Formamide Sensitivity: A Novel Conditional Phenotype in Yeast

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ABSTRACT
Yeast mutants unable to grow in the presence of 3% formamide have been isolated in parallel with mutants sensitive to either 37° or 6% ethanol. The number of formamide-sensitive mutations that affect different genes that can be identified from yeast cells is at least as large as the number of thermosensitive or ethanol-sensitive mutations. These mutations are of two types: those that are sensitive to formamide, temperature and/or ethanol simultaneously; and those that are specific for formamide sensitivity and show no temperature or ethanol sensitivity phenotype. Those genes susceptible to giving rise to formamide-sensitive alleles include the structural gene for DNA ligase, CDC9, and the structural gene for arginine permease, CAN1. The results indicate that formamide sensitivity can be used as a novel conditional phenotype for mutations on both essential and nonessential genes. This work also confirms that ethanol-sensitivity can be used as a conditional phenotype to identify mutations in at least as many genes as those susceptible to temperature or formamide sensitive mutations.

THE understanding of any biological process requires the identification of the proteins and genes involved in it. The contribution of genetic methods to this understanding through the isolation of mutants is very important. However, there are obvious limitations for the identification of mutations in essential genes. Conditional mutations, that is mutations that only express the mutant phenotype under some restrictive conditions (HOROWITZ 1948), serve to overcome this problem.

Temperature has been used extensively to isolate conditional mutants since first proposed by HOROWITZ and LEUPOLD (1951). The mutant phenotype can be expressed at either high (thermosensitive) or low (cryosensitive) temperatures. This technique has allowed the identification of many genes involved in essential biochemical and cellular processes, such as DNA replication, cell division, cytoskeleton structure, etc., since the first thermosensitive mutants were reported in phage (EDGAR and LIELAUSIS 1964; EPSTEIN et al. 1965), bacteria (EIDIC and NEIDHART 1965, KOHIYAMA et al. 1966) and yeast (HARTWELL, 1967). Different mutant proteins contain changes in charge, hydrophobicity, solvent structure and hydrogen bonding that influence its secondary or tertiary structure and that can confer a thermolability phenotype (ALBER et al. 1987).

There are several reasons to believe that alternative conditional phenotypes are necessary. First, not all proteins are equally susceptible to amino acid changes that make them thermolabile; the extensive work of HARTWELL (1967) on yeast suggested that the isolation of thermosensitive mutants is not random among the different biological processes studied. Second, not all amino acids of a protein are equally susceptible to lead to a thermosensitive phenotype (ALBER et al. 1987). Third, the identification and genetic analysis of extragenic suppressors of thermosensitive mutations in essential genes require a second conditional phenotype (MOIR and BOTSTEIN 1982). And, fourth, the use of different temperatures is not possible for homeothermic eukaryotes.

Recently BARTEL and VARSHAVSKY (1988) used heavy water (D2O) as a conditional mutant phenotype in yeast. Their work provides an important tool to identify novel conditional mutations and open new ways to isolate conditional mutants in homeothermic organisms.

A characteristic that can also be used to isolate conditional mutants is sensitivity to ethanol, which has been characterized at the genetic and physiological level (AGUILERA and BENITEZ 1986). A great many genes can be mutated to produce an ethanol-sensitive phenotype. A number of these mutations also lead to thermosensitivity. That study suggests that many genes, not necessarily involved in glucose or lipid metabolism, are susceptible to mutations that lead to an ethanol-sensitivity phenotype for growth. However, the use of ethanol-sensitivity as a conditional phenotype may be restricted to organisms such as yeast that tolerate ethanol concentrations of 6%.

In this study, formamide sensitivity as a novel conditional phenotype in yeast was investigated for the following reasons: (1) formamide is an ionizing solvent that destabilizes noncovalent bonds such as hydrogen bonds; (2) formamide is not metabolized by cells and
there are no reasons to believe a priori that yeasts have evolved as highly formamide-tolerant organisms as is the case for ethanol; and (3) formamide is a small molecule (HCONH₂) that should enter cells easily. We have isolated some mutants that are simultaneously formamide, ethanol and temperature sensitive for growth, and some others that are exclusively formamide sensitive. We have also identified formamide-sensitive alleles of the CDC9 and CAN1 genes.

The genetic analysis of the formamide sensitive mutants isolated in this study suggests that any type of gene, whether essential or not, is susceptible to formamide sensitive mutations.

MATERIALS AND METHODS

Strains: The yeast strains used in this study are the congenic strains W303-1B (MATa ade2-1 leu2-3, 112 ura3-1 trp1-1 his3-11, 15 can1-100), AYW3-3A (MATa ade2 leu2-3, 112 ura3 trp1 his3), and AYW3-4B (MATa ade2 leu2-3, 112 ura3 trp1 his3), 5a-P3631 (MATa his7-1 lys2-18), and C9Y-1B (MATa leu2 his3 cdc9-1). Twelve other strains carrying 11 different thermosensitive mutations in 10 cell cycle genes were used. These mutations were hpr6 (a CDC2 allele), cdc4, cdc5, cdc6, cdc8-1, cdc9-1, cdc13-1, cdc14, cdc17-1, hpr3 (a CDC17 allele) and cdc28. The cdc alleles used are original from L. Hartwell, C. Newlon and H. Klein laboratories. (The allele number is not given when it is not known.)

Media and growth conditions: Standard media such as rich medium YEPD, synthetic complete medium with bases and amino acids omitted as specified, and sporulation medium were prepared according to published procedures (SHERMAN, FINK and HICKS 1986). L-Canavanine sulfate was added to synthetic medium at concentrations of 60 µg/ml.

Formamide or ethanol were added to the medium at the absence from L. Hartwell, C. Newlon and H. Klein laboratories. The viability of the cells after mutagenesis was approximated with a final concentration of 20 µg/ml N-methyl-N'-nitro-N-nitrosoguanidine (nitrosoguanidine) for 15 min according to CALDERON and CERDA-OLMEDO (1983). Mutations were allowed to segregate by culturing the mutagenized cells in liquid YEPD for 6 hr at 30°C prior to plating. The viability of the cells after mutagenesis was approximately 90%. Mutagenized cells were plated on YEPD plates to isolate single colonies and on SC-can and SC-can-3% formamide plates to select for CanR colonies in either the absence or the presence of 2% formamide, respectively. After 2 days at 30°C, colonies from YEPD plates were replica-plated onto YEPD-3% formamide, YEPD-6% ethanol and YEPD. Those plates containing formamide or ethanol were cultured at 30°C and the simple YEPD plates at 37°C.

Genetic analysis: Genetic analysis was performed according to SHERMAN, FINK and HICKS (1986).

RESULTS

Isolation of formamide-sensitive mutants: Wild-type cells grow very poorly at formamide concentrations above 3% v/v and not at all at a concentration of 10% (data not shown). Strains AYW3-3A and AYW3-4B were mutagenized with nitrosoguanidine as described in MATERIALS AND METHODS. A total of 2635 surviving colonies (1887 from strain AYW3-3B and 748 from AYW3-3A) were picked onto new YEPD plates and after 2 days at 30°C, they were replica-plated onto YEPD-3% formamide, YEPD-6% ethanol and YEPD. The first two groups of plates were cultured at 30°C, and the YEPD plates at 37°C, in order to score for a formamide-sensitive (fs), ethanol-sensitive (es) or thermostensitive (ts) phenotype for growth. After three rounds of scrutiny, a total of 41 mutants (17 from strain AYW3-3A and 24 from AYW3-4B) with an fs, es or ts phenotype were selected. From Table 1 two results can be highlighted: of the 41 conditional mutants isolated 9 were simultaneously fs, es and ts; and of 32 fs mutants isolated, 12 were exclusively fs, and the other 20 were also ts or es or both. These results indicate that: first, the number of mutations leading to a formamide-sensitive phenotype arose at a frequency similar to that of mutations leading to a ts phenotype or an es phenotype; second, mutations conferring a fs phenotype could lead simultaneously to a ts and/or es phenotype; and third, many mutations were specific for an fs, es or ts phenotype.

Genetic analysis of formamide-sensitive mutants: Twenty formamide-sensitive mutants (12 from strain AYW3-3B and 8 from AYW3-3A) were selected for further genetic studies. All of them were crossed with either strain AYW3-3A or AYW3-4B to determine the recessivity or dominance of the fs phenotype. In all cases, diploid strains were able to grow on YEPD-3% formamide like the wild-type haploid parental strains, indicating that all 20 mutations were recessive. That most of the mutations were monogenic was confirmed by a genetic analysis of 12 of the selected fs mutants (10 from strain AYW3-4B and 2 from strain AYW3-3A). Between 9 to 14 tetrads were dissected and subjected to genetic analysis. Nine of the 12 crosses analyzed showed the 2:2 segregation ex-

<p>| TABLE 1 |
| Classification of 41 conditional mutants isolated |</p>
<table>
<thead>
<tr>
<th>Conditional phenotype</th>
<th>AYW3-3A</th>
<th>AYW3-4B</th>
<th>Total mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>fs ts es</td>
<td>3</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>fs ts</td>
<td>2</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>fs es</td>
<td>0</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>fs</td>
<td>7</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>ts es</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ts</td>
<td>4</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>es</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>24</td>
<td>41</td>
</tr>
</tbody>
</table>

*fs: sensitivity to 3% formamide; ts: sensitivity to 37°C; es: sensitivity to 6% ethanol. Sensitivity is defined in this table as the inability to form colonies.*
cosegregate with the ts phenotype; the rest were only.

Also, in eight mutants derived from strain AYW3-3A were those cases in which the thermosensitive phenotype crossed with each of the 12 mutants derived from AYW3-4B. All combinations studied complemented the same mutation is responsible for the ts phenotype, which indicates that the same mutation is responsible for the ts and fs phenotypes.

To establish complementation groups, each of the eight mutants derived from strain AYW3-3A were crossed with each of the 12 mutants derived from AYW3-4B. All combinations studied complemented as shown by the fact that all 96 diploids obtained were able to grow on YEPD-3% formamide. Thus, as far as shown by the fact that all 96 diploids obtained were able to grow on YEPD-3% formamide, indicating that they were not conditional for formamide. One mutant, however, was unable to grow on SC-can without formamide. This mutant also showed a wild-type CanS phenotype when grown in the presence of 6% ethanol or at 37°C.

To determine if this mutation maps at the CAN1 locus, the mutant strain A3A-CAN2F was crossed with W303-3B carrying the nonconditional allele can1-100. Tetrad analysis of dissected spores showed that the formamide-conditional CanR mutation of strain A3A-CAN2F was linked to the CAN1 locus (Figure 1). A 4 CanR:0 CanS segregation was obtained on SC-can-2% formamide for the seven complete tetrads analyzed, whereas a 2 CanR:2 CanS was obtained on SC-can as expected for the can1-100 allele that is the only allele expressing the CanR phenotype on this latter medium.

Isolation of a can1 formamide sensitive allele: The results shown suggest that formamide could be used to isolate conditional mutations in essential genes. To confirm that formamide-sensitive alleles could be obtained from a wide range of genes, whether essential for growth or not, a procedure that identifies CanR mutants that express the canavanine resistance phenotype only in the presence of 2% formamide was carried out. Canavanine is an arginine analogue that is toxic to the cell. Mutations conferring a CanR phenotype map at the CAN1 locus, which is the structural gene for arginine permease, can be easily selected on SC-can media.

From the same mutagenesis mentioned above, 1.5 x 10^7 cells from strain AYW3-3A and 3.8 x 10^7 cells from AYW3-4B were plated on SC-can containing 2% formamide. A total of 641 CanR colonies was obtained (143 from AYW3-3A and 498 from AYW3-4B). Of these mutants, 640 were also CanR in SC-can without formamide, indicating that they were not conditional for formamide. One mutant, however, was CanR when the medium contained 2% formamide, but was unable to grow on SC-can without formamide. This mutant also showed a wild-type CanR phenotype when grown at 37°C. The wild-type parental strain used was either W303-1B or 5a-P3651.

TABLE 2

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Complete tetrads</th>
<th>fs segregationb</th>
<th>2 fs: 2 wt</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>AYW3-4B1f</td>
<td>11</td>
<td>11</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>AYW3-4B3f</td>
<td>9</td>
<td>9</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>AYW3-4B4</td>
<td>9</td>
<td>9</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>AYW3-4B5f</td>
<td>7</td>
<td>6</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>AYW3-4B6f</td>
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<td>8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>AYW3-4B10f</td>
<td>9</td>
<td>9</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>AYW3-4B12</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>AYW3-4B20</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>AYW3-4B21</td>
<td>7</td>
<td>6</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

The wild-type parental strain used was either W303-1B or 5a-P3651.

Tetrad analysis of dissected spores showed that the formamide-conditional CanR mutation of strain A3A-CAN2F was linked to the CAN1 locus (Figure 1). A 4 CanR:0 CanS segregation was obtained on SC-can-2% formamide for the seven complete tetrads analyzed, whereas a 2 CanR:2 CanS was obtained on SC-can as expected for the can1-100 allele that is the only allele expressing the CanR phenotype on this latter medium.

A thermosensitive cdc9 allele that is formamide sensitive: The large number of fs mutants obtained in this study that were simultaneously ts suggests that mutations initially isolated on the basis of a temperature-sensitive conditional phenotype may also be formamide-sensitive. To test this possibility, the sensitivity to formamide of 13 different mutant strains carrying 11 different thermosensitive mutations in 10 cell cycle genes was determined. The mutations were hpr6 (a CDC2 allele), cdc4, cdc5, cdc6, cdc8, cdc9, cdc13, cdc14, cdc17, hpr3 (a CDC17 allele) and cdc28; and all of them confer cell cycle arrest phenotype at 37°C. Only the two strains carrying the same cdc9 allele (original from L. Hartwell strain H9C1A1) were unable to grow on YEPD-3% formamide at 30°C. The fact that the cdc9 mutation itself led to the fs phenotype was confirmed by demonstrating that cdc9 strains showed the same cell-cycle arrest phenotype in liquid YEPD-2% formamide medium at 30°C and YEPD at 37°C (Figure 2).

DISCUSSION

In this work it has been shown that sensitivity to 3% v/v formamide can be used as a novel and comple-
mentary conditional phenotype for mutations on both essential and nonessential genes as suggested by the following results: (1) the number of formamide-sensitive lethal mutations affecting different genes that can be isolated from yeast cells is at least as large as that of thermosensitive or ethanol-sensitive lethal mutations; (2) these mutations either confer sensitivity to formamide, temperature and/or ethanol simultaneously or are exclusively formamide sensitive; and (3) those genes susceptible to formamide-sensitive mutations include the structural gene for DNA ligase, CDC9, and the structural gene for arginine permease, CAN1. This work also serves to confirm, together with a previous report (AGUILERA and BENITEZ 1986), that ethanol sensitivity can be used as a conditional phenotype to identify mutations in at least as many genes as those susceptible to temperature or formamide sensitive mutations.

The frequencies at which $fs$, $es$ and $ts$ mutants can be obtained by mutagenesis are very similar. The same overlap shown for $ts$ and $fs$ phenotypes exists for $fs$ and $es$ and for $ts$ and $es$ phenotypes. Nine of a total 32 $fs$ mutants obtained show sensitivity to temperature and ethanol, but 18 were not $ts$ and 17 were not $es$ (Table 1). This suggests that formamide, temperature and ethanol sensitivity share common molecular bases but that there are differences that give rise to proteins sensitive to only one agent. In this sense, BARTEL and VARSHAVSKY (1988) did not find a high degree of overlap between heavy water sensitivity and thermosensitivity. Also, it was previously shown (AGUILERA and BENITEZ 1986) that the lower the ethanol concentration $es$ mutants could resist, the less likely to be $ts$. Of 10 cdc9 alleles tested in this study, only one was sensitive to 2% formamide. It could be that either the stringency of conditions used lead to a bias in the type of allele or the type of genes that can become sensitive to a particular solvent or that lethal mutations classified with only one conditional phenotype such as $fs$, share other phenotypes such as $ts$ or $es$, but that at the conditions used (37°C or 6% ethanol) they are partly functional so that cells are viable.

The formamide sensitivity phenotype can be obtained for any type of gene independent of its function. A DNA-ligase allele, cdc9, initially isolated as $ts$ has been shown to be also $fs$, and a new can1 allele has been isolated which expresses the CanR phenotype only in the presence of 2% formamide. The fact that a protein involved in DNA metabolism and a protein involved in transport across the membrane can become formamide sensitive suggests that the sensitivity to formamide should be related to the structure of the protein itself more than to the type of function which is involved.

Many different types of noncovalent interactions (ion pairs, hydrogen bonds, van der Waals contacts, hydrophobic interactions) contribute to the stability of a protein (MATTHEW, WEAVER and KESTER 1974). Changes in charge, hydrophobicity, hydrogen bonding and folding of a protein will depend either on the nature of the amino acid changed or the structural context where that amino acid is located (i.e., an amino acid may be located in a substructure inaccessible to solvents). Formamide and ethanol contain chemical groups (H, O, OH, NH) that compete for noncovalent bonds with the amino acids of the protein so that the functional conformation of the protein can be disrupted. This, together with the fact that the denaturation properties of a number of chemical agents are different (GORDON and JENCKS 1963), can explain that some amino acid changes lead to proteins that are unstable at high-temperature, ethanol or formamide concentrations, whereas others lead to proteins that are unstable only under one or two of the conditions mentioned.

Finally, one conditional phenotype may not be enough to isolate mutations in all essential genes of an organism (KABACK et al. 1984). Mutations leading
to one conditional phenotype may not be distributed randomly among all genes. HARTWELL (1967) observed that not all biological processes are equally affected by ts mutation. Some macromolecular structures or biochemical reactions may be more sensitive to a particular agent than others. High order macromolecular structures or their associated biological processes (cytoskeleton, ribosomes, replication, transport, etc.), and nonproteic structures (membranes), could be specially sensitive to a particular agent. If this were the case the conditional phenotype would be caused by the lability of the entire structure (i.e., cryosensitivity of yeast cytoskeleton, MOIR and BOTSTEIN (1982) or biological process (i.e., cryosensitivity of prokaryotic protein export, POGLIANO and BECKWITH, 1993) and not by the lability of the mutated protein itself. The fact that the ts and es phenotypes correlate with the ts phenotype in a number of mutants suggests that the conditions used in this work affect the lability of the protein itself. However, it cannot be discarded that some macromolecular structures could be specially sensitive to a particular solvent.

In summary, experimental conditions can be established under which a particular amino acid change will make a protein susceptible to selective inactivation by a specific chemical agent, such as formamide or ethanol, to a degree that leads to expression of the mutant phenotype. The use of different conditional phenotypes should contribute greatly to the identification of conditional lethal mutations in most of the essential genes of an organism and to isolate novel conditional alleles of known genes. Formamide and temperature could be used to create a more stringent condition to identify conditional mutations with a profound phenotype. Finally it should open new perspectives to the isolation of conditional mutants in other organisms.

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