Parasexual Hybridization in Cellular Slime Molds.
II. The Appearance and Characterization of Interspecific Hybrids between Dictyostelium discoideum and D. mucoroides

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ABSTRACT. Three drug resistant mutants of Dictyostelium discoideum and D. mucoroides were employed to demonstrate the occurrence of interspecific hybridization. Interspecific hybrids appeared at a frequency of $4 \times 10^{-5}$, whereas intraspecific hybrids appeared at a frequency of $1 \times 10^{-3}$. Nineteen interspecific hybrids were serially subcultured to investigate their genetic traits. The results showed that the progenies of the interspecific hybrids retained the genetic markers of both parents in a manner similar to intraspecific hybrids. The characterization of the interspecific hybrids demonstrated that parasexual hybridization occurred even between two different species. The present study showed that genetic analysis between different species of cellular slime molds could be performed by applying this interspecific parasexual system.

A novel sexual process has been established in the cellular slime mold Dictyostelium discoideum, and various genetic markers have been revealed to recombine during this process (10, 12, 15). Recently, linkage analysis was performed and mitotic crossing-over was confirmed (11, 13). This exchange process of genetic materials in this organism differs from that of ordinary sexual reproduction, and a useful term to describe this process is “parasexual,” i.e., “a process of genetic recombination other than a regular alteration of karyogamy and meiosis as in sexual reproduction” (17).

Investigations of the parasexual cycle so far in this organism have revealed the following points: (a) the frequency of appearance of heterozygous cells was $10^{-4}$ to $10^{-6}$ in standard selective conditions (10, 14, 23); (b) Mendelian segregation resulting from meiosis was not evident while chromosome elimination was suggested to occur (1, 10); (c) the diploid state of heterozygous cells was unstable and rapid haploidization occurred during the course of progeny multiplication (20, 22); and (d) all mutants of parasexual genetics employed so far were derived from the common strain NC-4, indicating that no mating types are involved in this process.

The occurrence of true sexuality involving meiosis was demonstrated during the process of macrocyst formation in Polysphondylium violaceum and D. mucoroides (3, 16). This process involves mating type systems, and a strain not forming macrocysts is heterothallic strain (2, 4). The frequency of gene recombinations occurring through
the parasexual cycle is about 500 times smaller than that through the orthodox sexual cycle (17). Thus, the parasexual cycle contributes to only a limited number of gene recombinations in comparison to the sexual cycle. However, the parasexual cycle can still replace the orthodox sexual cycle where the latter is not occurring (6).

The present study demonstrated that interspecific hybridization occurs between *D. discoideum* and *D. mucoroides*. The characteristics of the interspecific hybrids were studied in terms of drug resistance, ploidy and developmental morphology. It was shown that these hybrids were derived through the parasexual cycle similar to that of the intraspecific system of *D. discoideum*.

**MATERIALS AND METHODS**

*Chemicals.* Two antifungal antibiotics, Naramycin® (cycloheximide, Tanabe Seiyaku Co. Ltd., Osaka, Japan) and Trichomycin® (cabimicina, Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan) were used. The N-methyl-N'-nitro-N-nitrosoguanidine used was a product of Aldrich Chemical Co. (Milwaukee, Wisconsin, U.S.A.).

*Genetic notations.* The notation of genotypes was according to Fukui (7) as follows: nar¹, Naramycin resistant; nar², Naramycin sensitive; tri¹, Trichomycin resistant; tri², Trichomycin sensitive; alb, no pigment in fruiting body; and yel, yellow pigment(s) in fruiting body.

*Organisms.* Three haploid drug resistant mutants employed were isolated after treatment with nitrosoguanidine: (a) Nar 22, a Naramycin resistant (nar¹ tri² yel) which was derived from the wild type NC-4 of *D. discoideum* (10); (b) tri 11, a Trichomycin resistant (nar² tri² alb) which was derived from an albino mutant of NC-4 (10); and (c) mtri 21, a Trichomycin resistant (nar² tri² alb) which was derived from the wild type No. 11 of *D. mucoroides*.

*Culture methods.* Amoebae and food-bacteria (*Escherichia coli, B/r*) were cultured and harvested, as described previously (10).

*Isolation of genetic hybrids.* Genetic hybrids were selected after cell incubation at optimum conditions for inducing high frequency hybridization, i.e., non-growing primary culture for 6 h in buffered salt solution followed by secondary culture with bacteria during which cells doubled in number (9). In the present study, equal numbers (1 × 10⁷ cells/ml each) of amoebae of Naramycin and Trichomycin resistant mutants were mixed and cultivated by liquid shake culture. Secondary culture was performed on a lactose-peptone agar medium [0.1% lactose, 0.1% Bacto-peptone and 2% Bacto-agar (Difco Laboratories, Detroit, Michigan, U.S.A.)] (18), since mtri 21 did not grow in the liquid shake culture. An aliquot containing 5 × 10⁴ cells was finally inoculated onto a selective plate in association with food-bacteria. The selective medium employed for the isolation of hybrids from the control cross, nar 22 × tri 11, was 2% agar containing 75 µg/ml of Naramycin and 50 units/ml of Trichomycin. The selective medium for the interspecific cross, nar 22 × mtri 21, contained 125 µg/ml of Naramycin and 50 units/ml of Trichomycin. No sensitive cell could grow on such selective media (9, 10).

**RESULTS**

*Interspecific hybrids between *D. discoideum* and *D. mucoroides.* Parasexual hybridization was conducted by mixing the two drug resistant mutants and selecting the double resistant cells according to the method of Fukui and Miyake (9). Nar 22 (*D. discoideum*) and mtri 21 (*D. mucoroides*) were employed for the interspecific system, while the two mutant strains of *D. discoideum*, nar 22 and tri 11 were used for the intraspecific cross as a control. The interspecific hybrids were obtained at the frequency of 4 × 10⁻³, whereas the intraspecific hybrids appeared at the frequency of 1 × 10⁻³. Nineteen interspecific hybrid clones appearing on selective plates
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were designated as \textit{mdh} 1–19 and the characteristics of their progenies were examined with respect to drug resistance, ploidy and developmental sequence.

Drug resistances of progenies of interspecific hybrids. Previous studies (9, 10) revealed that the progenies of the intraspecific parasexual hybrids retained drug resistances to varying degrees, and the modes of resistance were classified into three types: (a) those which retained resistance to Naramycin more stably than to Trichomycin; (b) those which retained resistance to Trichomycin more stably than to Naramycin; and (c) those which retained resistances against both drugs to a similar degree. Thus, the drug resistances of the progenies of the interspecific hybrids were investigated in comparison with the modes of the intraspecific hybrids. It should be noted that a clone appearing on an original selective plate was composed of such cells multiplied at least into \(5 \times 10^5\) cells, i.e., about 20 cell generations from the original heterozygous cell. Moreover, the cells underwent 10 cell generations during each subculture on the lactose-peptone plate.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Strain & Spore size & Drug resistances \textsuperscript{a} \\
no. & after the 1st subculture on lactose-peptone & Original cells \textsuperscript{b} & Cells after the 8th subcultured \textsuperscript{c} \\
(\(\mu m\)) & & & S.P. & C.P. & N.P. & T.P. & S.P. \\
\hline
\textit{nar} 22 & 7.0\(\pm\)0.8 & 2 & & & & & \\
\textit{mdh} 1 & 7.7\(\pm\)0.9 & 10 & 100\textsuperscript{j} & 100\textsuperscript{j} & 13\textsuperscript{j} & & 0.6\textsuperscript{j} \\
\textit{mdh} 2 & 7.5\(\pm\)0.9 & 43 & & & & & \\
\textit{mdh} 3 & 7.6\(\pm\)0.8 & 20 & & & & & \\
\textit{mdh} 6 & 9.6\(\pm\)1.4 & 8 & 100 & 100 & & 0.01 & 0.01 \\
\textit{mdh} 7 & 9.3\(\pm\)0.9\textsuperscript{f} & 7 & 100 & 100 & & 0.01 & <0.01 \\
\textit{mdh} 8 & 10.0\(\pm\)1.2 & 37 & 100 & 100 & & 0.01 & 0.01 \\
\textit{mdh} 11 & 7.0\(\pm\)0.7 & 1 & 100 & 100 & & <0.01 & 0.02 \\
\hline
\end{tabular}
\caption{Spore size and drug resistance of interspecific hybrids \textsuperscript{a}}
\end{table}

S.P., Selective plate; C.P., control plate; N.P., Naramycin plate; T.P., Trichomycin plate.
\textsuperscript{a} An apparent \textit{D. discoideum} type determined subjectively according to morphology.
\textsuperscript{b} The numerals show the relative numbers of clones appearing on each plate (%).
\textsuperscript{c} Cells on the original selective plates.
\textsuperscript{d} Cells subcultured 8 consecutive times on lactose-peptone agar plates.
\textsuperscript{e} \(2\%\) agar with no drugs.
\textsuperscript{f} \(2\%\) agar with Naramycin (150 \(\mu g/ml\)).
\textsuperscript{g} \(2\%\) agar with Trichomycin (50 units/ml).
\textsuperscript{h} \(2\%\) agar with both Naramycin (125 \(\mu g/ml\)) and Trichomycin (50 units/ml).
\textsuperscript{i} Spores formed on the original selective plate.
\textsuperscript{j} Cells serially subcultured 14 times on lactose-peptone agar plates.

First, cells of hybrid clones on the original selective plates were examined for drug resistance against both drugs according to the following procedures: (a) cells from large clones on the original selective plates were directly inoculated onto the test medium, and (b) cells from small clones on the original selective plates were inoculated onto the test medium subsequent to subculturing once or twice on the lactose-peptone medium. Tables 1 and 2 show that cells inoculated onto plates containing both drugs retained resistances of varying degrees. This indicates that the resistances were in-
inherited in a similar manner to intraspecific hybrids (9, 10) with respect to cells of the original population.

Second, resistance level against Naramycin or Trichomycin or both drugs was examined after the progenies were serially subcultured on the lactose-peptone plates for 8 or 14 consecutive times (Table 1 and 2). The obtained results indicate that the modes of resistance could be classified into three types: (a) *D. discoideum* type which retained nar more stably than tri (mdh 2, 6, 7, 9 and 11); (b) *D. mucoroides* type which retained tri more stably than nar (mdh 12, 13, 14 and 16); and (c) recombinant type which retained both nar and tri stably (mdh 10). Thus, the results demonstrated that the progenies of the interspecific hybrids between *D. discoideum* and *D. mucoroides* retained resistances in a similar manner to the intraspecific hybrids of *D. discoideum*.

**TABLE 2. SPORE SIZE AND DRUG RESISTANCE OF INTERSPECIFIC HYBRIDS**

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Spore size after the 1st subculture on lactose-peptone (μm)</th>
<th>Drug resistances</th>
<th>Original cells</th>
<th>Cells after the 8th subculture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S.P.</td>
<td>C.P.</td>
<td>N.P.</td>
</tr>
<tr>
<td><em>mdh</em> 10</td>
<td>6.5±0.6</td>
<td>100</td>
<td>100i</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><em>mdh</em> 12</td>
<td>6.4±0.7</td>
<td>0.04</td>
<td>100i</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><em>mdh</em> 13</td>
<td>6.2±0.7</td>
<td>0.7</td>
<td>100i</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><em>mdh</em> 14</td>
<td>6.4±0.7</td>
<td>0.01i</td>
<td>100</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><em>mdh</em> 16</td>
<td>6.1±0.6</td>
<td>57i</td>
<td>100</td>
<td>4.4</td>
</tr>
</tbody>
</table>

S.P., C.P., N.P., T.P., see footnote of Table 1.

*a* An apparent *D. mucoroides* type determined subjectively according to morphology involving one intermediate type (*mdh* 10).

*b, c, d* Same as footnotes of Table 1.

*c* Cells subcultured once on lactose-peptone agar plates.

*i, j* Cells serially subcultured 14 times on lactose-peptone agar plates.

**TABLE 3. CHANGES IN SPORE SIZE DURING SERIAL SUBCULTURES OF INTERSPECIFIC HYBRIDS**

<table>
<thead>
<tr>
<th>No. of subcultures</th>
<th>Interspecific hybrid strains</th>
<th><em>mdh</em> 6</th>
<th><em>mdh</em> 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original</td>
<td>11.1±1.3 μm</td>
<td>10.2±1.8 μm</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9.6±1.4</td>
<td>10.0±1.2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>9.5±1.3</td>
<td>10.0±1.2</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>7.2±0.8</td>
<td>7.2±0.8</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>7.0±0.6</td>
<td>7.0±0.6</td>
<td></td>
</tr>
</tbody>
</table>

*a* The values are the mean major diameter of spores ±S.E.

*b* Cells were serially subcultured on lactose-peptone agar plates. Cells divided ten times on the average during each subculture.

Changes in spore size of interspecific hybrids. The spore size of the progenies of interspecific hybrids was determined by measuring the length of the main diameter of spores, since spore size and ploidy were verified to be interrelated (1, 21). All *D. mucoroides* type clones (*mdh* 12, 13, 14 and 16) examined after the first subculture on the lactose-peptone medium formed spores as small as those of a haploid parent
mtri 21 (Table 2). This indicates that they were haploid after being subcultured one time corresponding to 30 cell generations. On the contrary, the D. discoideum type clone examined by similar methods formed large as well as small spores, i.e., mdh 6, 7 and 9 formed large spores while mdh 1, 2, 3 and 11, small spores (Table 1). Mdh 6 and 9, which initially formed large spores on the original selective plates were investigated further for spore size changes during serial subcultures (Table 3). The results showed that the average spore size decreased gradually during serial subcultures for eight times (80 cell generations) and settled into the spore size range of a haploid parent, nar 22.

It should be noted that spore size changes had a relationship to drug resistance. Clones which initially formed large spores on the selective plates (e.g., mdh 6 and 9) had a tendency to lose drug resistance during serial subcultures (Table 1) and a parallel decrease in spore size was observed (Table 3). On the other hand, clones which initially formed small spores (e.g., mdh 2) retained drug resistances more stably during subcultures (Table 1).

Morphogenesis of interspecific hybrids. Fifteen among 19 hybrid clones appearing on the original selective plates were picked up with a platinum loop and were subcultured on the lactose-peptone medium to investigate their morphogenetic patterns. Ten clones (mdh 1-9 and 11) formed fruiting bodies similar to those of nar 22, while 4 clones (mdh 12-14 and 16) formed fruiting bodies similar to those of mtri 21. Clone mdh 10 formed fruiting bodies different from either parental strain.

Clone mdh 2 and 6 were picked up from the original selective plates for possible segregation of morphogenetic traits among progenies. The clonal culture was conducted. Ten amoebae on the average were inoculated on a lactose-peptone plate in as-

![Diagram of morphogenetic patterns](image)

Fig. 1. Diagrammatic representation of the morphogenetic patterns of two parental and a hybrid clone.
sociation with bacteria and were allowed to grow clonally. Five days after inoculation, when morphogenesis was completed, the morphology of the fruiting bodies was investigated under a dissecting microscope. All 100 examined clones of *mdh* 2 and 63 examined clones of *mdh* 6 formed fruiting bodies similar to those of *nar* 22. The results indicate that there was no segregation of morphogenetic traits among progenies of those hybrid clones, suggesting that these traits had already settled into one parental type when examined. (It should be noted again that the cells increased to about $5 \times 10^5$, i.e., 20 cell generations, when they were picked up from the original selective plates).

The morphogenetic sequence of *mdh* 10 was investigated in comparison to the parental strains and is represented graphically in Fig. 1. The morphogenesis of *mdh* 10 was similar to that of *mtri* 21 in the following points: (a) small aggregates with loose streams and (b) fruiting bodies without yellow pigment(s). On the contrary, the stalks constructed by migrating slugs of *mdh* 10 were thick and straight, being similar to those of *nar* 22; although as in *mtri* 21, they were constructed during migration of slugs and had no basal disks. Thus, it seemed that the progeny of *mdh* 10 retained the morphogenetic traits of both parents and constructed fruiting bodies with intermediate characteristics. The fruiting bodies of *mdh* 10 and both parents are shown in microphotographs (Fig. 2).

**DISCUSSION**

The present study revealed that double resistant clones appeared from the inter-specifically mixed culture of two drug resistant mutants of *D. discoideum* and *D. mucoroides*. The frequency of appearance of these clones was $4 \times 10^{-5}$ when the secondary culture for one cell division was conducted subsequent to the non-growing primary culture for 6 h; while it was $1 \times 10^{-5}$ with respect to the intraspecific system of *D. discoideum*. This indicates that the interspecific transmittance of genetic materials occurred between cells of different drug resistances, although the frequency was low compared with the frequency in the intraspecific cross.
It has been demonstrated that *D. discoideum* cells spontaneously fused at a frequency of $6 \times 10^{-3}$ when the non-growing primary culture was conducted for 6 h (9), and it was suggested that cell fusion occurred among cells in the agglutinates formed in the liquid shake culture (8). Raper and Thom (19) demonstrated that cells of *D. discoideum* and *D. mucoroides* constituted an integral parts of the same communal organization and a complete separation of the two species occurred during the later stages of aggregation. Furthermore, Fukui and Miyake (9) revealed that the transmittance of the resistant markers was completed by the end of aggregation. These observations suggest that spontaneous interspecific cell fusion occurs between *D. discoideum* and *D. mucoroides* cells in common agglutinates, and their genetic materials intermingle, resulting in the appearance of interspecific hybrid cells.

The occurrence of parasexual hybridization has been revealed in the intraspecific system of *D. discoideum* (11, 12, 20). It has also been demonstrated in the intraspecific hybrid clones that the modes of drug resistance change were comparable with those of ploidy, suggesting that these markers were chromosomal, and chromosome elimination occurred during the course of haploidization (8, 10). In this connection, Fukui and Takeuchi (10) showed that the modes of resistances could be classified into two types. The first type maintained genetic markers derived from one parent more stably than markers from the other parent; and the second type inversely maintained markers from the other parent more stably. The occurrence of a third type was also recently demonstrated, i.e., the genetic markers of both parents were maintained stably in a recombined manner (9). The present study verified that progenies of interspecific hybrids bore characteristics similar to those of the intraspecific parasexual hybrids: the parallel response between changes in drug resistances and ploidy and the three modes of resistance. This analogy suggests that parasexual hybridization occurred in the interspecific system of *D. discoideum* and *D. mucoroides*.

The possibility remains that the morphological aberration of the interspecific hybrid *mdh 10* was due to a spontaneous mutation. The fruiting bodies of *mdh 10* had some similarities to those of “MV” mutant of *D. mucoroides* described by Filosa (5). However, *mdh 10* was different from the MV mutant in that the stalks were simultaneously constructed with migration in *mdh 10*. Furthermore, *mdh 10* retained resistant markers of both parents in a recombined manner. Thus, it is likely that the morphological aberration of *mdh 10* was due to a mixing of genes of both parents in a way that the intermediate morphology was manifested.

The present study demonstrated that parasexual hybridization occurred in the interspecific system of *D. discoideum* and *D. mucoroides* in an identical manner with the intraspecific system of *D. discoideum*. This confirms the idea by Fukui and Takeuchi (10) that the parasexual cycle of this organism is more irregular than the parasexuality of fungi. Although the parasexual genetics studied in this organism were limited to the intraspecific system of NC-4, the present study indicated that the parasexual cycle could be performed in the interspecific system as well.

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