Evidence for Mitochondrial DNA Polymorphism and Uniparental Inheritance in the Cellular Slime Mold *Polysphondylium pallidum*: Effect of Intraspecies Mating on Mitochondrial DNA Transmission

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ABSTRACT

Restriction fragment length polymorphisms (RFLPs) were used as markers to monitor mitochondrial inheritance in the cellular slime mold, *Polysphondylium pallidum*. When two opposite mating types (*mat1* and *mat2*) of closely related strains were crossed, all the haploid progeny regardless of mating type inherited their mitochondrial DNA from the *mat2* parent only. When opposite mating types from more distantly related strains were crossed, most of the progeny also inherited their mitochondrial DNA from the *mat2* parent, but some inherited their mitochondrial DNA from the *mat1* parent. In both cases however, the transmission of mitochondrial DNA was uniparental, since in every individual progeny only one type of mitochondrial DNA exists. Moreover, in crosses involving more distantly related strains all the progeny of a single macrocyst were shown to contain the same type of mitochondrial DNA. These findings are discussed in regard to mechanisms of transmission and the possible involvement of nuclear genes in the control of transmission of mitochondrial DNA in Polysphondylium.

FEATURES of organelle heredity both in plants and animals have drawn the attention of biologists, since the mechanism of organelle gene transmission is distinct from that of genes in the nucleus. (WHATLEY 1982; BIRKY 1983). Mitochondrial genetics and transmission is of special interest due to the central role of mitochondrial respiration in cell metabolism and the reliance of most tissues on mitochondrial energy production (WALLACE *et al.* 1988). Mitochondrial DNA (mtDNA) shows uniparental inheritance in animals, and in some plants and fungi (DAWID and BLACKER 1972; FRANCISCO, BROWN and SIMPSON 1979; GILES *et al.* 1980; HAYASHI *et al.* 1978; HUTCHISON *et al.* 1974; BOYNTON *et al.* 1987; TAYLOR 1986; KAWANO *et al.* 1987; SILLIKER and COLLINS 1988). On the other hand, there have also been reports of biparental mtDNA transmission in vegetative diploids of Chlamydomonas (BOYNTON *et al.* 1987) and in junction line heterokaryons of the fungus Coprinus (BAPTISTA-FERREIRA, ECONOMOU and CASSELTON 1983; ECONOMOU *et al.* 1987).

Cellular slime molds have been very attractive to biologists, especially their asexual cycles which have provided simple models for eukaryotic cell differentiation and morphogenesis (LOOMIS 1982; SPUDICH 1987). Some species of slime molds also have a sexual or macrocyst cycle, but investigators have been discouraged from studying this, due to the poor germination levels and long maturation periods of the macrocysts. The species which are sexually competent form macrocysts when cells come together and make aggregates. In the sexual cell aggregate, two cells of opposite mating type fuse and form a diploid. The diploid, acting as a cytophagic cell, engulfs the peripheral cells in the aggregate and forms thick walls around the young macrocyst. The macrocyst, now considered a true zygote (MACINNES and FRANCIS 1974), matures and goes through meiosis to generate haploid progeny, which are released upon breakage of macrocyst walls during germination (NICKERSON and RAPER 1973; ERDOS, RAPER and VOGEN 1975; FRANCIS 1975; WALLACE and RAPER 1979; Okada *et al.* 1986).

Using the cellular slime mold *Polysphondylium pallidum* as a model we made an attempt to determine the transmission pattern of mtDNA in these organisms. *P. pallidum* was chosen because it has a higher level of germination than other sexually competent heterothallic species of slime molds, like *Dictyostelium discoideum* (FRANCIS 1980). This report shows that mtDNA polymorphisms (RFLPs) exist in heterothallic strains of *P. pallidum*. Furthermore, mtDNA transmission is uniparental, with mating type 2 being dominant to mating type 1 with respect to the mtDNA transmission pattern. Our results also imply that the progeny of a single macrocyst all have the same type of mtDNA. These observations will be discussed in

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TABLE 1

Genotypes and mtDNA types of the haploid strains in *P. pallidum*

Strain	Genotype	Description of genotypes	Mating type	mtDNA type
PN500	*wild type*	Isolated from wild	*mat1*	A
PN529	*tsg-17*	Mutant of PN500 unable to grow at 31.5°	*mat1*	A
PN543	*naf-2*	Mutant of PN500 resistant to sodium fluoride	*mat1*	A
PN550	*can-3, cyc-8*	Mutant of PN500 resistant to canavanine and cycloheximide	*mat1*	B
PN600	*wild type*	Isolated from wild	*mat2*	B
PN614	*tub-7*	Mutant of PN600 resistant to tubercidin	*mat2*	B
MF2-14	*wild type*	Isolated from wild	*mat1*	C
MF2-21	*wild type*	Isolated from wild	*mat1*	D
MF2-5	*wild type*	Isolated from wild	*mat2*	C
MF2-6	*wild type*	Isolated from wild	*mat2*	C

relation to possible mechanisms of mtDNA inheritance in the cellular slime molds.

MATERIALS AND METHODS

Organisms and culture conditions: *Polysphondylium pallidum* PN strains were kindly given to us by D. FRANCIS and the MF2 strains by R. EISENBERG (both strains were obtained through D. FRANCIS, University of Delaware). The genotypes of all haploid strains used in this work are listed in Table 1. All strains were grown on *Escherichia coli* strain B/r as food source on LP agar plates (0.1% lactose, 0.1% peptone and 1.5% Difco agar) and had a doubling time of 3.5–4 hr.

Macrocyst formation and germination: Macrocysts were generated by mixing cells of opposite mating type under the conditions recommended by FRANCIS (1980). After allowing 6 weeks for maturation, macrocysts were rinsed, diluted with sterilized distilled water, and vortexed extensively to separate macrocyst clumps. Single macrocysts (about 30) were then picked up under a microscope (200×) and put on agar plates for germination under the conditions described by FRANCIS (1980). The plates were incubated under light at 22° and checked for germination daily.

Screening of the progeny of a single macrocyst: Screening of the progeny was carried out under two different conditions. First, after germination, released amoebae were allowed to form diminutive fruiting bodies. Individual sori were then harvested and plated clonally. Second, agar blocks containing the newly germinated amoebae were cut out, the amoebae suspended in phosphate buffer (17 mM KH_2PO_4-Na_2HPO_4 pH 6.8), then plated to form single cell clones. After 3–7 days, amoebae from the edge of the clones were taken and further cultured for amplification. On the average 1–5 clones per macrocyst were analyzed.

Progeny culture and total DNA extraction: Amoebae from the edge of single clones were plated on LP agar plates with *E. coli* B/r. After allowing 3–4 days for growth, cells were harvested and washed with the phosphate buffer to eliminate bacteria. Cells were then starved (for complete digestion of ingested bacteria) in the same buffer for 4–5 hr in half the original culture volume, pelleted by centrifugation and used for total DNA extraction.

Cells were lysed in 0.4 M EDTA (pH 9) and 4% sarcosyl prewarmed to 55–60°. Proteinase K (Boehringer Mannheim) was added to a concentration of 0.1 mg/ml and the lysate was incubated at 55–60° for another 3 hr. Proteins were removed by two sequential extractions of phenol:chloroform:isoamyl alcohol (25:24:1), and DNA was precipitated with ethanol. After centrifugation, the pellet was dissolved in a minimal volume of 10 mM Tris-HCl, 1 mM EDTA; RNase A (Boehringer Mannheim) was added at a concentration of 70 μg/ml and the sample incubated at 37° for 2 hr. Next, proteinase K was added to a concentration of 0.1 mg/ml and the sample incubated for 2–3 hr at 55–60°. DNA was then extracted with phenol:chloroform:isoamyl alcohol and chloroform:isoamyl alcohol (24:1) and precipitated with ethanol.

Preparation of mitochondrial DNA: *P. pallidum* strains (PN600 or PN500) were grown to a density of 5–8 × 10^6 cells/ml in association with *E. coli* B/r in phosphate buffer (BOZZARO and GERISCH 1978) at 22–25°, and shaken (120 rpm). Cells were harvested, washed several times to remove bacteria and resuspended in phosphate buffer for starvation (4 hr). They were then pelleted and resuspended in 0.38 M sucrose, 20 mM Tris-HCl (pH 8), 1 mM EGTA, and 0.2% bovine serum albumin (BSA), at a density of 10^8 cells/ml and disrupted using a Waring blender type homogenizer (Nissei Ace, Japan) at 10,000 rpm. Homogenization was continued until ~90% of the cells were lysed based on aliquots examined under an inverted microscope at 3 min intervals. The homogenate was centrifuged at 1,000 × *g* for 10 min twice and the supernatant was then centrifuged at 10,000 × *g* for 15 min. The crude mitochondrial pellet was further purified by centrifugation on a discontinuous Percoll gradient (NISHIMURA, DOUCE and AKAZAWA 1982) as follows, it was resuspended in SHB buffer (0.25 M sucrose, 20 mM Hepes-KOH (pH 7.5), and 0.1% BSA), overlaid on a discontinuous gradient of 60%, 45%, 28%, and 5% Percoll, and centrifuged at 12,500 rpm for 1 hr, at 4° in a Beckman SW28 rotor. Mitochondria were recovered at the 28–45% interface and washed with excess SHB buffer to remove Percoll. Purified mitochondria were then treated with DNase I (10 μg/ml) at 4° for 1 hr, after which EDTA was added to a final concentration of 25 mM and mitochondria were recovered by centrifugation at 10,000 rpm for 15 min. DNA was extracted by lysing mitochondria in 1% SDS in 10 mM Tris-HCl (pH 8), 1 mM EDTA prewarmed to 55°. The sample was kept at 55° for an additional 5–10 min. mtDNA was then extracted by phenol, phenol:chloroform:isoamyl alcohol and chloroform:isoamyl alcohol and precipitated with ethanol. Large RNAs were removed by treatment with 2.5 M final concentration of LiCl (PELHAM 1985).

Enzyme digestion and electrophoresis of DNA: Purified mtDNA or total DNAs were digested with restriction endonucleases under the conditions recommended by the suppliers (Toyobo, Takara, Boehringer). In the case of mtDNA, RNase A was also added (50 μg/ml) in the reaction mixture. Total DNAs digested with restriction enzymes were separated by 1% agarose gel electrophoresis in 0.04 M Tris-

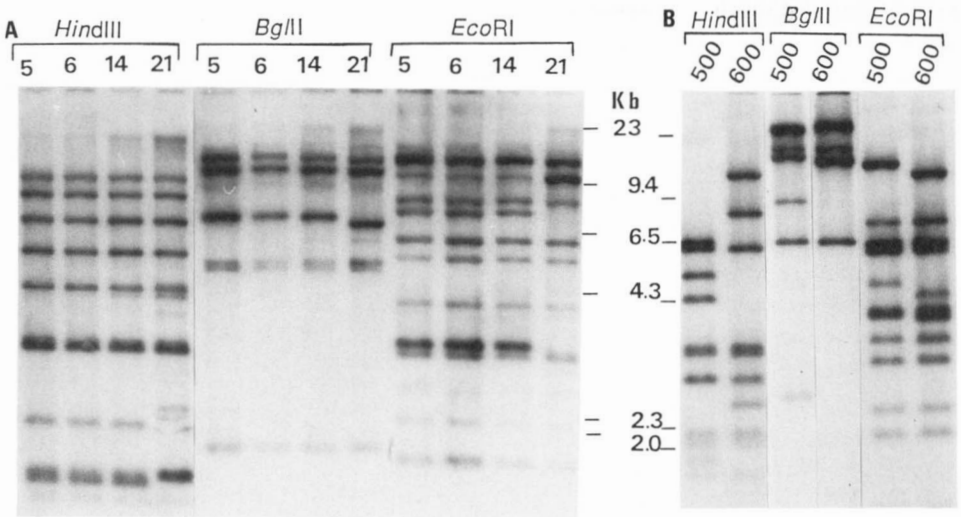

FIGURE 1.—RFLPs of *P. pallidum* strains. (A) MF2 strains (5, 6, 14 and 21); and (B) PN strains (500 and 600). Purified mtDNAs were blotted and probed with labeled PN600 mtDNA as described in MATERIALS AND METHODS.

acetate, 0.01 M EDTA (pH 8). Electrophoresis was carried out at 4 V/cm for 4–5 hr.

Blotting and probe hybridization: The gels were denatured in 0.4 N NaOH, 0.6 M NaCl for 30 min and blotted on to nylon membrane (GeneScreen Plus, NEN products). mtDNA probes (from either PN600 or PN500) were prepared using a random primed labeling kit (Boehringer Mannheim), and hybridized to blots in prehybridization buffer containing 100 μg/ml denatured salmon sperm DNA. Prehybridization, hybridization and washing conditions were according to the GeneScreen Plus (1987) protocol. Filters were then exposed to Fuji X-ray films.

RESULTS

In order to monitor the mtDNA inheritance in *P. pallidum* we needed genetic or physical markers. In contrast to yeast, mitochondrial mutants are not available in the cellular slime molds. Therefore, we searched for mtDNA polymorphisms in the heterothallic strains of *P. pallidum*. Both PN and MF2 strains of *P. pallidum* exhibited RFLPs with *Bgl*II, *Hin*dIII, and *Eco*RI (Figure 1) but no RFLPs were seen with *Bam*HI or *Pst*I (data not shown). Mitochondrial DNA of MF2-14 digested with different enzymes exhibited the same pattern as MF2-5 or MF2-6 mtDNA although it is of opposite mating type. Similar analyses using *Dictyostelium discoideum* heterothallic strains, HM1 and NC4, also revealed RFLPs (our unpublished observations). By adding the molecular weights of restriction fragments, we estimated the size of both the MF2 and PN mitochondrial genomes to be 56 ± 4 kb. Furthermore, the extent of methylated cytosine residues, if any, appears to be the same in both mating types since, mtDNA patterns of *Hpa*II and *Msp*I digests from both mating types were the same (data not shown).

Macrocyst germination in these strains was 20–30%, as compared to a previous report of 10% germination (FRANCIS 1980). These higher germination levels may have resulted from our efforts to select mature macrocysts with visible cells inside them and less condensed secondary walls. Subsequently we found that the germination rate could be raised to 50% if plates were incubated at 27° instead of 22°, without any influence on the transmission mode of mtDNA. We also noticed that infection of the macrocysts with *Trichoderma* sp. facilitates germination as recommended earlier by FRANCIS (1980). This treatment reduces macrocyst clumping, making isolation of single macrocysts easier. Cellulase secreted by *Trichoderma* sp. (SHIKATA and NISHIZAWA 1975) very likely digests the primary wall of macrocyst, which is reported to be rich in cellulose and can be removed by treatment with cellulase and macerating enzymes (FUKUI 1976).

We screened mtDNA of single progeny isolated from spores after fruiting body formation and from amoebae immediately after germination. The first method was convenient, since progeny of single macrocysts could be clonally plated at the same time even though macrocysts did not germinate synchronously, and because it eliminated any free amoebae which might not have been rinsed away during washings. However, as we were concerned about possible effects of selection or change in mtDNA constitution which could occur from amoebae to spore differentiation, we also selected amoebae immediately after germination and plated them clonally. In some plates we observed that amoebae, from germinated small macrocyst, formed microcysts instead of fruiting bodies; but under all conditions the progeny produced the same mtDNA transmission pattern.

mtDNA of the progeny were examined using Southern blot analysis. Figure 2 shows a typical pattern (*Hin*dIII digest) of mtDNA inheritance in the progeny from PN500 (*mat1*) × PN600 (*mat2*) cross from which three to five progeny per macrocyst and 60 single macrocysts were analyzed. All the progeny exhibited only the PN600 type mtDNA pattern. A MF2-21 (*mat1*) × MF2-6 (*mat2*) cross (Figure 3) and a MF2-21

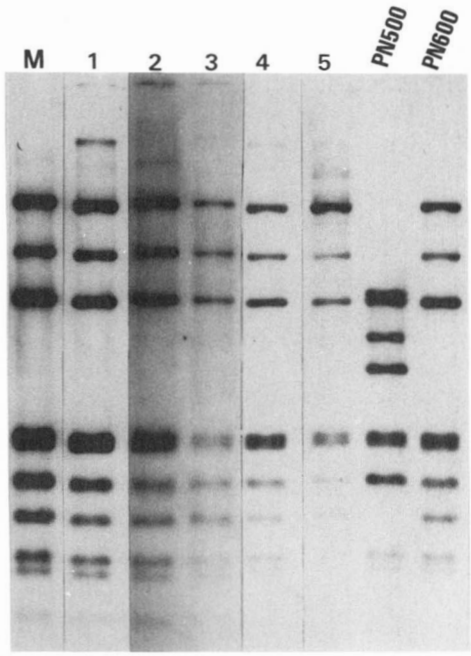

FIGURE 2.—Autoradiograph of *Hin*dIII cleavage patterns of mtDNA from PN500 × PN600 progeny. Total DNAs were digested with the enzyme, blotted onto nylon membranes and hybridized to PN600 or PN500 mtDNA probes labeled with [α-^{32}P]dATP. PN500 and PN600 lanes are parental mtDNAs. Lanes 1–5 represent single progeny of five different macrocysts. The four single progeny clones examined from each of 60 macrocysts (240 clones), all showed only B type mtDNA (see Tables 1 and 2).

(*mat1*) × MF2-5 (*mat2*) cross (Table 2) also showed the typical uniparental mtDNA inheritance pattern when 20 single macrocysts were examined. In each cross the MF2-6 (or MF2-5) *mat2* mtDNA pattern is seen in the progeny. Since we wanted to make certain that the relatively small number of samples screened had not left any *mat1* type mitochondrial DNA undetected, we analyzed the progeny of a mixture of macrocysts (150–200) after germination. The results for both crosses are shown in the lanes marked M (Figures 2 and 3) for mass screening. If mtDNA from the *mat1* parent was also transmitted, we would have expected to see a mixture of parental type mtDNAs. However, this was not the case and only *mat2* type mtDNAs (PN600 or MF2-6 type) were transmitted in these crosses.

Next we intercrossed the more distantly related *P. Pallidum* strains of MF2 and PN, defined on the basis of their different regions of isolation and different mtDNA restriction patterns (compare Figure 1, A with B). The latter is probably a more important criterion since the RFLP variation in mtDNA within and between species can reveal the genetic divergence of closely related strains (UPHOLT 1977; AVISE, LANSMAN and SHADE 1979). PN × MF2 combinations formed macrocysts and germinated at expected rates (20–30%) except in the case of PN600 × MF2-21 where both macrocysts formation and germination

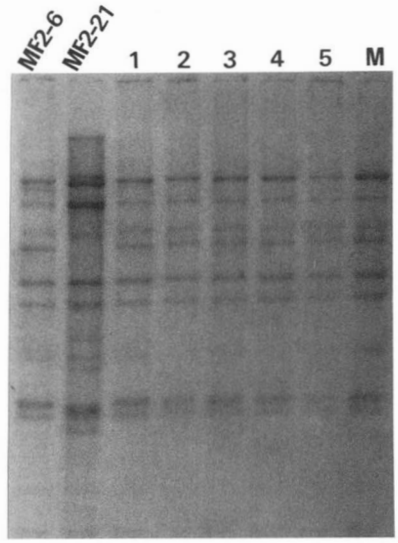

FIGURE 3.—Autoradiograph of *Eco*RI cleavage patterns of mtDNA from MF2-21 × MF2-6 progeny. Hybridization and screening were as described in Figure 2 and in MATERIALS AND METHODS. MF2-21 and MF2-6 lanes are parental mtDNAs. M is mtDNA from a mixture of the germinated macrocysts. Lanes 1–5 are typical mtDNA pattern of the progeny showing uniparental inheritance of C type (see Tables 1 and 2).

level were reduced. In the PN500 (*mat1*) × MF2-6 (*mat2*) cross (Figure 4), both parental mtDNA types are uniparentally transmitted to the progeny with that from MF2-6 (*mat2*) predominant (lanes 3, 4, 5, and 6). The lane marked M representing DNA from a mixture of germinated macrocysts shows bands from both parents. Other combinations such as PN600 × MF2-14, PN600 × MF2-21, and PN500 × MF2-5 also showed uniparental transmission of both types of mtDNA (Table 2A). While mtDNA from the *mat2* parent was predominant to mtDNA from the *mat1* parent the mating alleles and mitochondrial alleles of the same strains did not necessarily cosegregate. Furthermore, all progeny of a single macrocyst had the same type of mtDNA, either from the *mat1* or the *mat2* parent, but not both (Table 2, A and B).

Among the strains used in these experiments, a cycloheximide and canavanine resistant double mutant strain PN550 (*mat1*) exhibited a similar mtDNA restriction fragment pattern as PN600 (*mat2*) (Figure 5). The PN550 strain was found subsequently to have arisen from the cross PN654 (*cyc*^{r}) (*mat2*) × PN541 (*can*^{r}) (*mat1*) (D. FRANCIS, personal communication), consistent with our observation that PN600 mtDNA is uniparentally transmitted in crosses. In contrast, the mtDNA pattern of other mutants selected directly from a parental strain always had the parental type mtDNA of that strain.

DISCUSSION

Taking advantage of RFLPs as physical markers, we demonstrated that mtDNA in the cellular slime

TABLE 2

Type of transmitted mtDNA in the progeny

A				B. Mating type of the progeny[a]				
Cross			No. and type of transmitted mtDNA[c]					
mat1	*mat2*	No. macrocysts[b]		No. macrocysts	No. clones/ macrocyst	Total tested	*mat1*	*mat2*
PN500 × PN600		60	60 B	10	3	30	9	21
PN500 × MF2-5		20	18 C, 2 A	5	3	15	6	9
PN500 × MF2-6		20	17 C, 3 A	5	2	10	4	6
MF2-14 × PN600		20	19 B, 1 C	5	3	15	6	9
MF2-21 × PN600		20	20 B, (D)[d]	5	3	15	6	9
MF2-21 × MF2-6		20	20 C	5	2	10	6	4
MF2-21 × MF2-5		20	20 C	5	3	15	3	12
PN541 × PN654[e]			B				1	

[a] Only a fraction of the progeny was randomly selected for mating type analysis.
[b] From 1 to 5 clones per macrocyst were examined, all showed the same type of mtDNA pattern.
[c] mtDNA type is according to the nomenclature used in Table 1.
[d] All 20 macrocysts tested showed B type mtDNA only, but when the progeny of a mixture of macrocysts were tested, faint hybridizing bands belonging to D type mtDNA were also detected.
[e] This cross was done by D. FRANCIS and yielded strain PN550; for details see the text.

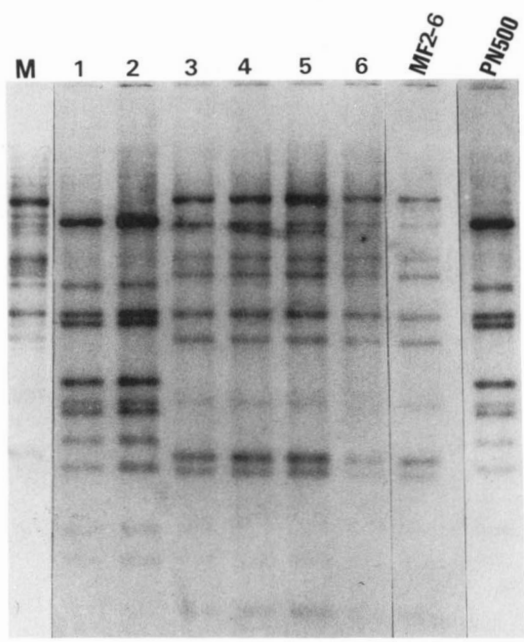

FIGURE 4.—Southern blot analysis of mtDNA (*Eco*RI digest) of PN500 × MF2-6 progeny. Hybridization conditions were as described in Figure 2. PN500 and MF2-6 lanes are parental mtDNAs. Lanes 1–6 are the progeny showing C type (lanes 3–6) and A type (lanes 1 and 2) mtDNAs, but the progeny of a single macrocyst all have either C or A and not both (for more details see Table 2). In lane M (mixture of the germinated zygotes), bands from both types can be seen.

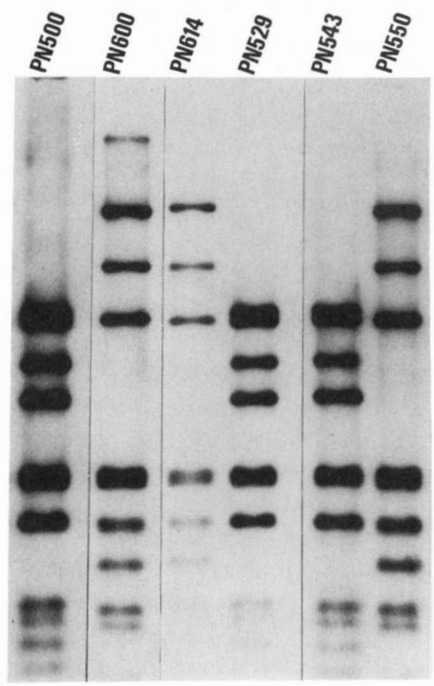

FIGURE 5.—Restriction patterns of mtDNAs from several mutants in *P. pallidum*. PN500, PN529, PN543 and PN550 are of *mat1* mating type. PN600 and PN614 are of *mat2* mating type.

mold, *P. pallidum*, is uniparentally transmitted to the progeny. In crosses of closely related *P. pallidum* isolates of opposite mating type randomly selected meiotic progeny all inherited mtDNA from only the *mat2* parent regardless of their mating types. In none of the progeny did we detect *mat1* type mtDNA, nor a mixture of the two parental type mtDNAs. Nevertheless, in a cross between more distantly related strains of opposite mating types, a small portion of progeny transmitted *mat1* type mtDNA uniparentally (Table 2), but progeny containing a mixture of the two parental mtDNA types were never detected.

During vegetative segregation in yeast, resulting in the partitioning of organelles at cell division, both parental mtDNAs present initially in the heteroplasmic diploid cells segregate to form homoplasmic progeny after about 20-cell generations (DUJON 1981). In *P. pallidum* however, when parents of opposite mating types are from closely related strains, one type of parental mtDNA is selectively eliminated

at some stage between zygote formation and germination. Uniparental inheritance of organelle DNA can be achieved by several mechanisms. It can be result from the failure of gametes from one parent to transmit organelle genomes to the zygote, *e.g.*, monogametic organelle transmission (MOGENSEN 1988), or when very few organelles from one parent are contributed in relation to those from the other parent, *e.g.*, in the case of sperm and egg (for review see BIRKY 1983). In the case of *P. pallidum* we do not suppose that there is any selective advantage at cellular level for one type of mtDNA. The number of mitochondria per cell in all the strains is estimated, by 4′,6-diamidino-2-phenylindole (DAPI) staining, to be 70-90/cell (our unpublished data). Besides, both parental mtDNAs are physically the same size, and the rate of cell multiplication is almost identical for both cell types. Moreover, the extent of methylation is the same in the gametes of heterothallic strains of *P. pallidum*, contrary to that seen for chloroplast DNA from gametes of opposite mating type in *Chlamydomonas* (ROYER and SAGER 1979; BURTON, GRABOWY and SAGER 1979).

The elimination process may also involve nucleases causing the extensive destruction of the mitochondrial genomes from one parent (SAGER and RAMANIS 1973; SAGER, SANO and GRABOWY 1984; KUROIWA 1985). We have actually observed extensive degradation of mtDNA in young 3–7-day-old macrocysts (our unpublished observations), but do not yet know whether both mtDNA types are destroyed to the same extent. However, in crosses of heterothallic strains having RFLPs, we did not observe any recombination between the two parental mtDNAs. Both mtDNA types are physically of the same size, and the amount of mtDNA extracted from the same number of cells was almost equal, suggesting the same amount of mtDNA/cell. In the zygote, both parental mtDNA types have the opportunity to co-exist in the macrocyst. Therefore, it would be reasonable to expect fusion and subsequent recombination of mtDNA similar to that seen in yeast (*e.g.*, FONTY *et al.* 1978). Nevertheless, none of the progeny showed extra bands which did not correspond to the patterns of one or the other parental type. Hence, we think that mtDNA from the *mat1* gametes of *P. pallidum* is probably destroyed early in zygote formation, perhaps by nucleases. However, we cannot rule out the possibility that only a small fraction of the mtDNA population recombines with the majority fraction and therefore is undetected by Southern blot analyses.

mtDNA segregation in *P. pallidum* may not be strictly mating type dependent (Table 2), since both parental mtDNA types are transmitted in crosses between more distantly related strains. Thus we hypothesize that the *mat2* allele is dominant to the *mat1* allele with respect to mtDNA transmission pattern, in crossing similar strains, but in the crosses of more distantly related strains the two alleles are codominant. mtDNA transmission probably may not be under control of mating type locus per se; but, as in *C. reinhardtii* (GALLOWAY and GOODENOUGH 1985; MATAGNE 1987; MATAGNE, RONSVAWY and LOPPES 1988), the gene(s) involved in organelle inheritance may be in a complex region closely linked to the mating type locus. Unfortunately our attempts to make parasexual diploids by artificial fusion of strains of opposite mating type (*e.g.*, PN500 and PN600) in order to study the mechanism of mtDNA transmission were unsuccessful, and zygotes were formed instead of parasexual diploids, probably due to the vegetative incompatibility as reported earlier (ROBSON and WILLIAMS 1979).

Although the sample sizes used in the present study are relatively small, our results from both progeny of single macrocysts and mass progeny of several macrocysts, strongly suggest that mtDNA is uniparentally inherited in *P. pallidum*. The mechanism of uniparental transmission is under further investigation.

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