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Peroxisome biogenesis in the yeast *Hansenula polymorpha* is controlled by a complex set of interacting gene products

(methanol/peroxisome-deficient mutants/unlinked noncomplementation/protein–protein interactions)

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ABSTRACT **We have studied the genetic interactions between mutant alleles in 12 genes, designated *PER1–PER12*, which are essential for peroxisome biogenesis in the yeast *Hansenula polymorpha*. Recessive mutations in any of these genes determined three different morphological phenotypes: (*i*) complete absence of peroxisomes (Per⁻); (*ii*) presence of small peroxisomes in conjunction with a major fraction of peroxisomal matrix proteins in the cytosol (Pim⁻); and (*iii*) presence of peroxisomes with aberrant crystalline matrix substructure (Pss⁻). Extensive complementation analysis showed many cases of noncomplementation—that is, diploids that contained both wild-type and mutant alleles of two different *PER* genes were unable to grow on methanol and showed peroxisomal defects. The observed cases of unlinked noncomplementation appeared to be gene and allele specific and were predominantly observed at lower temperatures (cold sensitive). The genetic results obtained were used to formulate a model of *PER* gene product interactions. In this model, five *PER* gene products are key or core components of the complex. Other *PER* gene products appear to play a more peripheral role.**

In recent years, much progress has been made in our understanding of the molecular mechanisms involved in biogenesis/function of subcellular entities in eukaryotic cells. These studies indicated that protein sorting, (un)folding, and assembly in mitochondria (1), vacuoles (2), and nuclei (3) and protein transport through the secretory pathway (4) are mediated by complex systems composed of numerous components that interact both physically and functionally.

In contrast, comparatively little is known about the biogenesis and function of microbodies (peroxisomes; ref. 5). Yeasts are attractive model organisms for studies of peroxisomes. Indeed, the proliferation and enzymic contents of these organelles can be precisely prescribed by manipulating growth conditions (5). Also, the organisms are readily accessible to advanced biochemical and genetic methods (6). Moreover, a series of peroxisome-deficient mutants has recently been obtained from different yeast species—e.g., *Saccharomyces cerevisiae* (*PAS* genes; ref. 7), *Hansenula polymorpha* (*PER* genes; refs. 8 and 9), and *Pichia pastoris* (*PER* and *PAS* genes; refs. 10 and 11). These studies have revealed numerous genes essential for peroxisome biogenesis and function (7–9). A comparable genetic complexity has been demonstrated in human cell lines from patients with peroxisome disorders (12).

Here we report on specific physical and functional interactions that appear to exist between protein products of several *PER* genes in *H. polymorpha*. Evidence for their interactions was obtained through an unusual complementation behavior—namely, unlinked noncomplementation—in which specific pairs of *per* mutants bearing single recessive mutations in different genes failed to fully complement as diploids. The number of specific cases of unlinked noncomplementation is to our knowledge unprecedented and has provided insights into the functional organization of *PER* products.

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MATERIALS AND METHODS

Strains. All mutants were derived from wild-type *H. polymorpha* CBS 4732. Repeatedly backcrossed auxotrophic mutants of *H. polymorpha* NCYC 495 (*ade11-1*, *met6-1*, *ade11-1 met6-1*, and *leu1-1*; ref. 13) were used for backcrossing and genetic analysis of mutants obtained.

Growth Conditions. Solid media used for mating and complementation analysis, sporulation of hybrids, and random spore analysis were described (13). For induction of peroxisomes and enzymes involved in methanol metabolism mutant strains were pregrown at 37°C in yeast extract/peptone/dextrose (YEPD) medium. After two transfers (14), the cultures were diluted 1:4 in fresh mineral medium (14) containing 0.5% methanol as carbon source and subsequently incubated for 24–36 h.

Different representatives of the various complementation groups were also grown in carbon-limited continuous cultures at 37°C on glucose/methanol mixtures as described (15).

Mutant Isolation and Genetic Methods. Chemical mutagenesis by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and isolation of methanol-nonutilizing (Mut⁻) mutants of *H. polymorpha* were described by Cregg *et al.* (8). Two hundred sixty Mut⁻ strains were pregrown in YEPD, diluted in methanol-containing medium as described above, and incubated for 24–36 h at 37°C. Putative peroxisome-deficient mutants, selected by phase-contrast light microscopy (9), were further screened by transmission electron microscopy (8). Mating of strains, complementation testing, sporulation of diploids, and random spore analysis were performed as described by Gleeson and Sudbery (13) with minor modifications as described (9).

Electron Microscopy. Whole cells were fixed with 1.5% (wt/vol) $KMnO_4$ for 20 min at room temperature. Spheroplasts, prepared according to Douma *et al.* (16), were prefixed in 6% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 60 min at 0°C, followed by postfixation in a mixture of 0.5% OsO_4 and 2.5% (wt/vol) $K_2Cr_2O_7$ in the cacodylate buffer for 90 min at 0°C. After dehydration in a graded ethanol series, the samples were embedded in Epon 812; ultrathin sections were cut with a diamond knife and examined in a Philips EM 300.

Immunocytochemistry was performed on ultrathin sections of Lowicryl-embedded cells (14) using specific antibodies against alcohol oxidase and protein A/gold (16).

RESULTS

Mutant Isolation and Morphological Analysis. All mutants described in this paper were identified within a collection of 260 methanol-nonutilizing (Mut$^-$) mutants of *H. polymorpha* as described (8). After incubation of the mutants in methanol-containing media, 60 strains were identified by direct electron microscopic examination of ultrathin sections as having one of the following peroxisomal defects: (*i*) complete absence of peroxisomes (Per$^-$ phenotype; 38 strains), (*ii*) presence of an abnormally low number of small peroxisomes in conjunction with the presence of the bulk of the peroxisomal matrix proteins in the cytosol (Pim$^-$ phenotype; 20 strains), and (*iii*) aberrations in the peroxisomal substructure—i.e., presence of electron-dense inclusions in the crystalline peroxisomal matrix (Pss$^-$ phenotype; 2 strains). All mutants were nonconditional—i.e., displayed a Per$^-$, Pim$^-$, or Pss$^-$ phenotype during incubation on methanol, independent of the temperature. All but two of these mutants were due to recessive monogenic mutations. Characteristic examples of these phenotypes are shown in Fig. 1.

Genetic Analysis. It was initially assumed that each phenotype was gene specific, and therefore the *per* mutants were divided into groups by phenotype for complementation analysis. Pim$^-$ mutants were organized into five complementation groups as described (8, 9). The remaining Per$^-$ and Pss$^-$ mutants were examined and found to be monogenic; most of them harbored mutations that were recessive to their respective wild-type alleles. However, two mutations were dominant. Backcrossed derivatives of each Per$^-$ and Pss$^-$ mutant were subjected to complementation analysis against each other and against the Pim$^-$ mutants. The results suggested that the total *per* mutant population represented mutations in 14 genes. To precisely establish the number of genes affected, representatives of each group were subjected to linkage analysis. Table 1 shows the linkage relationships and map positions of the corresponding genes, as deduced from random spore analysis. Twelve genes were defined (*PER1–PER12*). Genes *PER4–PER7* and locus *MET6* were mapped to linkage fragment 1, previously identified by Gleeson and Sudbery (13). A gene order (*MET6–PER5–PER7–PER4–PER6*) was determined by three-point cross analyses. Genes *PER1*, *PER2*, and *PER9* mapped to linkage fragment 2, while genes *PER3*, *PER10*, and *PER11* were located in linkage fragment 3 with relative map positions as presented in Table 1. The two other genes, *PER8* and *PER12*, were unlinked to each other, to any of the *PER* genes indicated above (see Table 1), or to *LEU1* and *ADE11* loci (data not shown). In addition to defining the number and relative positions of *PER* genes, the combined complementation and genetic linkage analysis also made clear that Pim$^-$, Per$^-$, and Pss$^-$ phenotypes were allele specific and not gene specific, since in at least two instances, different alleles of one gene displayed different peroxisome-defective phenotypes (17).

Unlinked Noncomplementation Between *per* Alleles. As indicated in Table 2, some mutant alleles of *PER7* failed to functionally complement mutations in *PER1* and *PER4*, while certain mutant alleles of *PER11* failed to complement mutations in *PER2–PER6* and *PER9* with respect to restoration of the ability of diploids to grow on methanol as the sole carbon source at standard growth conditions (37°C). These thus represent unlinked noncomplementing mutations, as observed in *Saccharomyces cerevisiae* and the fruit fly *Drosophila melanogaster* (18–20), and suggest that the products of these genes interact. As demonstrated in previous studies (21, 22), the use of physiologically suboptimal conditions may permit identification of additional gene product interactions. Therefore, we analyzed the complementation properties of selected

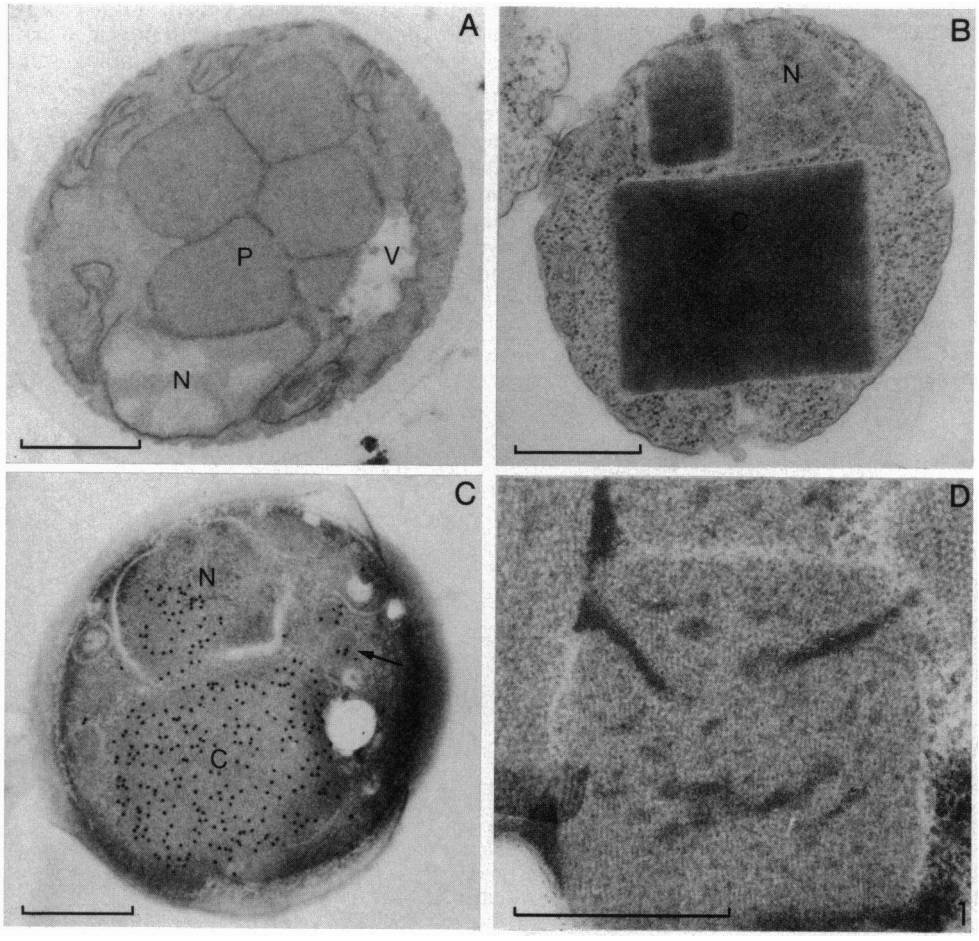
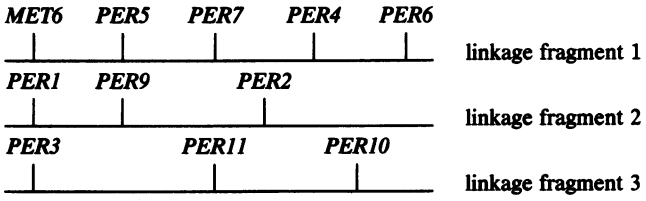


FIG. 1. (*A–D*) Overall morphology of wild-type *H. polymorpha* NCYC 495 ($KMnO_4$-fixed cells) and characteristic peroxisomal mutants (*B–D*) of fully derepressed, methanol-incubated cells. (*B*) *per7*, mutant 125/2E, displaying the Per$^-$ phenotype (protoplast: glutaraldehyde OsO_4). (*C*) *per1*, mutant 124/2D, showing the Pim$^-$ phenotype (glutaraldehyde, Lowicryl; specific antibodies against alcohol oxidase/protein A/gold; arrow indicates small alcohol oxidase-containing peroxisomes). (*D*) Single peroxisome of *per6*, mutant 210, showing the Pss$^-$ phenotype (spheroplast; glutaraldehyde OsO_4). C, alcohol oxidase crystalloid; N, nucleus; V, vacuole; P, peroxisome. (Bars = 0.5 μm.)

Table 1. Linkage data from random spore analysis of *per1–per12* mutants of *H. polymorpha*

Gene or gene pair	Observed number of segregants		Distance, % recombination
	Parental	Recombinant	
MET6–PER5	3102	582	15.8
PER5–PER7	269	79	22.7
PER7–PER4	343	11	3.1
PER4–PER6	334	53	13.7
MET6–PER7	826	396	32.4
MET6–PER4	948	513	35.1
PER5–PER4	494	156	24.0
PER7–PER6	346	61	15.0
PER5–PER6	161	96	37.3
PER1–PER9	689	34	4.7
PER9–PER2	247	92	27.1
PER1–PER2	268	112	29.5
PER11–PER3	336	174	34.1
PER11–PER10	983	89	8.3
PER10–PER3	304	211	41.0

Gene order



Number of recombinants was scored on MM medium supplemented with methanol (Mut$^+$ recombinants) and by complementation tests of Mut$^-$ segregants with appropriate tester strains bearing *per* mutations of the parental strains. Gene order was determined by three-point analysis. *PER8* and *PER12* showed no linkage to each other or to any of the other *PER* genes or to *LEU1* and *ADE11* loci.

alleles of *per1–per12* at various temperatures (25°C, 31°C, 37°C, and 43°C) in order to identify additional examples of interacting gene products. The results presented in Table 2 revealed many cases of unlinked noncomplementation, especially at low temperatures. Several pairs of mutants showed unlinked noncomplementation at all temperatures tested (complete or nonconditional noncomplementation). Other mutant pairs failed to complement each other only at lower temperatures (25°C, 31°C, and, in some cases, 37°C) but complemented normally at elevated temperatures (43°C and, for some hybrids, 37°C) (cold-sensitive noncomplementation).

Ultrastructural analyses indicated that each case of functional noncomplementation was also reflected in aberrations in peroxisome morphology. Double-heterozygous hybrids bearing noncomplementing (cold sensitive; Cs) mutations showed normal wild-type peroxisome proliferation (in size, number, and crystalline substructure of the organellar matrix) when incubated at a permissive temperature. However, upon incubation at a restrictive temperature, specific disturbances in peroxisome morphology were observed. In certain cases, these defects were identical to those in the parental mutant pairs (Per$^-$, Pim$^-$, or Pss$^-$ phenotype; see Table 2). However, in many other cases, defects different from those of the parental strains were observed. These "constructed" phenotypes included (*i*) *p*eroxisomes with *i*rregular *s*hape (Pis$^-$), (*ii*) *p*eroxisomes *a*ffected in their bounding *m*embranes (Pam$^-$), (*iii*) *p*eroxisome *p*roliferation *d*efective (Ppd$^-$), (*iv*) *p*eroxisome *s*uper *p*roliferation (Psp$^-$), and (*v*) *c*ytosolic proteinaceous *ag*gregate accumulation (Cag$^-$). Characteristic examples of each of these phenotypes are shown in Fig. 2. Immunocytochemical experiments (Fig. 2*B Inset*) indicated that the cytosolic aggregates in Cag$^-$ mutants contained alcohol oxidase (23). In some of the hybrids, a combination of the above defects was observed (Table 2; Fig. 2).

Data from genetic analysis indicated that in all cases in which unlinked noncomplementation occurred, it was due to the same monogenic recessive mutations that caused the Mut$^-$ and specific peroxisome morphology phenotype of the parental mutants. In the meiotic progeny of diploids formed by any of these mutants with the wild-type strain, the phenotype unlinked noncomplementation demonstrated 1:1 segregation and complete cosegregation with the Mut$^-$, Per$^-$, Pim$^-$, or Pss$^-$ phenotype. Genetic analyses also revealed that the Cs phenotypes observed in heterozygous diploids were caused by the specific *per* mutations and not by other conditional recessive mutations passed on to the homozygous state during formation of the hybrids. For instance, Cs noncomplementation observed in hybrids formed between *per5* and any mutant in *per1–per4* (see Table 2) was not caused by a masked (or cryptic) conditional recessive mutation because hybrids formed between any of the *per1–per4* mutants were not Cs (data not shown).

Table 2. Complementation grid for *per* mutants

Mutant allele	*per5-112*	*per6-210*	*per7-125*	*per10-108*	*per11-60*
per1-124	Cs$^-$ Psp$^-$	Cs$^-$ Pis$^-$ Pss$^-$	−Per$^-$	Cs$^-$ Pis$^-$ Pam$^-$	−Ppd$^-$ Cag$^-$
per1-172	Cs$^-$ Pis$^-$	Cs$^-$ Pis$^-$	−Per$^-$ Cag$^-$	++	++
per2-15	Cs$^-$ Pis$^-$	Cs$^-$ Pis$^-$	Cs$^-$ Pis$^-$	Cs$^-$ Pis$^-$	−Pim$^-$ Psp$^-$
per3-188	Cs$^-$ Pis$^-$	Cs$^-$ Pis$^-$	Cs$^-$ Pis$^-$	Cs$^-$ Pis$^-$	−Pim$^-$ Cag$^-$
per3-162	Cs$^-$ Pis$^-$	Cs$^{+/-}$ Pis$^-$	++	++	++
per3-220	Cs$^-$ Pis$^-$	Cs$^-$ Pis$^-$	−Pis$^{+/-}$ Pam$^-$	Cs$^-$ Pis$^-$	++
per4-152	Cs$^-$ Pim$^-$ Pam$^-$	Cs$^-$ Pis$^-$	−Pim$^-$ Cag$^-$	Cs$^-$ Pis$^-$ Cag$^-$	−Pim$^-$ Cag$^-$
per5-112	−Pim$^-$	Cs$^-$ Pis$^-$	Cs$^-$ Ppd$^-$	Cs$^-$ Ppd$^-$	−Ppd$^-$ Cag$^-$
per6-210	Cs$^-$ Pis$^-$	−Pss$^-$	Cs$^-$ Pis$^-$	Cs$^-$ Pis$^-$	−Pis$^-$
per7-125	Cs$^-$ Ppd$^-$	Cs$^-$ Pis$^-$	−Per$^-$	Cs$^-$ Ppd$^-$ Cag$^-$	−Per$^-$ Cag$^-$
per8-214	Cs$^-$ Psp$^-$	++	++	++	++
per9-73	Cs$^-$ Pam$^-$	Cs$^-$ Pis$^-$	++	++	−Pim$^-$
per10-108	Cs$^-$ Ppd$^-$	Cs$^-$ Pis$^-$	Cs$^-$ Ppd$^-$ Cag$^-$	−Per$^-$	Cs$^-$ Pis$^-$
per11-60	−Ppd$^-$ Cag$^-$	−Pis$^-$	−Per$^-$ Cag$^-$	Cs$^-$ Pis$^-$	−Per$^-$
per12-3	Cs$^-$ Pis$^-$	Cs$^-$ Pis$^-$	++	++	++

Growth of diploids on MM medium supplemented with methanol was scored as follows: −, no growth at any temperature tested; Cs$^-$ and Cs$^{+/-}$, normal growth at 43°C and (for many diploids) at 37°C, no growth and weak growth, respectively, at 25°C, 31°C, and (for some diploids) at 37°C; ++, diploids that grew normally on methanol at all temperatures and were morphologically characterized by the presence of intact peroxisomes. For simplicity, only some mutant alleles of corresponding genes are included. Mutants *per1-124*, *per2-15*, *per3-188*, *per4-152*, and *per5-112* showed a Pim$^-$ phenotype; *per6-210* showed a Pss$^-$ phenotype; all other mutant alleles had a Per$^-$ phenotype.

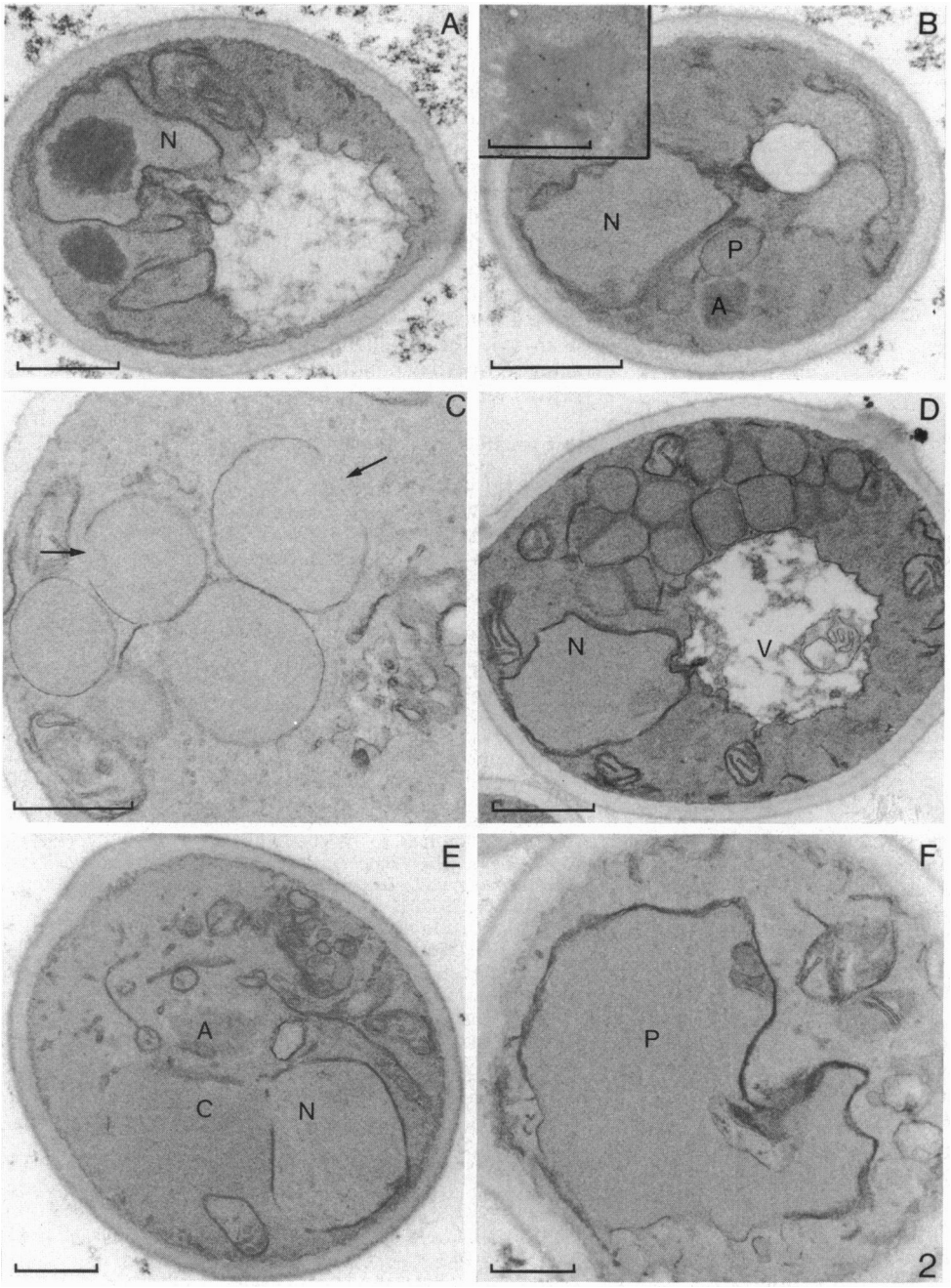


FIG. 2. (*A–D*) Characteristic examples of morphological mutant phenotypes of different double-heterozygous diploids, aberrant from those of the parental mutant strains in Fig. 1. All micrographs are of cells incubated in methanol-containing medium at restrictive temperatures. (*A*) *per5-112/+ per11-60/+* (Cs^- Per^- Cag^-) characterized by the presence of cytosolic aggregates; aggregates are also occasionally observed in the nucleus. (*B*) *per7-125/+ per10-108/+* (Cs^- Ppd^- Cag^-) showing few small peroxisomes together with cytosolic aggregates; these aggregates were positively labeled after incubation of thin sections with anti-alcohol oxidase and protein A/gold (*Inset*). (*C*) *per3-220/+ per7-125/+* (Cs^- Pam^-); sites of disruption of the peroxisomal membrane are indicated by arrows. (*D*) *per1-124/+ per5-112/+* (Cs^- Psp^-) showing the presence of numerous peroxisomes. (*E*) *per4-152/+ per7-125/+* (Cs^- Pim^- Cag^-) characterized by the presence of both small peroxisomes, cytosolic crystalloids, and cytosolic aggregates. Due to permanganate fixation the substructure of the crystalloid is poorly preserved. (*F*) *per2-15/+ per7-125/+* (Cs^- Pis^-) showing the typical aberrant morphology of the peroxisomes contained in such mutant cells. A, proteinaceous aggregate; C, N, V, and P, see legend to Fig. 1. (Bars = 0.5 μm.)

Together, our observations indicated that unlinked noncomplementation of the alleles tested was (*i*) predominantly a Cs phenomenon; (*ii*) gene specific—i.e., occurred only between specific pairs of genes (for instance, between *PER5* and any of the *PER1–PER4* genes, but not within *PER1–PER4* genes; compare Table 2); and (*iii*) allele specific, since it was often observed between only a few alleles of certain mutant pairs of genes (for instance, the double heterozygote *PER3/per3-188 PER7/per7-125* showed Cs noncomplementation, whereas the double heterozygote *PER3/per3-162 PER7/per7-125* showed complementation at all temperatures). Using this information on noncomplementation, we have constructed a diagram that summarizes our observations on genetic interactions between the *PER* gene products (Fig. 3).

DISCUSSION

This paper describes a detailed genetic analysis of mutations in 12 genes (*PER1–PER12*) essential for peroxisome biogenesis/function in the methylotrophic yeast *H. polymorpha*. Like genes encoding major peroxisomal proteins in *Candida tropicalis* (24, 25), most of the *PER* genes of *H. polymorpha* appear in genetically linked clusters (Table 1). Three linkage groups exist: (*i*) *PER5–PER7–PER4–PER6*, (*ii*) *PER9–PER1–PER2*, and (*iii*) *PER3–PER11–PER10*. The functional role of the products of each *H. polymorpha PER* gene remains to be determined. Analysis of Pim^- mutants suggests that *PER1–PER5* probably specify components of a general import mechanism of peroxisomal matrix proteins (9).

Interestingly, as also observed in human peroxisome disorders (12), different alleles of *H. polymorpha PER* genes (e.g., *PER1* and *PER3*; ref. 17) display different peroxisome morphology phenotypes. Thus, the peroxisome morphology phenotypes are allele specific. At present, it is not clear how these phenotypes relate to each other. It is conceivable that the Pim^- phenotype is a consequence of a partial impairment of peroxisome function, while Per^- phenotypes result from a complete block of that function. However, like Per^- mutants,

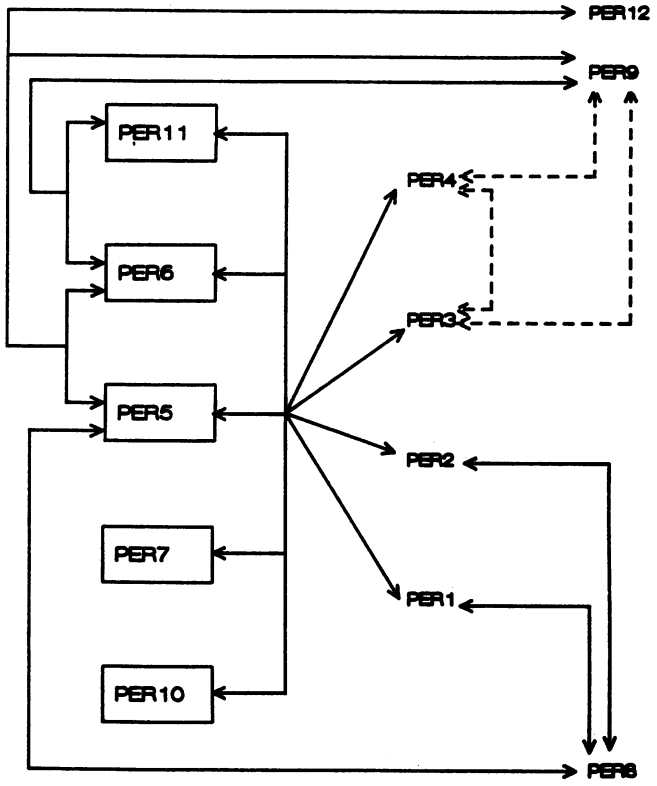


FIG. 3. Interactions between products of *PER1–PER12* genes deduced from unlinked noncomplementation data. Dashed lines indicate weak interactions (leaky growth of the corresponding double-heterozygous hybrids at restrictive temperatures).

Pim$^-$ mutants are totally defective in methanol utilization (Mut$^-$). Further insights are likely to be gained from an analysis of the primary sequence of *PER* gene products. Toward this goal, seven *PER* genes have now been cloned; their DNA sequences remain to be determined (V.I.T., H.R.W., and J.M.C., unpublished data).

The unusual complementation behavior—that is, the failure of recessive mutations in different genes to complement—suggests distinct functional relationships between the protein products of these genes. Analysis of the patterns of unlinked noncomplementation indicates that *PER* gene products interact in a specific manner. A map of interactions of products of these genes is depicted in Fig. 3. Since products of the genes *PER5*, *PER6*, *PER7*, *PER10*, and *PER11* appear to interact to some degree with all or most of the other *PER* gene products, they may be key or core components in a large multicomponent complex controlling import and/or other essential peroxisome functions. Several comparable cases of unlinked noncomplementation have been reported in *S. cerevisiae* (18, 19) and *D. melanogaster* (20). In each of these cases, it was proposed that they are a consequence of interacting gene products. In a number of cases, this hypothesis has been confirmed through biochemical studies that demonstrate the existence of these products in a multicomponent complex. For example in *D. melanogaster* and *S. cerevisiae*, interaction of α- and β-tubulins, which form α/β heterodimers in polymerized microtubules, was predicted from noncomplementation of specific alleles (19–26).

Three features of our unlinked noncomplementing *per* mutant pairs suggest that they reflect specific interactions between proteins. First, the unusual complementation behavior is gene and, often, allele specific. This property was also observed for genes encoding the major microtubule-associated proteins (19). Second, the most frequently observed phenotype is cold sensitivity. Cs phenotypes are believed to often reflect defects in protein–protein interactions (19). Third, aberrations in peroxisome morphology always coincide with the restrictive temperature for growth on methanol. In some instances, the peroxisomal aberration is the same as that in the parental haploid mutants, and in other instances it is different from either parent.

Identification of interactions between *PER* gene products by means of unlinked noncomplementation is a powerful tool in elucidating the molecular mechanisms of peroxisome biogenesis/function. In conjunction with recent major advances in the molecular genetics of *H. polymorpha* (27) and the extensive knowledge on the physiology/biochemistry of the organism (5), the availability of large numbers of well-characterized peroxisomal mutants renders *H. polymorpha* very attractive for these studies.

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