer information in the future about bifidobacteria.

MATERIALS AND METHODS

**Organism and culture conditions.** *Bifidobacterium bifidum* Es5 used here is one of the single cell clones derived from *B. bifidum* E which was reported earlier (19, 20).

The organism was grown anaerobically at 37°C in a nutrient broth consisting of 35 g of lactose, 2 g of ammonium acetate, 25 g of sodium acetate, 2.5 g of K₂HPO₄, 0.2 g of L-cysteine HCl, 1 g of ascorbic acid, 5 g of yeast extract (Difco laboratories, Detroit, Mich.), 4 g of beef extract (Difco), and 10 g of proteose peptone (Difco) per liter and 5 mM CaCl₂·2H₂O (pH 6.8). For solid medium, 1.5% agar (Difco) was added. This is a modified version of the complete medium previously reported (19), but it is a primary one which was employed for cultivation of bifidobacteria prior to the establishment of the latter. A major difference between the two is the use of ascorbate, instead of pyruvate, as a reductant in this nutrient medium. It should also be mentioned that strain Es5 used throughout the work has been successively maintained with this modified medium since its isolation (20).

The anaerobic condition was maintained by CO₂ gas-filling in a test tube or by a Gas Pak system (Becton Dickinson and Co., Cockeysville, MD).

**Protoplasting.** For the preparation of exponentially growing cells, overnight cultures were diluted 10-fold in fresh nutrient broth and then incubated anaerobically at 37°C for 3 hr. The cells were collected by centrifugation at 3,000 rpm for 5 min, then resuspended in the same volume of prewarmed (37°C), fresh broth. Incubation was started after addition of 2 volumes of the cell suspension to 3 volumes of the prewarmed broth containing various concentrations of PCG (penicillin G potassium salt, Meiji Seika Ltd., Tokyo). The cell density corresponded approximately to 2×10⁶ colony forming units (CFUs) per ml. PCG-treatment was carried out anaerobically at 37°C with or without sucrose (0.1-0.5 M).

The turbidity of the cultures was measured by absorbancy at 650 nm. Viable
cells (bacterial CFUs) were enumerated by dilution of PCG cultures with APC buffer followed by plating onto nutrient agar media with a glass rod and counting colonies after 2 days of incubation. APC buffer contains (per liter) 12.5 g of sodium acetate, 2.5 g of K$_2$HPO$_4$, and 0.2 g of L-cysteine HCl (pH 6.8). The rate of protoplast formation is the ratio of the number of spherical cells to that of total cells expressed as a percentage. The cells were counted under a phase contrast microscope.

RESULTS

Susceptibility of *B. bifidum* Es5 to PCG

Exponentially growing cells of *B. bifidum* Es5 were inoculated into prewarmed, fresh nutrient broth containing 0.1, 1 or 10 units of PCG per ml and incubated anaerobically at 37°. Figure 1 shows the changes in turbidity of those cultures during PCG treatment, with typical results from several experiments. Decrease in turbidity (lysis) of the culture was observed at a concentration of PCG above 0.1 unit/ml, and was the greatest at 1 unit/ml.

In our preliminary experiment, the pH of the culture appeared to have an important effect on the PCG activity. This idea came from the observation that decrease in turbidity was less when PCG was added intermediately (without renewing the culture broth) to a growing culture. It seemed that the acidic pH of the culture was responsible for the weakened effect of the PCG on the cells under such condition, since bifidobacteria produce large quantities of acetate and lactate via lactose fermentation thus lowering the pH of culture. In fact, the decrease in turbidity was prevented when cells were incubated with PCG in broth adjusted to pH 6.0 (with Tris-maleate or with addition of acetate), though the growth in the

Fig. 1. Growth inhibition in *B. bifidum* Es5 by PCG.

Exponentially growing cells were inoculated into prewarmed nutrient broth (pH 6.8) containing PCG at 0 (×), 0.1 (△), 1 (○) and 10 (□) units/ml, then incubated anaerobically at 37°. Dotted lines represent the growth of the cultures at pH 6.0.
absence of PCG was scarcely affected in such acidic medium (Fig. 1).

*Morphological conversion of cells of strain Es5 into spherical forms by PCG*

In the process of cell lysis caused by PCG, spherical bodies appeared in the nutrient broth without the addition of any osmotic stabilizers. These spheres burst on suspension in deionized water and they lacked apparent cell walls under electron microscopy (Fig. 2). Thus it may be said that the spheres induced by PCG are protoplasts. Optimal concentrations of PCG for protoplasting were 0.25 to 1 unit per ml, though protoplasts were induced by various concentrations of PCG ranging from 0.1 to 100 units/ml. The ability of PCG at each of the concentrations to induce protoplasts seemed to correspond with its lytic efficiency. As shown in Fig. 3, when PCG was present in the broth at 1 unit/ml, the rate of protoplast formation (PFR) after 6 hr incubation was about 50%, showing maximal yield. At 0.1 unit/ml, the PFR was less than 10%.

The protoplasts induced in the absence of supplement for osmotic stabilization gradually swelled in the course of further incubation. The PFR was raised to 80% by supplementing with 0.2 M sucrose. Remarkable enhancement of the PFR was observed in the presence of 0.1 unit of PCG per ml with 0.2 M sucrose. Optimal sucrose concentrations for enhancement of protoplasting were from 0.1 to 0.25 M.

![Fig. 2. An electron micrograph of protoplast of B. bifidum Es5. The protoplast was induced by PCG (1 unit/ml) after 5 hr incubation. For electron microscopy, the protoplast was fixed with 4% glutaraldehyde in a nutrient broth, then with 1% OsO4 in veronal buffer (pH 6.0). The thin section was stained with uranyl acetate and lead citrate. Scale represents 1 µm.](#)
Under these conditions, the protoplasts did not swell. However, in a higher concentration of sucrose (0.5 M) fewer protoplasts were induced by PCG and they shrunk.

We experimented with other substances, known as osmotic stabilizers, to see whether they also enhance protoplast induction and stabilization. NaCl (0.1–0.5 M) had no effect on either induction or stabilization of protoplasts. Polyethylene glycol 4000 (5–20%) was ineffectual. When added to a nutrient broth, it made a precipitate, which however did not influence protoplast induction by PCG. In addition, it caused strong aggregation of protoplasts. Neither did MgSO₄ (0.01–0.5 M) enhance induction of protoplasts by PCG, but it stabilized them as described later.

**Effects of PCG and sucrose on viability of B. bifidum Es5**

During PCG treatment with or without sucrose, the number of viable cells was assessed. As shown in Fig. 4, viability of the cells decreased slightly even with
sucrose alone, but no protoplasts were observed, up to the 6th hr. PCG at 0.1 unit/ml caused first a slight loss of viability, then a continuation of growth. However, in the presence of 0.2 M sucrose with 0.1 unit of PCG per ml the cells drastically lost their viability, while protoplasting was greatly promoted (Fig. 3). PCG at 1 unit/ml caused significant loss of viability while a moderate rate of protoplasting occurred (Fig. 3). Sucrose (0.2 M) with 1 unit of PCG per ml enhanced both the loss of viability and the PFR (Fig. 3).

Turbidity of the cultures was also monitored during PCG treatment in the presence of sucrose (Fig. 5). Turbidity in the cultures did not decrease, when the cells were treated with 0.2 M sucrose and each of the PCG concentrations. Therefore, the changes in optical density were inadequate for measuring protoplasting under the existing conditions.

**Development of L-colonies on nutrient agar media**

It was shown that the conditions for induction of protoplasts in liquid media were applicable to the development of L-colonies (L-forms) on solid media. Cells of strain Es5, not necessarily exponential-phase cells, were diluted with APC buffer, and overlaid with small amounts of melted soft agar media onto nutrient agar plates containing either PCG or PCG with 0.2 M sucrose. They were hardened and incubated anaerobically at 37°C. After 5 days of incubation, tiny (ca. 0.1 to 1.0 mm in diameter), semi-transparent colonies appeared in the soft agar layer. They were quite different from the bacterial ones of strain Es5, and proved under microscopic observation to be L-colonies (Fig. 6). These colonies developed on both kinds of plates, but on the plates with PCG and sucrose grew the most colonies. In addition, the number of L-colonies decreased with increasing concentration of PCG; maximal colony yield was obtained from the plates containing 0.1 unit of PCG per ml and 0.2 M sucrose.
L-colonies developed also from the FLU-treated cultures (i.e. protoplast suspensions) diluted with APC buffer containing 0.5 M sucrose and/or 20 mM MgSO₄. Not all of the L-colonies appearing on these plates seem to come from the protoplasts, since bacterial cells still remain in the suspensions. However, the protoplasts probably are able to grow to L-forms on the solid media, since the number of L-colonies from the samples diluted in stabilized conditions (with the buffer containing sucrose and Mg ion) were much greater than those from the shocked conditions (with APC buffer).

It was possible to subculture the L-forms by transferring them suspended in the buffer containing sucrose and Mg ion. This was done every 5 days on the nutrient agar plates containing PCG alone or with 0.2 M sucrose. The L-forms reverted to bacterial states when the PCG and sucrose were removed from the culture media, which occurred both on solid media after one more subculturing and in liquid media after 48 hr incubation.

**DISCUSSION**

Somewhat unusual relationships exist in protoplast formation, loss of viability and cell lysis in strain Es5 during PCG treatments. Loss of cell viability occurred...
more strongly than the yield of protoplasts and more quickly than the morphological conversion into protoplasts. In the process of protoplasting by PCG alone, turbidity of the cultures gradually decreased (Fig. 1), but not in the induction by PCG and sucrose (Fig. 5). From the above findings, it is clear that loss of viability does not parallel perfect cell lysis. TOMASZ et al. (17) have suggested that penicillin gives “limited” damage to bacteria which is not sufficient to cause culture lysis, but which may be irreparable under the conditions of viability assay. Our findings appear to be in agreement with their hypothesis. However, the reason for the increase in turbidity of the culture with PCG and sucrose is still unclear.

Sucrose is conventionally used to make hypertonic conditions and to protect osmotically fragile cells from bursting. On the other hand, it is known also that sucrose causes plasmolysis in Escherichia coli (21, 22), or triggers autolysis in several bacterial species (23–26). In the latter, cell lysis caused by sucrose was followed mostly by formation of autoplasts. It is suggested that in B. bifidum Es5 sucrose causes certain cellular modifications of the bacteria and stabilizes the protoplasts. Also sucrose leads to cells more susceptible to the killing and lytic effects of PCG: drastic enhancement of protoplast induction and loss of viability were brought about by treatment with 0.1 unit of PCG per ml and 0.2 m sucrose, either of these alone induces protoplasts hardly at all and they have weak bactericidal effects. In addition, the findings (data not shown) that, in the presence of sucrose, reversion of the L-forms to the walled state was prevented even by removal of PCG, as seen in Bacillus subtilis (18), suggest that sucrose acts on the regulation of autolysin (s) of the organism, which have not yet been found in bifidobacteria but perhaps exist in view of their hypothetical roles in bacterial metabolism (27, 28).

In the lytic effect of PCG on the cells of strain Es5, environmental pH was important (Fig. 1). Suppression of lysis was observed at pH levels below 6.2. GOODELL et al. (29) have reported similar pH dependency in various bacteria, and they suggested the relation to autolysins. However, in strain Es5, the mechanism needs further investigation.

Treatment with PCG failed to achieve 100%-yield in protoplast formation. PCG together with lysozyme was also of no avail, being different from the effect on clostridia (30). Deprivation of CaCl₂ from the nutrient broth did not influence protoplast induction by PCG, though it did in Lactobacillus casei (31). Recently, it was ascertained by us (unpublished data) and by UEDA et al. (32) that N-acetyl-muramidase SG derived from Streptomyces globisporus was available for high yield production of protoplasts in B. bifidum Es5. Using that enzyme, nearly 100% protoplast formation was achieved within 60 min. Therefore, the enzyme is more useful than PCG in protoplast formation.

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REFERENCES