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Induced symbiosis: Distinctive *Escherichia coli-Dictyostelium discoideum* transferable co-cultures on agar

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Abstract

Despite the near ubiquity of symbiosis, only a few new symbiotic associations have been reported. The establishment of the unique amoeba-bacterial symbiosis observed by Jeon and his colleagues has been difficult to retrace experimentally mainly because of the failure to grow both partners in pure culture. The details of symbiosis origin and especially laboratory induction are unknown in all cases. Here, we present an experiment in which specific strains of four-year subculture *Escherichia coli* and *Dictyostelium discoideum* evolved interdependently to produce a new morphological entity on agar plates. The cocultured organisms lost their pure culture identities under the conditions in which both control organisms retained their independent culturability. Between days 32 and 101 of culturing of *E. coli* and between days 259 and 645 in *D. discoideum* pure culture identity was lost. Yet through the four years both organisms could always be cocultured and stored frozen. We traced the emergence of characteristic changes toward a repeatedly inducible symbiotic relationship in pure cultures of both cocultured organisms. Since both the enteric bacterium and the cellular slime mold are free-living and culturable in pure culture, genetically well-characterized. We provide a useful model for the laboratory study of symbiotic evolution.

Keywords: Experimental symbiology, *Escherichia coli*, *Dictyostelium discoideum*

1. Introduction

Historically, until the beginning of the 19th century, the experimental creation of symbiotic relationships have been focused on natural organism associations (Fred et al., 1932; Ahmadjian, 1962; Paracer and Ahmadjian, 2000). These experiments were generally carried out by separating symbiotic partners (symbionts). These were then grown independently and subsequently reunited. Successful experiments have been performed in which host symbionts coexist with free-living organisms (Bomford, 1965; Rahat and Reich, 1985), and some new symbioses are observed (Lazo, 1966; Jeon, 1995).

However, in cases of such novel symbioses, observations were limited due to the inability to grow either one or both symbionts as pure cultures. Therefore, it was not possible to eliminate the contaminating effects of other organisms, and difficult to experimentally retrace the course of symbiogenesis. Further, limited changes occurring in these organisms as a result of evolution have been reported. In light of this information, the sequence of events that constitute symbiotic evolution remains insufficiently documented.

One of the authors (M.T.) discovered that the common bacterium *Escherichia coli* and the bactivorous cellular slime mold *Dictyostelium discoideum*, whose genetic characteristics have been thoroughly investigated (Blattner et al., 1997; Eichinger et al., 2005), can coexist stably when grown on minimal nutrient agar at 22°C (Todoriki et al., 2002). Their coexistence in a colony (translucent and dome-shaped appearance) was associated with the production of mucus by *E. coli*; this phenomenon is not observed in *E. coli* in the non-symbiotic state of yellowish and prostrate with lusterless surfaces colonies.

As previously reported (Margulis, 1993; Jeon, 1995), long-term coexistence is expected to increase characteristic changes that favor interdependency. In September 1998, a long-term successive subculture of the colonies was commenced on minimal nutrient agar plates.

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2. Material and Methods

Culture conditions

The cell concentrations of both pre-cultured single-cloned plasmid-transformed cells of *Escherichia coli* that contain genes for green fluorescent protein (GFP) and ampicillin resistance, and single-cloned cells of *Dictyostelium discoideum*, an aggregation-defective slime mold mutant (Todoriki et al., 2002), 4×10^7/ml *E. coli* and 10^5/ml *D. discoideum*, were adjusted with liquid medium C (Kashiwagi et al., 2001) supplemented with 0.5 mM glutamine (CG medium). One milliliter aliquots of the diluted cells were spread onto 1.5% agar (CG agar), dried and incubated at 22°C to produce the first generation. One month later, ~100 µl (containing ~10^6 *D. discoideum* cells and an undetermined number of *E. coli* cells) of the resulting mucoidal colonies was collected with a flame-sterilized spatula and resuspended in 1 ml CG medium. The cell suspensions were adjusted to 10^6/ml *D. discoideum* cells measured using a hemocytometer (Nitirin) with a DIAPHOT-TMD phase contrast microscope (Nikon Inc.). Twenty-five 10 µl aliquots of cell suspension were placed dropwise onto each of two 90×15 mm CG agar plates. The dried plates, incubated at 22°C, produced the second generation. The suspension was prepared for frozen storage by mixture with filter-sterilized liquid medium C containing 0.1 M glutamine and 10% dimethyl sulfoxide, modified method of Laine et al. (1975). Two 600 µl aliquots of the sample were dispensed into 2 ml vials and stored at –80°C. The frozen cells were recovered by rapid thawing and used for several experiments. Well-developed second generation colonies arising from the single droplets were designated for the next subculturing.

Thereafter, colonies were subcultured every month replicating the procedure used to cultivate the second generation. When observations of colonies under the phase contrast microscope revealed a retardation in cell multiplication, the subculturing cycle was extended to two months, until a resumption of normal cell multiplication was detected. When the number of *D. discoideum* cells in the colonies appeared insufficient to initiate the next generation, undiluted colonies were spread onto fresh CG agar, and then incubated until a sufficient number of organisms was obtained. As a control experiment, a pure culture of *E. coli* was grown by spreading 1 ml CG medium containing 4×10^7 cells onto CG agar, and then incubating dried plates at 22°C for one month. Approximately 10 µl (10^9–10^7 cells) of the resulting *E. coli* growth was collected with a flame-sterilized spatula and incubated to spread directly onto the CG agar every month. A further ~10 µl of the *E. coli* cells was retained as a frozen sample using the same method described for the cocultured sample. Collaterally, at the end of the growth phase, 100 µl (10^9–10^7 cells) of pure culture *D. discoideum* cells was subcultured into 10 ml of a modified HL5 liquid medium (Todoriki et al., 2002), containing 50 µg/ml ampicillin and 50 µg/ml kanamycin (HL5-ak medium) every two weeks.

Observations

The frozen cocultures were rapidly thawed and separated into three samples; two of these were used to monitor *E. coli* cell properties. The pure cultured stocks were divided into two samples. First sample of both cocultured and *E. coli* pure cultured cells was used to set up a shaking culture by inoculation into LBG-a medium (Luria-Bertani medium (Sambrook et al., 1989) containing 2 mM glutamine (LBG medium) and 50 µg/ml ampicillin) and incubation at 37°C. The mean number of *E. coli* cells during maximum growth was measured using a BioSpec-mini (Shimadzu) at 600 nm optical density (OD_{600}). Dilutions of a second frozen sample of both cocultured and pure cultured *E. coli* cells were incubated on LBG and LBG-a both containing 1.5% agar (LBG agar and LBG-a agar, respectively) at 37°C. The *E. coli* ampicillin resistance was calculated as the percentages of colony forming units (CFUs) on LBG-a divided by CFUs on LBG. The maximum colony diameter, an expression of the degree of colony proliferation, was also measured. Using a fluorescence microscope (Todoriki et al., 2002), the GFP expression ratios for the LBG-a samples were calculated as the percentages of GFP fluorescent CFUs divided by the total CFUs. A third thawed sample (cocultured only) was spread onto CG agar and incubated at 22°C until sufficient *D. discoideum* cells were obtained for experimental purposes. The colony was resuspended in 10 ml HL5-ak medium, adjusted to a concentration of 10^4/ml *D. discoideum* cells, and was incubated without shaking at 22°C. *D. discoideum* cell numbers were measured under the phase contrast microscope.

3. Results

Characteristic changes of E. coli *during the coculture*

The *E. coli* phenotype was assessed by transferring it to a liquid medium containing ampicillin and growing it at 37°C. *D. discoideum* cells were grown under nonpermissive temperature conditions. It was observed that the maximum cell density of the cocultured *E. coli* cells decreased soon after the subculturing was started, and by day 75, the proliferation was undetectable (Fig. 1a). Despite this result, these cells could be stably subcultured in the mucoid form of growth under the coculture conditions.

Further examination assessed the properties of *E. coli* on agar plates. In the case of *E. coli*, these cells were transferred onto a nutrient agar plate at 37°C and incubated alone. The percentage of ampicillin-resistant cells was

assessed by comparing the number of colony forming units (CFUs) on the agar plates with and without ampicillin. The CFUs obtained from the plate lacking ampicillin, which was assumed to be the total number of cells, were maintained in the order of 10^5–10^9/ml. The CFU percentage of the control cells on day 0 was 99.4% of the total cell population; it then decreased to and was maintained at 0.3% (±0.5 s.d.; $n = 12$) on and after day 14, i.e., the first day on which the CFUs were counted, and subsequently during the experiment. Both the colony diameter and GFP expression were examined among emergent colonies under ampicillin selection conditions. It was observed that the diameters of coculture colonies decreased by approximately half (Fig. 1b) of that on day 75. This reduction in the colony diameter is correlated with the decline in cell density from day 75, as shown in Fig. 1a. Further, GFP expression was determined as the percentage of GFP-expressing cells among the total number of ampicillin-resistant cells in the CFUs. The observations revealed that GFP expression declined dramatically from 96.0% on day 75 to 0% on day 101 (Fig. 1c).

Based on these results for the coculture, we investigated whether similar changes had also occurred in the pure culture. The results showed that the pure culture exhibited properties different from those of the coculture. Unlike the coculture, which lost ampicillin resistance on day 75, ampicillin resistance in the liquid medium (Fig. 1a) was maintained until day 382 and was eventually lost on day 552. Further, the colony diameters on the nutrient agar had decreased to half the initial size by day 552 (Fig. 1b); for the coculture, a similar reduction was observed on day 75. GFP expression on nutrient agar (Fig. 1c) disappeared on day 552, and for the coculture, it disappeared by day 75. However, the percentage of ampicillin resistance was maintained at the low level of 3.2% (±3.9 s.d.; $n = 9$) from day 101, the first day on which ampicillin resistance of the pure culture was measured. It is clear that the changes exhibited by cocultured and pure-cultured *E. coli* are different, and this difference can be attributed to the presence of *D. discoideum*.

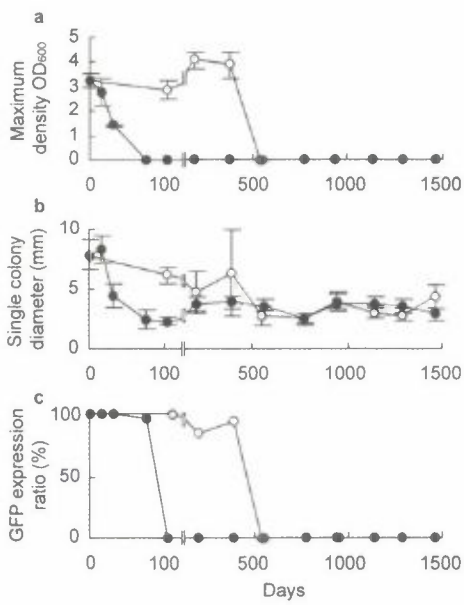

Figure 1. *E. coli* cells during long-term culture; coculture (filled circles) and pure culture (open circles) assay for transfer to other media. Control cells provided the media conditions for day 0. (a) The maximum growth level of ampicillin-resistant cells is represented by the mean optical density of liquid medium containing ampicillin at 600 nm (OD_{600}) (see Methods). The values represented as the means (±s.d. $n = 2$). b, c, Cell properties in nutrient agar plates containing ampicillin (see Methods). Diameters of pure cultures colonies at maximum growth (b). Values of b represented as means ± s.d. ($n = 5$). The percentages of fluorescent cell numbers determined by green fluorescent protein (GFP) expression in colony forming units (CFU) as compared with the total number of ampicillin-resistant cell numbers (c).

Characteristic changes of D. discoideum *the coculture*

In response, *D. discoideum* properties were traced to its proliferability (Fig. 2) and cell appearance (Fig. 3) when these cells were transferred into a liquid medium selective for *D. discoideum* and nonconducive to *E. coli* growth. The appearance of the cocultured cells in the mucoid colony clearly indicated vigorous proliferation on minimal nutrient agar throughout the experiment (Figs. 3a, b). From the beginning of the experiment to day 259, the cells were observed to undergo normal proliferation (Fig. 2) and were irregularly shaped (Fig. 3c) in the liquid medium. The same cell morphology was observed when cells were grown under the pure culture conditions in the liquid medium. From day 297 to day 552, the maximum proliferation decreased to less than one-tenth of the value on day 259 in the liquid medium (Fig. 2), although the cells were still active as in Fig. 3c. On day 645 and thereafter, they exhibited no growth (Fig. 2), and the cells shrunk, became rounded, and died approximately 10 days after the transfer (Fig. 3d). Thus, the growth characteristics of *D. discoideum* appear to occur in two phases; prior to day 645, *D. discoideum* cells existed in the free-living state, whereas after 645 days, they showed evidence of *E. coli* dependence.

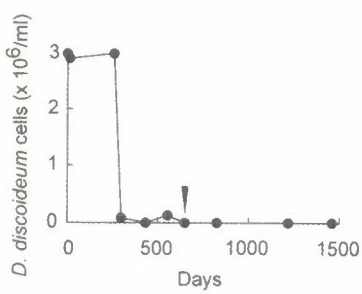

Figure 2. *D. discoideum* number of cells during long-term co-culturing 15 days after transfer into liquid. Day 0 data from control. Change in appearance from normal (Fig. 3c) to shrunken morphology (Fig. 3d) at arrow.

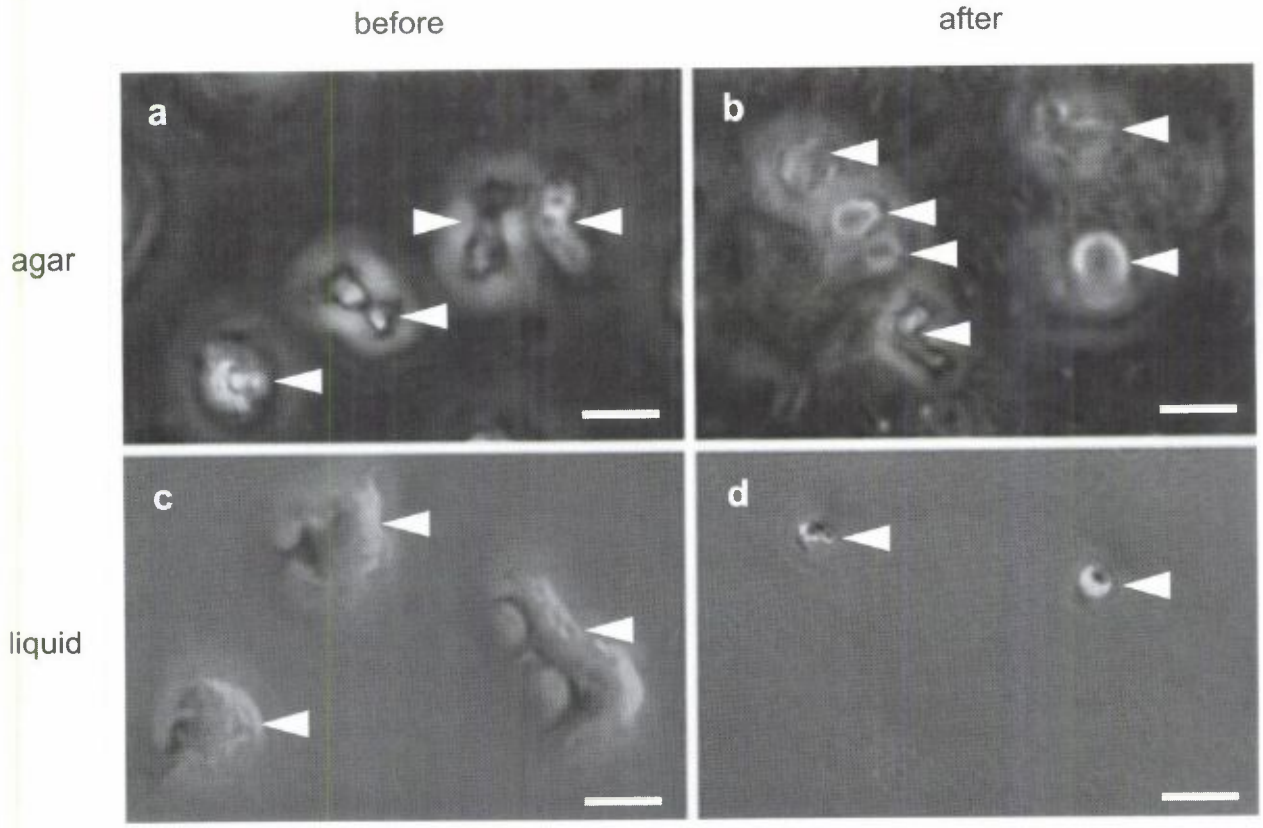

Figure 3. The appearance of *D. discoideum* cells during long-term co-culturing before (day 259; a and c) and after (day 645; b and d) culture identity was lost. Individual cells are indicated by arrows. The cocultured cells were observed on minimal nutrient CG agar (a and b). Cells were transferred from the coculturing CG agar to HL5-ak liquid medium in the case of *D. discoideum*, (see Methods) and were observed after 15 days (c and d). Scale bar = 10 μm.

4. Discussion

The results from *E. coli* of GFP expression and ampicillin resistance in the liquid medium suggest that phenotypic changes were caused by its plasmid loss. However, plasmid loss would not explain why ampicillin resistance on nutrient agar was maintained at a constant low level during the course of the experiment since the *E. coli* strain without the resistance plasmid should be ampicillin sensitive. Our group has yet to initiate research comparing the gene expression profiles of *E. coli* cells using GeneChip technology as in Matsuyama et al. (2004). This could reveal the changes in the genetic characteristics of the subculture. It is evident that the changes observed in the cocultured *E. coli* cells in the presence of *D. discoideum* occurred approximately one year earlier than in the pure culture. Therefore, it appears that these changes were attributable to the presence of *D. discoideum*.

Pure-cultured *D. discoideum* cells grown in the liquid medium maintained their proliferative potential and were active as in Fig. 3c throughout the four-year-long culturing period (data not shown). This condition is different from that of the coculture as *D. discoideum* cells cannot be cultured independently under the same conditions. Nevertheless, simple aging does not provide an explanation for *D. discoideum* alteration. The laboratory strain that was grown as a pure culture in liquid media is known to carry three mutations, *axe*A, *axe*B and *axe*C (Williams et al., 1974; North and Williams, 1978). Therefore, the alterations in the *D. discoideum* cells conceivably caused this reversion. Nonetheless, coculturing has clearly led to changes in the characteristics of *D. discoideum*, and these changes are most likely to result in a growth dependency on *E. coli*.

The cocultured organisms lost their pure culture identities; this occurred between days 32 and 101 (2nd to 4th generation) in the case of *E. coli* and between days 259 and 645 (8th to 13th generation) in the case of *D. discoideum*, during the four years of the coculture (29 generations). When it assumed doubling time of *E. coli* as ~one hour, and of *D. discoideum* as ~10 hours, both are well according, the cell-conversion was happen in 500–2500 generations of cell-cycle for *E. coli* and 500–1500 for *D. discoideum*.

This corresponds to the possible course of symbiotic

evolution from autonomy to tight integration that has been traced for many varied symbioses (Margulis, 1993). We believe our repeatable experiment can be used to trace the path toward a symbiotic relationship and can be considered as a useful model of symbiotic evolution.

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