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Volume II

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FOREWORD

One of the major goals of the representatives from Upjohn and Labatt Brewing Company, Ltd. in planning this symposium* was to bring together, in one forum, industrial and academic scientists. It was concluded that both the topic and timing of the symposium were appropriate for disproving several "myths" surrounding the relationship between academic and industrial research. These myths range from industrial research being solely applied, to the widely held feeling that the focus of academic and industrial research differ significantly. The organizers of the symposium wished to address these misconceptions in several ways. First, it appeared that the topics of interest were common both to academic and industrial researchers, and that both the title and content of the symposium should reflect the attempt to bridge the usually presumed "academic-industrial gap" in research interests. Next, the participants and their contributions should reflect the full range of research interests, from studies of the basic mechanisms of yeast molecular biology to application of yeast expression technologies in industries as diverse as brewing, oil production and pharmaceuticals. Finally, it was concluded that the state-of-the-art was of interest, and not review-type papers, and the participants were requested to prepare their presentations accordingly.

The results of the experiment, at least as measured by the anecdotal observations of both participants and organizers, indicated that a successful experiment has been carried out in essentially all respects. International participants from both industrial and academic laboratories engaged in lively presentations and debate throughout the symposium, and new, unpublished research results were at the core of most presentations. Furthermore, research results presented as posters received equal weight to oral presentations and are included as full manuscripts in this current publication.

It is a great pleasure, therefore, to thank both the sponsors of the symposium, The Upjohn Company and Labatt Brewing Company, Ltd., and the participants for a stimulating and scientifically important symposium. It is our hope that the proceedings of this symposium will not only reflect the exciting synergism that occurred during the symposium, but will also contribute substantially to the scientific literature in this area.

Ralph E. Christoffersen, Ph.D.
Vice President
Biotechnology and Basic Research Support
The Upjohn Company

* Symposium on the Biochemistry and Molecular Biology of Industrial Yeasts, Brook Lodge, Kalamazoo, Mich., October 25 to 30, 1985, sponsored jointly the The Upjohn Company, Kalamazoo and Labatt Brewing Company, Ltd., London, Ontario, Canada.

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Dr. Stewart, as Chairman of the International Commission for Yeasts, organized the 5th International Symposium for Yeasts held in London, Ontario in July, 1980. He is presently Vice-Chairman of the International Commission for Yeasts and Secretary of the Biotechnology Commission of IUPAC. He is co-editor of the CRC Press journal, *Critical Reviews in Biotechnology,* is on the editorial board of the *Journal of Food and Microbiology* and of *Biotechnology Letters,* as well as being on the advisory board of *Microbiological Sciences.* In addition to editing a number of books, Dr. Stewart has published over 100 original papers, patents, and reviews.

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Chapter 1

DEVELOPMENT OF YEAST TRANSFORMATION SYSTEMS AND CONSTRUCTION OF METHANOL-UTILIZATION-DEFECTIVE MUTANTS OF *PICHIA PASTORI* BY GENE DISRUPTION

James M. Cregg and Knut R. Madden

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ABSTRACT

The methylotrophic yeast *Pichia pastoris* is being developed as a system for the expression of heterologous genes. Host/vector systems for DNA transformation which are based on auxotrophic mutants, as well as vectors which contain the complementing biosynthetic genes, have been developed. *P. pastoris* autonomous replication sequences have been isolated; therefore, both autonomously replicating and integrative vectors are available. Our experiences with *P. pastoris*, along with reports on other yeast systems, are critically reviewed to provide insights which might assist in the development of transformation systems in other yeasts.

The development of the *P. pastoris* system has been extended to include the use of site-specific gene disruption techniques. We describe the use of gene disruption to construct *P. pastoris* mutants which are defective in the expression of alcohol oxidase, the first enzyme in the methanol-utilization pathway. Examination of the mutant phenotypes revealed that *P. pastoris* has two functioning alcohol oxidase genes: a primary gene *(AOX1)*, whose product is the major methanol oxidase in methanol-grown cells; and a secondary gene *(AOX2)*, whose product may be expressed at lower levels or may be less enzymatically active on methanol.

I. INTRODUCTION

A. DNA Transfer in Yeasts

The ability to transfer and maintain desired DNA sequences in an organism is an essential step in establishing the function of these DNA sequences. Since the transformation of *Saccharomyces cerevisiae* was first described,[1] transformation has been reported for only a few additional yeast species. In addition to *S. cerevisiae* and other closely related species, such as *S. uvarum*,[2-4] the list includes: the yeasts, *Kluyveromyces lactis*,[5] *K. fragilis*,[6] and *Candida utilis*;[7] the *n*-alkane-utilizing yeasts, *C. maltosa* and *Pichia guilliermondii*;[8] the methanol-utilizing yeast, *P. pastoris*;[9] and the fission yeast, *Schizosaccharomyces pombe*.[10]

In developing a transformation system, the first and often most difficult step is the identification of a strong selectable marker, i.e., a gene which confers a phenotype upon cells, which thereby allows them to be identified and selectively grown in the presence of a vast majority of untransformed cells. Two types of selectable markers or, more accurately, host/selectable-marker systems are most often described for yeast transformations. The first type is called a positive or dominant marker system and frequently involves an antibiotic to which the yeast is sensitive, as well as a gene which confers resistance to the drug. The second type is a complementing marker system and usually involves a recessive auxotrophic mutant host and the biosynthetic gene which complements the host's defect. For initial transformation attempts, each of the various host/selectable-marker systems has advantages and disadvantages, which will be discussed.

Once a strong selection system has been established, the fate of transformed sequences in the cell can be considered. Plasmids which contain sequence homology with a yeast genome can recombine and integrate into the genome.[1,5,9,10] Alternatively, in most yeasts described to date, plasmids can be maintained as autonomous elements if an autonomous replication sequence (ARS) exists within the plasmid.[1,5-10] The isolation and characterization of ARS elements and the behavior of ARS-based plasmids in *P. pastoris* and other yeasts will be described.

The tendency of yeast vectors to integrate at sequences of homology shared by the vector and host genome has been exploited by what are referred to as site-specific gene disruption or gene replacement techniques. The techniques first employ recombinant DNA methods to restructure a cloned DNA fragment in vitro. This is followed by transformation of the recombinant molecule in order to take advantage of the homologous recombination system

of the host organism so that a portion of the native genome can be replaced with the altered recombinant fragment. The techniques were originally developed for experiments involving *Saccharomyces cerevisiae*.[11-14] Other than *S. cerevisiae*, examples of gene replacement have been reported for *Schizosaccharomyces pombe*[15] and the filamentous fungi, *Neurospora crassa*[16] and *Aspergillus nidulans*.[17] Even though the gene disruption methods have been applied to only a few fungal species, the relatively broad evolutionary spectrum of these species suggests that the methods will be of great value in the molecular genetic research of most, if not all, fungi.

The first application of a site-specific gene disruption method to the methyltrophic yeast, *P. pastoris*, is described in the second section of this report.

B. Methylotrophic Yeasts

The first report of a eukaryotic organism, a yeast, which could utilize methanol as the sole source of carbon and energy, did not appear until 1969.[18] This relatively recent discovery has been followed by extensive research, focusing primarily on the microbiological, physiological, and biochemical aspects of methanol metabolism in yeasts.[19-21] Only a few representatives in only four yeast genera (*Candida, Hansenula, Pichia,* and *Torulopsis*) are known to be capable of growth on methanol. The methanol-utilization pathway in yeasts is well defined. In the first step of the pathway, methanol is oxidized to formaldehyde, a reaction which is catalyzed by the enzyme alcohol oxidase (AOX). At this point, formaldehyde can be oxidized further to create formate and then carbon dioxide by a dissimilatory pathway which generates energy for the cell. Alternatively, formaldehyde can be assimilated to form cellular constituents by a pathway which starts with dihydroxyacetone synthase (DAS), an enzyme which catalyzes the condensation of formaldehyde with xylulose 5-monophosphate.

Methylotrophic yeasts are interesting to molecular biologists for at least three reasons. The first interest is based on the observation that many of the enzymes involved in the methanol-utilization pathway are present at higher levels in methanol-grown cells than in cells grown on other carbon sources. For example, in *P. pastoris*, AOX is not present in glucose-grown cells, but comprises as much as 30% of the total protein from methanol-grown cells.[22] Thus, one interest is to study the mechanisms by which this family of genes appears to be regulated by methanol. For *P. pastoris*, three of these methanol-regulated genes have been isolated, including an AOX gene (*AOX1*) and a DAS gene (*DAS*).[23] Recently, control sequences from *AOX1* and *DAS* have been identified and used to construct *Lac*Z fusion vectors.[24] Studies on *P. pastoris* cells have demonstrated that these two promoters are tightly regulated at the level of transcription and highly active in methanol-grown cells.[23,24]

The second interest in methylotrophic yeasts is based on the discovery that several of the C_1-pathway enzymes, such as AOX, catalase, amine oxidase, and DAS, are sequestered in a subcellular organelle termed the microbody, or peroxisome.[19-21,25-27] In cells grown on methanol, AOX and DAS are synthesized in great quantities. In these cells, the "microbodies" become enormous, occupying up to 80% of the cell volume.[20] Sequence information from the *P. pastoris* AOX protein and its gene indicate that, unlike many other compartmentalized enzymes, packaging of AOX does not involve a processed amino terminal signal sequence.[23] Thus, it would be most interesting to understand the mechanism by which the cell compartmentalizes such enzymes in peroxisomes. Highly expressed enzymes such as AOX are ideal for such studies. Of course, the possibility of employing the peroxisomal packaging process as a method for avoiding the potential toxic effects of high internal concentrations of foreign products in eukaryotic hosts is attractive.

A third interest in methylotrophic yeasts is in the application of these cells as hosts for the expression of heterologous proteins. Strains of *P. pastoris* have been selected for growth at high cell density. Concentrations of as much as 130 g (dry cell weight) per liter can be

harvested in a continuous fermentation process.[28] Thus, the utilization of methanol-regulated promoters in conjunction with the fermentation technology developed for this yeast is attractive as a production system for the synthesis of valuable foreign proteins.

II. DEVELOPMENT OF YEAST TRANSFORMATION SYSTEMS

A. The Selectable Marker

1. Dominant Marker Systems

For transformation of *Saccharomyces cerevisiae* and other yeasts which are closely related to *S. cerevisiae*, the successful use of several dominant markers has been reported. The list of markers includes the following genes from *Escherichia coli:* the aminoglycoside phosphotransferase gene from the transposable element Tn601 (Tn903), which confers resistance to the eukaryotic antibiotic G418;[29-31] the hygromycin B phosphotransferase gene, which confers resistance to hygromycin B;[32] the chloramphenicol acetyltransferase gene, which confers resistance to chloramphenicol;[33,34] and the dihydrofolate reductase gene encoded on the plasmid R388, which confers resistance to sulfanilamide and methotrexate.[35] The list of dominant-selection genes from *S. cerevisiae* includes: the copper-chelatin-encoding gene, which confers resistance to copper;[36] the UDP-*N*-acetylglucosamine-1-P transferase and 3-hydroxy-3-methylglutanyl-CoA reductase genes, which confer resistance to tunicamycin and compactin, respectively, when present on multicopy plasmids;[37] and the acetolactate synthase gene (*ILV2*), which confers resistance to sulfonylurea herbicides.[38]

At present, only two reports exist which describe the successful use of dominant markers in initial transformation of a yeast species not belonging to the *Saccharomyces* genus. Ho et al.[7] transformed a strain of *Candida utilis* with a plasmid which contained an aminoglycoside phosphotransferase gene by direct selection for resistance to G418. However, it is difficult to access this dominant selection system, since the authors did not report either the transformation procedure which they employed or their results with regard to transformation frequencies, the state of the plasmid sequences in cells (integrated or autonomous), or the mitotic stability of the plasmid in cells grown under selective or nonselective conditions.

The second report of direct selection with a dominant marker in a non-*Saccharomyces* yeast was by Das and Hollenberg,[5] and involved transformation of *K. lactis* by direct selection for resistance to G418. Their initial plasmid PTY75-*LAC4* was about 25 kb in length and contained the following DNA fragments of interest: the entire 2-μm plasmid; the *K. lactis* β-galactosidase gene (*LAC4*); and the phosphotransferase gene from Tn601. A *lac4 K. lactis* strain was employed, which allowed the authors to distinguish spontaneous G418-resistant mutants from true transformants. They found that, on the average, only 5% of the G418-resistant colonies were Lac^+ and that the frequency of true transformants was about 4/μg of plasmid. PTY75-*LAC4* was reported to be autonomous in the *K. lactis* cells. The low transformation frequency appeared to be due, at least in part, to the low frequency of spheroplast regeneration. The authors then described the subsequent development of two complementation-type selective markers based on the *LAC4* and *S. cerevisiae TRP1* genes. These two latter selection systems did not have the problem of spontaneously resistant mutants, as did the G418 resistance-selection system.

Initial attempts to transform *P. pastoris* with vectors containing one of the aminoglycoside phosphotransferase genes from either Tn5 or Tn601 by selection for resistance to G418 were unsuccessful.[39] After establishing a *P. pastoris* transformation system based on a complementing marker system, transformation with some of these vectors was reexamined. We discovered that expression from the bacterial sequences of the phosphotransferase gene from Tn601 can be sufficient for direct selection of G418-resistant transformants, if the gene is contained on a multicopy plasmid. However, the 2-μm plasmid ARS which was present on the intitial plasmids does not maintain plasmids as autonomous elements in *P. pastoris*.[9]

Initial attempts to transform *P. pastoris* with a hygromycin B phosphotransferase gene-containing plasmid were unsuccessful as well.[39] The plasmid used, pLG89, contained the hygromycin B resistance gene under the transcriptional control of the *S. cerevisiae CYC1* promoter and terminator.[32] The plasmid also contains the 2-μm circle ARS and transformed *S. cerevisiae* by direct selection for resistance to hygromycin B. As with the G418 resistance plasmids, the inability of the 2-μm ARS to function in *P. pastoris* could be the cause of our failure to obtain hygromycin B-resistant transformants. However, recent studies in which expression of other *S. cerevisiae* promoter fragments has been examined in *P. pastoris* and vice versa have shown that, between these two species of yeasts, promoter fragments are often not expressed as well in the heterologous host.[24,39-41] Thus, poor expression of the *S. cerevisiae CYC1* promoter in *P. pastoris* may have contributed to the problem as well. In summary, with positive selection genes for intitial transformations of *P. pastoris*, we found ourselves in a situation in which promoters, transcription terminators, and/or autonomous replication sequences were required to express a drug resistance gene sufficiently, yet a transformation system was required to identify such sequences.

A recently described and promising dominant selection scheme for transformation of non-*Saccharomyces* yeasts involves the use of sulfonylurea herbicides like sulfometuron methyl (SM). In many pro- and eukaryotic organisms, this drug inhibits acetolactate synthase, an enzyme required for the synthesis of isoleucine and valine.[38,42] Falco and Dumas[38] have isolated the *S. cerevisiae* acetolactate synthase gene (*ILV2*) from mutant strains in which resistance to the drug is dominant. They suggest that one might be able to transform many fungal hosts by using SM to induce an auxotrophic-like state in the host and then transforming the cells to "prototrophy" with a vector which contains a dominant SM-resistant *ILV2* gene. This dominant selection scheme may be superior to others, since low expression levels of this *ILV2* gene in a heterologous host may be sufficient to relieve the auxotrophic-like sensitive state induced by the drug.

2. Complementing Marker Systems

The most frequently described type of host/selectable-marker system for fungi involves an auxotrophic host and a complementing biosynthetic gene. The development of this type of marker system has two separate steps. The first step is the identification of an auxotrophic mutant with a specific enzymatic defect. The use of one of the many mutant selection or enrichment techniques which have been developed for *S. cerevisiae* may facilitate this process. The second step is the obtainment of a complementing biosynthetic gene. If the *S. cerevisiae* gene is not available, then the isolation of the gene from either *S. cerevisiae* or other yeasts by complementation of an appropriate *S. cerevisiae* mutant should provide the desired gene.

The first step, the identification of an auxotroph with a known enzymatic defect, may be troublesome in a yeast whose sexual cycle has not been characterized and for which ploidy, i.e., haploid, aneuploid, diploid, or polyploid, has not been established. The determination of ploidy for a genetically uncharacterized yeast is not simple. (See Beckerich et al.[54] for a discussion of the problems and considerations involved in analyzing yeast sexual cycles and in determining ploidy.) Since auxotrophic mutations are almost always recessive and, therefore, phenotypically undetectable when heterozygous, one should ideally begin the mutant isolation process with a yeast strain which is haploid. For many yeasts which have been characterized genetically, the sexual cycle does appear to contain a reasonably stable haploid phase.[54] Therefore, if a yeast is not haploid or if its ploidy is in question, the sporulation of the yeast might be attempted as a means of obtaining a derivative of the yeast which is less likely to be diploid or polyploid.

To isolate specific types of auxotrophic mutants of *S. cerevisiae*, a number of positive mutant-selection and enrichment schemes have been described.[49-52] One of these methods

may produce the desired mutant host, particularly in yeasts which are closely related to *S. cerevisiae*. However, in yeasts less closely related to *S. cerevisiae*, many of these techniques may not yield the desired mutant. For example, we found that *P. pastoris* was not sensitive to ureidosuccinic acid, a drug which has been used to select pyrimidine auxotrophs of *S. cerevisiae*.[49] As another example, we found that *P. pastoris* was quite sensitive to α-aminoadipic acid, a drug which has been used in *S. cerevisiae* to select mutants in the *LYS2* and *LYS5* genes.[50] However, despite several attempts, we could not generate an α-aminoadipic acid-resistant mutant of *P. pastoris*, with or without prior mutagenesis. In the same experiments, the frequency of lysine-requiring *S. cerevisiae* mutants resulting from the selection procedure was high, even without mutagenesis.

Recently, a promising mutant-selection procedure for yeasts was described by Boeke et al.[52] Mutants of *S. cerevisiae* which were resistant to 5-fluoroorotc acid (5-FOA) were often defective in their orotidine-P decarboxylase or *URA3* gene product. In addition, the authors found that wild-type Ura^+ cells of both *Schizosaccharomyces pombe* and *E. coli* were sensitive to 5-FOA, while mutants of these same organisms which were defective in orotidine-P decarboxylase were not. Thus, this mutant selection procedure may be applicable to many organisms. Since the *Saccharomyces cerevisiae URA3* gene is well characterized and readily available,[53] the isolation of a *ura3*-equivalent mutant in an organism could result in the rapid establishment of an excellent auxotrophic host/biosynthetic gene-selection system.

For many non-*Saccharomyces* yeasts, it may be necessary to isolate auxotrophs without the aid of a selection technique and later identify the specific enzymatic defect in one of the mutants. A mutant which is defective in a commonly employed *S. cerevisiae* marker gene would be most convenient, since the appropriate *S. cerevisiae* gene should complement the defect in most yeast species (see discussion on cross-species complementations below). However, in some yeasts, the isolation of a spcific type of auxotroph may be difficult or impossible. Extensive mutagenesis of *P. pastoris* resulted in auxotrophic mutants which were affected in only a few amino acid biosynthetic pathways. None of these auxotrophs were defective in biosynthetic pathways whose genes included those commonly employed as selectable markers in *S. cerevisiae* (e.g., the leucine pathway, *LEU2;* the aromatic pathway, *TRP1*).[39] In addition, biochemical analysis of a collection of 20 histidine-requiring mutants of *P. pastoris* indicated that the mutants represented defects in only 2 of the 7 genes likely to be involved in the histidine biosynthetic pathway. This bias in the types of auxotrophs, along with the difficulty in selecting mutants which are defective in specific genes, such as *LYS2*, suggests that the *P. pastoris* strain which we mutagenized may be aneuploid. It was fortunate that one of the mutants was defective in histidinol dehydrogenase, which is encoded by the well-characterized *HIS4* gene of *S. cerevisiae*.[55,56]

Once a mutant strain with a known biosynthetic defect has been isolated and characterized, the second step in establishing a complementation-type selection system is to obtain a complementing biosynthetic gene. A large number of reports exist in which heterologous biosynthetic genes have complemented the defect in foreign yeast hosts. Cross-species complementation has two practical advantages with regard to obtaining a biosynthetic gene. The first is that the appropriate *S. cerevisiae* gene, if readily avialable, will probably complement the defect in most foreign yeast hosts. Several examples of cross-species complementations involving *S. cerevisiae* genes and heterologous yeasts have been reported.[5,6,8-10] The second advantage is that if the *S. cerevisiae* gene is not available, the isolation of the biosynthetic gene from the heterologous host by complementation of an *S. cerevisiae* mutant is, in most cases, likely to be successful. As examples, the *P. pastoris ARG4* and *HIS4*,[9] the *C. albicans URA3*,[44] the *Dictyostelium discoidium URA3* and *URA5*,[45] and the *Drosophila ADE8*[46] gene equivalents were all isolated from DNA libraries by transformation of an appropriate mutant *S. cerevisiae* host. In the past, complementation of *E. coli* auxotrophic hosts has been employed to isolate fungal biosynthetic genes with sporadic success.[47,48] Our