Polyethylene Glycol-induced Uptake of Bacteria into Yeast Protoplasts

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Cells of the nitrogen-fixing bacterium *Azotobacter vinelandii* and the unicellular cyanobacterium *Anacystis nidulans* were introduced into protoplasts of *Saccharomyces cerevisiae* by the polyethylene glycol (PEG) method. Factors influencing the uptake frequency were examined, and experimental conditions were established for maximizing the uptake frequency. Under optimal conditions, each protoplast took up a few bacterial cells. Electron-microscopic studies showed the localization of integrated bacterial cells in membrane-bound vesicles of the cytoplasm or large vacuoles. The protoplasts at the intermediate stages of uptake revealed two major mechanisms of uptake: (a) "endocytosis" by a single protoplast and (b) "cell fusion" between two or more protoplasts. Some bacterial cells disintegrated during the subsequent incubation period through a heterophagy-like process.

Since the molecular cloning technique has recently allowed successful transfer of nitrogen-fixation (*nif*) genes among several bacterial genera,^{1~6)} it may be considered to be possible to introduce *nif* genes into eukaryotic cells such as plants with viruses or plasmids. Even if this is done, there are some barriers before the actual expression of *nif* genes takes place in eukaryotes; for example, nitrogenase requires protection from oxygen and a supply of much ATP.⁷⁾

All naturally occurring eukaryotic nitrogen-fixing systems are endosymbioses in which host cells contain nitrogen-fixing prokaryotes in cytoplasmic vesicles such as in the cases of the legume-*Rhizobium* system,^{8,9)} Protozoa-cyanobacteria systems and cyanelles.¹⁰⁾ Therefore, the incorporation into eukaryotic cells of microorganisms capable of aerobic nitrogen-fixation as endosymbionts could be a possible means of extending nitrogen-fixation to eukaryotes.

It has been shown that polyethylene glycol treatment of plant and fungal protoplasts induces, as well as cell-to-cell fusion, uptake of external materials. Plant and fungal protoplasts were shown to take up organelles^{11,12)}

and microorganisms.^{13~17)} However, the uptake mechanisms, the cellular localization of symbionts, and the fate of these artificially forced associations have never been fully investigated. Only Giles and Whitehead¹⁵⁾ reported an association that was stable for a long period. They introduced cells of an aerobic nitrogen-fixing bacterium, *Azotobacter vinelandii*, into fungal protoplasts (*Rhizopogon* sp.). The nitrogen-fixation activity of the association was observed for up to 138 days.

In this work, we introduced nitrogen-fixing cells of *A. vinelandii* and photosynthetic cells of *A. nidulans* into yeast protoplasts by the PEG method, and examined factors affecting the uptake frequency, mechanisms of the uptake event, and the fate of the artificial association.

MATERIALS AND METHODS

Microorganisms and cultures. The heterothallic triploid wild type strain, *Saccharomyces cerevisiae* A71,¹⁸⁾ was used as a host. Cells were grown at 30°C with shaking in a medium containing 1% yeast extract, 2% peptone, and 2% glucose (YPG). *Azotobacter vinelandii* NCIB8789 (wild

type) was grown at 30°C with shaking in a modified Burk's nitrogen-free medium¹⁹⁾ containing: KH_2PO_4 , 0.2 g; K_2HPO_4 , 0.8 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.09 g; sucrose, 20 g; 1 ml of Fe-Mo solution (1% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.1% K_2MoO_4); in H_2O , 1,000 ml. The growth was measured with a Klett-Summerson photoelectric colorimeter. Exponentially growing cells of *A. vinelandii* were labeled by addition of a uniformly labeled ^{14}C -L-amino acid mixture (New England Nuclear) at 0.5–1.0 μCi per ml of the growth medium for 2–4 hr. Under the growth conditions described, the incorporation of the label was approximately 3×10^3 cpm per 10^8 cells. The cyanobacterium, *Anacystis nidulans* M-6, was obtained from the Institute of Applied Microbiology, University of Tokyo. The cells were grown at pH 8.0 in a modified Detmer medium (MDM) containing per liter: KNO_3 , 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g; K_2HPO_4 , 0.25 g; NaCl , 0.1 g; $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 0.01 g; 1 ml of Fe-solution (0.2% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$); 1 ml of A_5 solution.²⁰⁾ Cultures were incubated at 30°C under continuous illumination from daylight fluorescent lamps (ca. 3,000 lux).

Preparation of yeast protoplasts. Yeast cells in the late exponential phase of growth were harvested, washed, and suspended in a protoplast-forming medium (PFM) containing 0.6 M KCl, 0.01 M phosphate buffer (pH 7.5), and 0.6 mg/ml Zymolyase 5,000 (Kirin Brewery Co., Japan).²¹⁾ The cells were incubated at 30°C for 2 hr with gentle shaking. Prepared protoplasts were collected by centrifugation at $1,000 \times g$ for 10 min, washed, and suspended in 0.6 M KCl. The number of protoplasts was determined with a hemacytometer.

PEG treatment. The standard procedure was as follows. To 1 ml of a mixture of yeast protoplasts (2×10^7 cells) and bacterial cells (the labeled *A. vinelandii*, ca. 2×10^9 cells, 6×10^4 cpm), 0.5 ml of aqueous 45% (w/v) PEG 4,000 (Wako Pure Chemical Industries, Japan) solution containing 75 mM CaCl_2 was added, and the mixture was incubated at 30°C for 20 to 30 min. After centrifugation at $1,000 \times g$ for 15 min, protoplasts were resuspended in 0.05 M Tris-HCl buffer (pH 8.0) containing 20% sucrose, and incubated at 30°C for 1 to 2 hr. The protoplasts were then washed several times with the same buffer and collected on a Whatman GF/C filter by filtration. After drying, the filter was put in a scintillation vial containing 10 ml of a toluene scintillator solution. The radioactivity was measured with a Beckman LS-250 scintillation counter. For electron microscopy, cells of *A. nidulans* were used instead of *A. vinelandii* as a symbiont because, unlike *A. vinelandii*, *A. nidulans* has electron microscopically distinguishable cellular structures such as the lamella structure of thylakoids.

Electron microscopy. Cells for transmission electron microscopic examination were fixed for 2 hr at 4°C with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4)

containing 20% sucrose, post-fixed with 1% OsO_4 in the same buffer for 2 hr, dehydrated in a graded ethanol-series, transferred to QY-1 (*n*-butylglycidyl ether), and immersed in Epon. Thin sections were cut with a Sorvall MT-1 microtome, and stained successively with 2% uranylacetate for 30 min and a lead salts mixture²²⁾ for 5 min. They were examined under a JEM-100B (JEOL) electron microscope at 80 kV. Cells for scanning electron microscopy were fixed and dehydrated in the same manner as for the transmission microscopy, suspended in acetone, and dried at room temperature. A small portion of the dried material was attached to a conductive stub and sputter-coated with gold and palladium in an Eiko JB-3 Ion Coater. Samples were examined under a JEM-100CX (JEOL) scanning electron microscope.

RESULTS AND DISCUSSION

Factors influencing the uptake frequency

Cells of *A. vinelandii* were taken up into yeast protoplasts on PEG treatment (Fig. 1). The degree of the uptake was estimated by counting the radioactivity of the ^{14}C -labeled bacterial cells on a glass filter after the PEG treatment and the washings (see MATERIALS AND METHODS). Table I shows the radioactivity remaining after the washing of various combinations of yeast cells and PEG concentrations. The counts did not decrease anymore on successive washings or on digestion with lysozyme (1 mg/ml) at 30°C for 1 hr. Factors that influence the uptake frequency are listed in Table II. The maximal uptake, 2–3 bacterial cells per protoplast, was obtained when yeast protoplasts were mixed with ca. 100-fold bacterial cells, treated with 30% PEG 4,000 solution containing 50 to 60 mM CaCl_2 at 30°C for 30 min, and washed and incubated in 50 mM Tris-HCl (pH 8.0) containing 50 to 60 mM CaCl_2 . With higher concentrations of CaCl_2 in the PEG solution, lower concentrations of PEG were required for effective uptake (Fig. 2). The optimal conditions for the uptake were similar to those reported for fungal protoplast fusions,^{23,24)} which indicates some involvement of the membrane fusion mechanism in the uptake event.

FIG. 1. Electron Micrograph Showing a Cell Aggregate of Yeast Protoplasts and Cells of *A. vinelandii* after PEG Treatment.

Bacterial cells were taken up into the yeast protoplasts (arrows). Bar marker represents 1.0 μm .

TABLE I. UPTAKE OF ^{14}C -LABELED BACTERIAL CELLS BY YEAST PROTOPLASTS*

Yeast cells (2×10^7)	REG (%)	Radioactivity retained (cpm) ^a
None	30	76 ± 30
Whole cells	30	368 ± 85
Protoplasts	30	1170 ± 125
Protoplasts	0 ^b	163 ± 40

* Protoplasts or whole cells were mixed with labeled cells of *A. vinelandii* (2×10^9 cells) and treated with PEG solution containing 50 mM CaCl_2 at 20°C for 30 min.

^a Values from three experiments.

^b Protoplasts were treated with 0.6 M KCl solution containing 50 mM CaCl_2 .

TABLE II. FACTORS AFFECTING THE UPTAKE FREQUENCY*

Factors		Radioactivity retained (% of control)
PEG, 4000	10 (%)	24
	20	75
	30	100
	40	101
CaCl_2	20 (mM)	48
	40	75
	60	100
	80	100
Temperature	20 (°C)	85
	30	100
	40	80
pH ^a	6.0	55
	7.0	60
	8.0	100
	9.0	87
Cell ratio (Bacteria/Yeast)		
	1	0.1
	10	6
	100	100
	1,000	72

* The standard procedure (control experiment) is described in MATERIALS AND METHODS.

^a After the PEG treatment, cells were washed, re-suspended and incubated in 0.05 M Tris-Maleate buffer (pH 6 and 7) or 0.05 M Tris-HCl buffer (pH 7, 8 and 9) containing 20% sucrose.

Mechanisms of the uptake

A. nidulans was used as a symbiont in cytological studies because *A. vinelandii* possesses less characteristic structures and is sometimes indistinguishable from the host cells (Fig. 1). *A. nidulans* possesses a photosynthetic lamellar structure of thylakoids which can be used as a marker for long time experiments.

Yeast protoplasts and cyanobacterial cells treated with PEG instantaneously formed

FIG. 2. Effects of Concentrations of PEG and CaCl_2 on the Uptake of Labeled Bacterial Cells into Yeast Protoplasts.

PEG solutions of each concentration contained 10 mM (●), 50 mM (○), or 100 mM (●) CaCl_2 .

large non-specific aggregates (Figs. 3A and B). Cyanobacterial cells were in the aggregates and were taken up into yeast protoplasts through two mechanisms. In one mechanism, a cyanobacterial cell first adhered to the plasma membrane of a protoplast and formed a depression in the membrane (Fig. 3A). The plasmolysing effect of PEG then caused the depression to become a deep invagination containing the cell in the peripheral cytoplasm of the yeast protoplast (Figs. 4A and B). Subsequent sealing of the opening of the invagination by the fusion of the membrane resulted in the release of the cyanobacterial cell in the membrane bound vesicle into the cytoplasm of the protoplast (Figs. 4C, D, E, and F). The other mechanism of uptake was suggested by the electron micrographs shown in Fig. 5. In this case, the cyanobacteria were initially surrounded by two or more yeast protoplasts (Fig. 5A). The plasma membranes of the protoplasts surrounding the cyanobacterial cells subsequently fused with each other at the contact sites (Figs. 5B and C). After completion of the cell fusion, cyanobacterial cells were contained in membrane-bound vesicles within the cytoplasm of the yeast protoplasts (Fig. 5C). Figure 6 schematically shows these mechanisms.

The organisms taken up were localized in membrane-bound vesicles or sometimes in large vacuoles (Fig. 7). This is the case for most

naturally occurring endosymbioses²⁵⁾ as well as for experimentally established endosymbioses as reported by Ahn and Jeon²⁶⁾ and Jolley and Smith.²⁷⁾ Enclosure by the host membrane might be a prerequisite for the symbiont to establish a stable association.²⁵⁾

Stability of the association

Meeks *et al.*¹⁷⁾ observed for the association between tobacco protoplasts and a cyanobacterium, *Anabaena variabilis*, that most of the protoplasts expelled the cyanobacterium and simultaneously disintegrated. Apparent ejection, however, was not observed for the yeast-bacteria system, although some protoplasts were disrupted during the incubation period. The bacterial cells taken up may eventually be digested by hydrolyzing enzymes through a heterophagy-like process since several enzyme activities such as those of peptidases, proteases, RNase(s), and phosphatases are reported to be located in the vacuoles of yeast cells.²⁸⁾ In fact, the possibility of heterophagy was suggested by Fig. 8. It shows a yeast protoplast with cells of *A. nidulans* incubated for 24 hr at 30°C after PEG treatment. Two of three bacterial-cells in the protoplast are considerably degraded. Some of the endocytotic vacuoles derived from the invagination of the plasma membrane might be modified to acquire the lysosomal function; this can be attained through the fusion of vacuoles during the yeast growth cycle.²⁹⁾ The rapid disappearance of labeled bacteria in the growth medium might be caused by the heterophagy process and/or selective rupturing of the protoplasts preceding the subsequent growth.

So far, we have failed in forming a stable nitrogen-fixing association between *Saccharomyces* and *Azotobacter*. In order to establish a stable association, there would be, in addition to the digestion, at least three other conditions for microorganisms to satisfy after the entry into a host cell: (1) a supply of nutrient and growth factors from the host, (2) cell division in harmony with that of the host, and (3) harmlessness to the host.

This paper concerns the uptake stage of the

FIG. 3. Electron Micrographs of a Large Non-specific Cell Aggregate of Yeast Protoplasts and Cyanobacterial Cells (*A. nidulans*) Formed by PEG-Treatment.

(A) A thin section of the aggregate. The cyanobacterial cells are enclosed within yeast protoplasts (arrow 1) or sticking in invaginations of the protoplast membranes (arrow 2). Bar marker represents 10 μm . (B) Scanning electron micrograph of the aggregate. The cyanobacterial cells are indicated by arrows. Bar marker represents 10 μm .

FIG. 4. Electron Micrographs Showing Several Stages of Uptake of the Cyanobacterial Cells (*A. nidulans*) by Yeast Protoplasts.

The cyanobacterial cell initially adhering to the surface of the yeast protoplast enters deeply into the invagination of the protoplast (A and B). Subsequent sealing of the opening of the invagination (C) by fusion of the membrane (arrows in D and E) releases a membrane-bound vesicle enclosing the cyanobacterial cell into the cytoplasm of the protoplast (F). A; *Anacystis nidulans* cell, CW; cell wall, M; mitochondrion, N; nucleus, PM; plasma membrane, T; thylakoid of the cyanobacterial cell, V; vacuole. Bar marker represents 1.0 μm.

FIG. 5. Thin Sections Indicating Another Mechanism of Uptake by Yeast Protoplasts.

(A) A cyanobacterial cell is surrounded by three yeast protoplasts. Subsequently, the plasma membranes of the protoplasts surrounding the cyanobacterial cell fuse with each other at the contact sites indicated by arrows (B). (C) After the fusion, the cyanobacterial cell is released in a cytoplasmic vesicle. Abbreviations used are the same as in Fig. 4. Bar marker represents $1.0\ \mu\text{m}$.

establishment of an association. The detailed fate of the association and several influencing factors for establishing a stable system are now under investigation.

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FIG. 6. Diagrammatic Representation of the Two Mechanisms of Uptake of Cyanobacterial Cells (*A. nidulans*) by Yeast Proto-plasts.

The large circle and the small black circle represent the yeast protoplast and the cyanobacterial cell, respectively.

FIG. 7. A Thin Section Showing a Yeast Protoplast Containing a Cyanobacterial Cell in the Large Vacuole.

Bar marker represents 1.0 μm .

FIG. 8. Electron Micrograph Showing a Yeast Protoplast Containing Cyanobacterial Cells; after Incubation.

Two of three cyanobacterial cells in the cytoplasmic vesicles are disintegrated (arrows). Cell wall materials and the cytoplasmic debris can be seen. Bar marker represents 1 μm .

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