21.1. INTRODUCTION

Heterologous expression of biopharmaceuticals and enzymes is common, but increasingly, metabolic engineering of biosynthetic or biodegradative pathways requires the balanced expression of multiple proteins under complex regulatory conditions. The transformation and manipulation of well-studied hosts such as Escherichia coli or Saccharomyces cerevisiae draw on many genetic tools and strains and a plethora of research into regulatory responses and protein functions. These data on familiar workhorses, while essential, represent only a small fraction of the biochemistry and physiology present in the microbial realm. Moreover, with the advent of whole-genome sequencing, it is becoming possible to obtain particular advantages by using different microbial systems for specialized applications.

21.1.1. Yeasts as Expression Hosts

As unicellular organisms, yeasts are relatively easy to isolate, scale up, and manipulate genetically. They can achieve high growth rates. Their thick cell walls make them more resistant to osmotic shock. Because they are larger than bacteria, they are easier to harvest by filtration or centrifugation. They will grow at an acidic pH, which discourages the growth of most contaminants. They are not susceptible to most viral infections, and they lack lipopolysaccharide endotoxins. They generally have minimal nutritional requirements and can be cultivated at very high cell density. Cell and protein yields are often high. Specifically with respect to heterologous proteins for therapeutic use, yeasts have an advantage over bacteria in their capacity to produce N-glycosylated proteins with disulfide linkages (42).

Some of the physiological features of yeasts cause difficulties. Their thick cell walls can make release of intracellular proteins more difficult, and their natural glycosylation systems differ from those of mammals. However, even these problems have been addressed with yeasts that secrete heterologous proteins and by the genetic development of strains having humanized glycosylation patterns. Within the dikaryotic Ascomycetes to which the budding yeasts belong, at least 20 species of yeasts have been examined for their industrial properties. Several have been used for heterologous protein expression or metabolically engineered for novel properties. S. cerevisiae is the best studied of the ascomycetous budding yeasts. Genetically it is the best understood eukaryote, which makes it very easy to manipulate. It is not as useful for heterologous protein production because it lacks respiration complex 1 and hence exhibits relatively low ATP and cell yields. It has relatively limited capacity to metabolize carbon sources other than glucose and sucrose. It tends to hyperglycosylate proteins, and it does not possess a strong secretion pathway, so proteins larger than about 30 kDa occur as intracellular or periplasmic products. Researchers and industrial processes have drawn on the features of nonconventional yeasts to overcome these problems.

21.1.2. Nonconventional Yeasts

Nonconventional yeasts can be roughly defined as any yeast other than S. cerevisiae. A number are used in industrial settings. If one says “yeast” without further clarification, S. cerevisiae is assumed. The realm of “yeasts,” however, is far more diverse. Closely related to S. cerevisiae are Kluyveromyces marxianus and Kluyveromyces lactis. Their expression vectors, selectable markers, and transformation systems are similar to those of S. cerevisiae. Nonconventional yeasts such as Pichia pastoris, Hansenula polymorpha, Arxula adeninivorans, and Yarrowia lipolytica cannot readily employ 2µm plasmid-based vectors and generally use selectable markers and autonomous replication sequences derived from autologous sources. These yeasts have found wide application in heterologous expression because they either produce very high levels of heterologous proteins, secrete proteins, or exhibit more versatile substrate utilization. The popularity of methylotrophic yeasts and particularly P. pastoris and H. polymorpha for heterologous protein production stems from the establishment of their Generally Recognized As Safe status, their capacity for cultivation at very high cell densities, and the existence of effective systems for transformation and expression.

Nonconventional yeasts are notable for their use of diverse carbon sources, physiological properties, and unusual biochemical pathways. Utilization of lactose and galactose is particularly important in the fermentation of whey (64), and xylose and cellobiose fermentations are important in the bioconversion of lignocellulosics (86, 141a). However, utilization of alkanes (117, 121) and particularly methanol (35) has proven significant because protein products can accumulate to high levels in these yeasts. Thermotolerance is also a valued trait that can suppress contamination.
or increase growth rates. A. adeninivorans will grow at temperatures up to 48°C (174), and K. marxianus, which will ferment cellobiose, will grow at temperatures up to 45°C, making it potentially useful for simultaneous saccharification and fermentation. Debaryomyces Hansenii shows unusual osmotolerance (129). Production of extracellular enzymes such as lipase (14, 135, 153), amylase (23, 40, 100, 154), and endoglucanases (123, 124) is facilitated in yeasts with effective secretion systems. Finally, nonconventional yeasts are particularly useful in the production of glycerol (27), lactic acid (131), citric acid (73, 99), or carotenoids such as astaxanthin (111, 113, 164, 185). The capacities of nonconventional yeasts to produce useful products other than ethanol or to produce ethanol from substrates other than glucose, sucrose, and fructose have led to efforts that focus on their metabolic engineering for improved product formation.

Several researchers have previously reviewed the general transformation and expression techniques for non-Saccharomyces yeasts (15, 20, 37, 60, 61, 108, 127, 171), so our emphasis here will be on more generalized features of expression systems in nonconventional yeast systems.

21.2. ELEMENTS OF TRANSFORMATION VECTORS

All yeast transformation and expression systems require ways to introduce engineered DNA into a cell, enable it to survive, assist its propagation, and promote its transcription. The most essential requirements are a selectable marker and a functional promoter. With these two elements alone, it is possible to introduce modified genes and effect transformations of the DNA. The efficiencies of such simple systems, however, are very low. Linearized DNA is susceptible to degradation by endo- and exonucleases, and the DNA cannot be transcribed until it has become integrated into the genome, which can occur only after the DNA enters the nucleus. To increase the probability of survival, replication, and expression, autonomous replication sequences (ARSs) are often incorporated into the transformation vector. In the case of S. cerevisiae and related genera, these are derived from the origin of replication (Ori) sequences of native 2µm plasmids that are found in Saccharomyces and Kluyveromyces species. While the 2µm origin of replication will function in many yeasts, it is often inefficient or poorly stable in yeasts outside of the Saccharomyces-Kluyveromyces clade. Occasionally an ARS identified through functional studies in S. cerevisiae can be used in another yeast (137); however, the efficiency of transformation is generally low. It is often necessary to isolate an ARS from the species of interest.

21.3. SELECTABLE MARKERS

A selectable marker enables the isolation of cells that have taken up the exogenous DNA. They can be based on complementation of genetic lesions that create nutritional deficiencies (auxotrophies) or on genes that enable acquired resistance to otherwise toxic medium constituents. The latter are termed dominant selectable markers. These are preferred since they can be employed with commercial yeast strains that are diploid or lacking auxotrophic deficiencies. Drug resistance markers are often used, but they should not be left in the final construct for commercial strains. This is especially true of yeasts that are used in producing food-grade products.

21.3.1. Auxotrophic Markers

By far the most common auxotrophic selectable marker employed is URA3 (13, 39, 52, 91, 120, 139, 184). Its popularity is attributable to the relative ease of obtaining ura mutations by selection for resistance to the orotic acid homolog 5'-fluoroorotic acid (5'-FOA). Also, if URA3 carried on an episomal vector is used to complement a ura3 mutation, it is very easy to cure the host by cultivation on medium containing 5'-FOA (13). Cells with a complete pathway for uracil biosynthesis take up the 5'-FOA antimetabolite and incorporate it into their RNA. The 5'-fluoro group causes mispairing, and cells prototrophic for uracil biosynthesis are killed. 5'-FOA selection plates also contain uracil or uridine at a concentration of 50 to 100 µg/ml. If a cell is deficient in either orotidine-5'-phosphate decarboxylase (URA3, pyrG) or orotate phosphoribosyltransferase (URA5) (39, 120), it will not take up the 5'-FOA, and the mutant survives by using the uracil or uridine provided in the medium. The ura3 mutant must be homozygotic for the trait in order to survive in the presence of 5'-FOA, so it is sometimes necessary to sporulate the culture prior to selection. Also, residual orotidine-5'-phosphate decarboxylase in the cell cytoplasm can be sufficient to incorporate 5'-FOA, so it is useful to cultivate sporulated or mutated cells on medium containing uracil for a few generations prior to selection on 5'-FOA plates.

Other popular auxotrophic markers include the multifunctional HIS4 gene (109, 119), the LEU2 gene for 3-isopropylmalate dehydrogenase (16, 41, 71a, 112, 142, 175), the TRP1 gene for phosphoribosylanthranilate isomerase (28, 154, 155), and the ADE1 gene for N-succinyl-5-aminoimidazole-4-carboxamide ribotide (SAICAR) synthetase (24, 71a, 109). Recipient hosts can be obtained either through random mutagenesis and screening, which is not recommended for industrial strains since it can result in other unwanted changes, or by targeted deletion using transposable elements. URA3, or some other selectable marker. Auxotrophs in general and Leu2 and Ade1 auxotrophs in particular often grow poorly—even when their deficiencies are compensated with nutritional supplements—and are not highly suitable for industrial applications. Backcrossing the mutated cells against a parental strain and subsequent screening and selection for the desired auxotrophic marker are recommended.

21.3.2. Drug Resistance Markers

Drug resistance genes are widely used as dominant selectable markers to transform yeasts, other fungi, and bacteria. Their extreme utility means that this practice will continue, but possible proliferation of drug resistance into human, plant, and animal pathogens requires good practices in biosafety containment. Where possible—and certainly when modified organisms are to be released into nature—dominant resistance markers that do not use or that compromise clinically important drugs should be employed. Alternatively, once the genetic modification is completed, the marker should be excised (see below).

To be effective for transformation, the target yeast must be susceptible to the antibiotic, and the protein must confer resistance even when expressed at a low level. Both of these requirements can present problems when developing transformation systems with nonconventional yeasts. Non-Saccharomyces yeasts often exhibit high levels of drug resistance. This means that high concentrations need to be used in the selection plate in order to kill the cells, and the specialized antibodies used for yeasts are often expensive.
Second, many of the drug resistance markers are derived from bacterial proteins, and expression of functional proteins occurs at only a low level in many yeasts. A significant fraction of yeasts belonging to the genus Candida, along with Debaryomyces Hansenii, Clavispora Lusitaniae, Lodderomyces elongisporis, Candida rugosa, and others, use CUG (CTG in DNA) to code for serine rather than leucine (51, 158). While the CUG codon is infrequently used by these species, bacteria use it regularly. The substitution of serine for a leucine can disrupt protein structure, so the CUG yeasts do not express the bacterial drug resistance markers properly. To get around this problem, correct expression of bacterial drug resistance markers in CUG yeasts requires changing CUGs to some other codon for leucine (101,102,161) or synthesizing the gene with optimum codon usage (see below).

When employing a drug resistance marker for transformation, it is necessary to add a short recovery incubation period (ca. 4 h) in complete medium without the antibiotic to allow expression of the drug resistance protein before exposing the cells to the selective agent.

The two most widely used resistance markers are genes that code for aminoglycoside-3-phosphotransferase, which confers resistance to Geneticin (G418) or kanamycin. The kan1 marker is widely available (53, 67, 145, 171). To inhibit yeast growth, Geneticin is typically used at a concentration of about 50 µg/ml in the final medium. It is soluble in water at 50 mg/ml and stable at room temperature. A 1-µg/ml solution in 0.1 M potassium phosphate buffer (pH 8.0) should be sterilized by filtration, stored at 2 to 8°C, and used within 30 days. Kanamycin is likewise used at about 50 µg/ml. It is much less stable than Geneticin and even less stable than penicillin at 25 to 37°C (50).

The gene for hygromycin phosphotransferase, hph (66, 187), confers resistance to hygromycin B. It is widely used as a selectable marker for yeasts and filamentous fungi (3, 7, 21, 68, 137, 152, 165, 186). At a concentration of 0.38 mM, hygromycin completely halts growth of eukaryotic cells even in rich medium by blocking chain elongation in ribosomes (65). Typical concentrations used to inhibit yeasts and fungi are about 0.2 to 1.0 mg/ml. Hygromycin B is stable in solution for about 2 years at 4°C and about 1 month at 37°C (77).

Some yeasts and fungi are notably resistant to gentamicin and hygromycin, so other drugs must be used. In recent years, Zeocin has proven to be very useful as an inhibitory antibiotic for yeasts and other organisms (1, 4, 102). Zeocin effectively inhibits growth at a concentration of 25 to 100 µg/ml. It is stable in a dry powder form for up to a year at 0°C, but in solution, it is light sensitive. Plates containing Zeocin can be stored for several weeks at 4°C in the dark. The mechanism of action of Zeocin is not known, but it is structurally similar to bleomycin and pheleomycin, which complex with Fe2+ to cleave DNA (18). The resistance factor ble codes for a protein that binds bleomycin, thereby eliminating its DNA cleavage activity (46). ble genes have been described from a number of bacterial sources (85). In addition, the bacterial gene for chloramphenicol acetyltransferase (cat) can impart resistance to pheleomycin when it is expressed properly in yeasts (31). It also has been widely used to transform various yeasts (85).

Most eukaryotic cells are sensitive to inhibition by the glutarimide antibiotic cycloheximide, but resistance to it is widespread among yeasts (156). Cycloheximide binds to the 80S ribosomal subunit, thereby inhibiting translation. The resistance mechanism is attributable to a specific amino acid difference in ribosomal protein L41. Yeasts that are susceptible to cycloheximide can be transformed with a mutated form of L41 to confer resistance (92).

### 21.3.3. Dominant Non-Drug Resistance Markers

With the spread of antibiotic resistance among clinical isolates, concern is rising over the use of drug resistance markers to transform medically important human, animal, or plant pathogens. One useful selectable marker that does not use drug resistance is the IMH3 gene for inosine monophosphate (IMP) dehydrogenase, which confers resistance to mycophenolic acid (MPA). IMP dehydrogenase catalyzes an NAD-dependent rate-limiting step in the biosynthesis of guanine nucleotides. Growth of Candida albicans is inhibited by 1 µg/ml of MPA, which is a specific inhibitor of the IMH1 form of IMP dehydrogenase. However, transformants overexpressing a different form of IMP dehydrogenase, the C. albicans IMH3 gene, will resist up to 40 µg/ml of MPA (11, 88, 95). The bioavailable form of MPA, mycophenolate mofetil, has a number of clinical applications, mainly as an immune suppressant for transplantation, but it is not widely used as an antimicrobial. The CalMH3 gene has been used successfully as a dominant selectable marker in Candida parapsilosis and C. albicans (11,44, 55).

Sulfite resistance has long been studied in yeasts since it is important in wine making. Two genes for sulfite resistance from S. cerevisiae, the transcription activator FZFI1-4 (RSU1-4) and to a lesser extent its regulated target, SSU1, have been used as dominant selectable markers in sulfite-susceptible strains (125). The latter is a plasma membrane sulfite pump, which enables cells to survive in the presence of sulfite.

### 21.4. EXCISION ELEMENTS

Most nonconventional yeasts have only a few selectable markers for genetic transformation. It is therefore very useful to be able to recover them for reuse. URA3 is almost unique in exhibiting a bidirectional selection. It is possible to identify URA3 transformants by selecting for growth on uracil (Ura) medium, and it is possible to select for URA3 by plating on 5-FOA. Normally the reverse selection is employed when the URA3 gene is carried on an autonomous plasmid. However, it is also possible to design URA3 and other selectable markers so that they will excise readily. Direct flanking repeat sequences on either side of the selectable marker will occasionally (ca. 10–4) recombine to excise the intervening sequence (169). For example, short (40-bp) flanking direct repeat sequences from A phage promoted the spontaneous excision of URA3 after it was used to disrupt LEU2 (112). The excised URA3 mutants were recovered on 5-FOA plates. In the case of selectable markers that do not have such sensitive means for identifying spontaneous excision reversions, two recombinase proteins can greatly enhance the frequency.

The Cre recombinase from bacteriophage P1 has been shown to excise intervening DNA that is flanked by direct repeats of its cognate loxP sequence. The efficiency of this excision approaches 70%, making identification of the resulting marker-deficient strains relatively easy (67). In a similar manner, the Flp recombinase, encoded by the 2µm

- **URA3**: Selectable marker for Ura+ phenotype, commonly used in yeast transformation.

- **HGPRT**: Selectable marker for hypoxanthine-guanine phosphoribosyltransferase, confers resistance to 6-thioguanine.

- **Nourseothricin (G418)**: Selectable marker for resistance to Geneticin, commonly used for yeast transformation.

- **Zeocin**: Selectable marker for resistance to zeocin, widely used in yeast transformation.

- **Hygromycin B**: Selectable marker for resistance to hygromycin B, commonly used for yeast transformation.

- **Cycloheximide**: Selectable marker for resistance to cycloheximide, commonly used in yeast transformation.

- **IMH3**: Selectable marker for resistance to mycophenolic acid, commonly used in yeast transformation.

- **RSU1-4**: Selectable marker for resistance to sulfite, commonly used in yeast transformation.

- **Flp recombinase**: Protein involved in recombination that can excise DNA flanked by loxP sites.

- **Cre recombinase**: Protein involved in recombination that can excise DNA flanked by loxP sites.
plasmid of *S. cerevisiae*, specifically recognizes 13-bp repeat elements found in the 2μm circle. When these are present as flanking direct repeats, the intervening sequence is excised (6). When these or the loxP sequences are in opposite orientations, the intervening sequence is inverted.

### 21.5. PROMOTERS

In the past, promoter selection for heterologous expression has been based on experience with a relatively limited set of genes. In recent years, however, as we have gained powerful tools for large-scale quantification of promoter expression profiles, selection has become tuned to specific applications. When faced with developing promoters for a new yeast system, little is likely to be known about promoter properties since the strength and expression profiles can vary widely from one organism to another. Genome-wide expression arrays provide the most comprehensive way to identify useful promoters. The induction patterns for *S. cerevisiae* genes can be obtained from the Saccharomyces genome database (http://www.yeastgenome.org/).

**ADH1** has been employed for strong heterologous expression in *S. cerevisiae*, and the **ADH2** promoter has been used for glucose-repressible expression. However, few other yeasts exhibit such high levels of fermentation. The galactokinase (**Gal1** or the bifunctional UDP-glucose-4-epimerase (Galactowaldensase), aldose-1-epimerase (Mutarotase) (**GAL10**) promoter can be used for regulated (glucose-repressed/galactose-induced) expression. Both of these are widely distributed among yeasts. The **TDH3** (GAP) promoter is often used for strong constitutive expression (25, 34, 71, 74, 81, 104, 146). Triose phosphate dehydrogenase has three isoforms in *S. cerevisiae* and between one and three isoforms in other yeasts. This is also true in *Pichia stipitis* (87). Alternatively, the translation elongation factor (**TEF1**) promoter (2, 15, 161) or the histone H4 (**AHSB4**) promoter has been used for strong constitutive expression (173).

In *P. pastoris* and *H. polymorpha*, promoters that are responsive to the presence of methanol are used. Even though these yeasts are frequently employed for heterologous protein production, only a limited set of promoters is in common use (146). The **AOX1** promoter for alcohol oxidase is most often employed. It is very strong, tightly controlled through glucose repression, and highly induced upon a shift to methanol as a carbon source (38). The promoter for formaldehyde dehydrogenase, **FDL1**, is sometimes used as an alternative to **AOX1** (147). Occasionally more precise expression levels are required for optimal protein production. For this purpose, a library of *P. pastoris* promoters has been characterized (69). The use of a constitutive promoter such as **PMAI** for plasma membrane H⁺-ATPase can avoid the fire hazard inherent in the use of methanol as a carbon source at large-scale use (35).

### 21.6. REPORTER GENES

Once a promoter is isolated, it is necessary to determine its relative strength. This is most conveniently done through the use of reporter genes. β-D-Galactosidase (**lacZ**) is commonly used due to the availability of a rapid screen (22, 136). Some yeasts, however, such as *P. stipitis*, *D. hansenii*, *Pichia guilliermondii*, *K. marxianus*, and *K. lactis* possess β-D-galactosidase that can interfere with heterologous expression assays. Also, CUG yeasts will not translate **lacZ** properly. For example, *E. coli lacZ* contains 54 CUG codons.

Even more popular than **lacZ** is green fluorescent protein (**GFP**). While the quantitative analysis of expression requires a measurement of fluorescence, this marker is particularly useful when expression occurs only in a fraction of the cell population, during a certain phase of the cell cycle, or in a region of the cell. The native GFP does not express well in yeast, and especially in CUG yeasts; however, a codon-optimized form of GFP, in which all of the CUGs have been replaced with TTG to specify leucine, expresses well in both *C. albicans* and *S. cerevisiae* (33).

### 21.7. INTEGRATED EXPRESSION

Integrated expression (expression of genes after integration into the chromosome) is critical for stable industrial production strains. Even with integrated expression, mutations that enable more rapid growth of the host to the detriment of product formation can proliferate. The use of episomal vectors can result in strain instability and inconsistencies from batch to batch (61).

Integration can be random or targeted to a particular site. Targeted integration is generally preferred since this yields better-defined transformants with potentially higher productivity. Regulatory certification is easier to obtain if the integration site is defined, and by knocking out genes that can form by-products, cell yields are increased.

Integration of exogenous DNA requires double-strand break (DSB) repair, which can occur either through homologous recombination (**HR**) or through nonhomologous end joining (**NHEJ**). The former is mediated by Rad52-related proteins (97) and the latter by Ku70/Ku80, Lig4, and a number of other gene products (5, 46, 143). **HR** requires relatively long regions of homology to repair a DSB, whereas **NHEJ** requires little or no homology to join two broken strands (Fig. 1).

**HR** predominates in *S. cerevisiae*, but in *P. stipitis*, *K. lactis* (97), fungi such as *Aspergillus* (160) and *Neurospora* (122) spp., and higher organisms (78), the frequency of **NHEJ** can be 1 to 4 orders of magnitude higher than targeted integration by **HR**. Mutating the **KU80** or **KU70** genes in *K. lactis* (97), *P. stipitis* (94, 114), and other fungi (122) greatly increases the proportion of targeted disruption by **HR**; however, the overall number of recovered transformants drops by more than an order of magnitude. Even though there are fewer total transformants, recovery of site-specific integrants is much easier because they represent 80 to 97% of the recovered mutants (Fig. 2A).

**FIGURE 1** Homologous recombination and nonhomologous end-joining mechanisms for recombination.

- **HR**
- **NHEJ**
- DNA synthesis
- 5' - 3'
- 3' - 5'
- FIGURE 1 Homologous recombination and nonhomologous end-joining mechanisms for recombination.
(A) The efficiency of integration for a selection marker is greatly increased by linearizing the vector within a sequence found in the target genome. Simple integration without deletion or modification of the cloned gene essentially doubles the gene copy. (B) By deleting a portion of the target gene before integrating the selectable marker, it is possible to disrupt the target. For this to be effective and to achieve high levels of transformation, it is necessary to excise the disruption cassette from the vector and transform with a fragment having sequences that overlap with the target gene. (C) It is possible to subsequently recover the selection marker by flanking it with direct repeats.
Aside from targeted integration, restricting the efficiency of the promoter of the selectable marker will also increase the recovery of transformants with multiple integration events. In *Arxula adeninivorans*, it was possible to obtain transformants with multiple integrational events for amylase production by reducing the length of the LEU2 promoter from its initial 565 bp to only 56 bp (154). Transformants recovered from the complete promoter contained only a single integration event, but transformants obtained with the abbreviated promoter contained up to eight integrated copies when both were targeted to the 25S RNA gene (Fig. 2B). Presumably, strains with more integrated copies are favored for growth because they achieve a functional level of LEU2 expression. Madzak et al. have reported similar results with the use of a defective *URA3* marker (115).

Restriction endonuclease-mediated integration has been reported to increase the frequency of site-specific integration (43, 166). While it does improve the overall frequency of integration events, linearization alone is effective as well (Fig. 2C). In fact, Maassen et al. (114) have suggested that the increase in integration events that they observed when transforming *P. stipitis* with plasmids along with the restricting enzymes was attributable to the prevention of recircularization rather than to any specific effect on the target genomic DNA.

The frequency of integration events can also be increased by incubating the host cells with sublethal doses of bleomycin just prior to transformation with linearized DNA. Bleomycin introduces DSBs in DNA, so exposure to sublethal doses of this antibiotic increases opportunities for recombination.

### 21.7.1. Multicopy Integration

More than 400 copies of the long terminal repeat elements of Ty1 and Ty2 are found in the *S. cerevisiae* genome. These have been used as targets for multiple integration events in order to increase the stable transmission of heterologous genes (106). Transformation frequencies are higher and stabilities are greater when two flanking copies of the δ-region are used (105). This technique has been fairly widely used in *S. cerevisiae* (29, 47, 93, 106), and use of the same sequence has been attempted in *K. lactis* (172).

Ylt1 is a repetitive element that exists in about 35 copies with an additional 30 copies of long terminal repeat elements in the *Yarrowia lipolytica* genome. Relatively high-copy-number integration events could be obtained when a defective *URA3* allele was used as the selectable marker. Most of these were direct tandem integration events (90, 126).

The ribosomal DNA (rDNA) locus has been used as a site for multiple integration events in *Y. lipolytica*, *K. lactis*, *S. cerevisiae*, *Candida utilis*, *Schizosaccharomyces pombe*, and *Phaffia rhodozyma* (19, 21, 33, 37, 39, 44).

### 21.8. Expression from Autonomous Plasmids

An ARS can greatly increase the efficiency of transformation by enabling replication of plasmid DNA in its target host. An autologous ARS can be obtained from the prospective host by cloning a library of short restriction fragments from the host into a vector bearing a selectable marker. The library is then transformed into the target host. The transformed yeasts are pooled, and a plasmid preparation is made from the yeast cells. Any rescued plasmids are transformed back into *E. coli*, and a number of clones are selected for characterization. After two or three cycles of transformation and rescue, it is possible to obtain an ARS that allows stable replication in both hosts (1, 17, 30, 96, 119, 157, 168, 184). Alternatively, it is possible to synthesize a library of random AT sequences and screen for ARS activities in the same manner (54). Centromeric sequences are needed for appropriate partitioning (168, 182, 183).

### 21.9. Transformation of *P. pastoris* and *H. polymorpha*

The logic for expression of recombinant genes in either *P. pastoris* or *H. polymorpha* is much the same. Both yeasts are strong methanol-inducible yeast species, and methanol-regulated promoters are most often utilized for expression. Thus, one clones the recombinant gene under control of a methanol-regulatable promoter such as that from the alcohol oxidase gene (known as the *AOX1* gene in *P. pastoris* and the *MOX* gene in *H. polymorpha*). Then one proceeds to culture the expression strains in glucose for growth and strain maintenance. Under these conditions, expression of the recombinant gene is “off,” minimizing selection against a strain that produces large amounts of foreign protein when induced. When production of the recombinant protein is desired, one switches to a methanol medium. A difference between the yeasts is that the *P. pastoris AOX1* promoter is strictly dependent on methanol and requires the alcohol for full expression levels, whereas the *H. polymorpha* MOX promoter can be derepressed to full expression levels by feeding of glycerol as a carbon source or by feeding glucose at a carbon limited rate. If ethanol is a problem for production with *P. pastoris*, then a variety of other promoters are available for this yeast, such as the *GAP* promoter, which is constitutive, or the *ADH* promoter, which can be derepressed to a high level of expression in response to growth rate-limiting amounts of glucose. Another difference between these yeasts is growth temperature. *P. pastoris* does not grow well above 30°C, whereas *H. polymorpha*’s optimal growth temperature is about 37°C. As a result, *H. polymorpha* grows significantly faster than *P. pastoris* and most other yeasts, and processes for *H. polymorpha* take a significantly shorter time. An advantage of *P. pastoris* is that the strains and vectors needed for recombinant gene expression are commercially available through Invitrogen (Carlsbad, CA) and other sources, whereas the *H. polymorpha* system is not sold commercially and is more difficult to obtain. In addition, the *P. pastoris* system has been much more extensively utilized, and therefore there is much more published literature available on expression in this yeast.

Techniques for culturing *P. pastoris* or *H. polymorpha* at the bench level are very similar to those used for *S. cerevisiae*. The most common rich medium for cultivation of either yeast species is YPD (1% yeast extract, 2% peptone, 2% dextrose), and defined medium is YNB (0.67% yeast nitrogen base with ammonium sulfate and without amino acids, 2% dextrose plus any amino acids or nucleotides required for growth at ~50 µg/ml each). Growth of *P. pastoris* or *H. polymorpha* on methanol requires that the dextrose be replaced with methanol to 0.5%. Incubations of *P. pastoris* are typically done at 30°C, whereas those for *H. polymorpha* are generally done at 37°C. In liquid YPD, *P. pastoris* has a generation time of ca. 90 min, and *H. polymorpha* at 37°C in the same medium has a generation time of approximately 1 h. With methanol as sole carbon source
and a defined culture medium, the generation time of P. pastoris is around 5 h and that for H. polymorpha is about 3 h per generation at 37°C.

The first consideration in the selection of a P. pastoris or H. polymorpha expression vector is whether you intend the yeast to secrete a protein product or produce it intracellularly. A general rule of thumb is to produce a recombinant protein in the same way that it is expressed in its native host: if a protein is produced intracellularly by its native host, one should also produce it intracellularly in the yeast host; if the protein is secreted from its native host, secrete it from the yeast system. Although there have been exceptions to this general rule, it is generally best to follow it since the intracellular and secretory environments are very different from each other and synthesis of a protein in the wrong compartment may result in improper folding or degradation. A number of vectors have been constructed for each yeast species; a list and detailed discussion of these vectors for P. pastoris can be found in Lin Cereghino et al. (109) and at www.Biogrammatics.com, and H. polymorpha is discussed in reference 61.

These days, genes to be expressed are usually obtained by PCR amplification from genomic DNA or cDNA, or by gene synthesis; either method facilitates the incorporation of convenient restriction sites, signal sequences, purification tags, etc. When the goal is efficient secretion of the gene product, the cloning can be a little trickier. Use of the native secretion signal sequence associated with the heterologous gene is the most straightforward, but those signal sequences may not be efficiently used by the yeast host. Alternatively, a yeast signal sequence, such as that for the S. cerevisiae alpha mating factor (αMF), can replace the heterologous signal sequence in the expression vector. The αMF secretion signal is commonly used because it has been proven to give efficient secretion of many types of recombinant proteins. Although the recombinant protein may be successfully secreted using the αMF signal, proper N-terminal processing of the heterologous protein may not occur, and modifications of the αMF signal, or the use of an alternative secretion signal sequence, may ultimately be necessary to obtain a properly processed protein.

### 21.9.1. Transformation by Electroporation

For transformation of either P. pastoris or H. polymorpha, electroporation is the most commonly used procedure, and therefore a modified version of that described by Becker and Guarente (10) will be related here. For the other procedures, readers are referred to either of the volumes of Methods in Molecular Biology: Pichia Protocols (36, 72); for H. polymorpha, please refer to Hansenula polymorpha: Biology and Applications by G. Gellissen (59).

### 21.9.2. DNA Preparation

For all transformation methods, linearized plasmid DNA is most commonly transformed into either yeast for integration into the yeast genome. The DNA sequences at the ends of the linear plasmid DNA stimulate integration by a single crossover recombination event into the locus shared by vector and host genome. Therefore, linearization of an expression plasmid is performed within a yeast sequence such as the promoter sequence. The final vector, prepared in E. coli, is cut with a restriction enzyme that linearizes the vector, and then the DNA is purified and concentrated to approximately 100 ng/µl prior to transformation. At this point the vector is ready for transformation into yeast.

### Preparation of Electrocompetent Cells for Transformation of P. pastoris

The procedure for growth and preparation of electrocompetent P. pastoris cells is taken from Lin-Cereghino et al. (110) and is described below. All solutions should be autoclaved except for the dithiothreitol (DTT) and HEPES solutions, which should be filter-sterilized.

1. Electroporation instrument: BTX Electro Cell Manipulator 600 (BTX, San Diego, CA). Parameters for electroporation vary considerably depending on the apparatus used. Check the instructions provided by the manufacturer for specifics for each instrument.
2. Inoculate 10 ml of YPD medium with a single fresh P. pastoris colony of the strain to be transformed from an agar plate and grow overnight with shaking at 30°C.
3. Use the overnight culture to inoculate a 500-ml YPD culture in a 2.8-liter Fernbach culture flask to a starting optical density at 600 nm (OD

### Electroporation Procedure

1. Add up to 1 µg of linearized plasmid DNA sample in no more than 5 µl of water to a tube containing 40 µl of cold water. At this stage and from here on, keep the cells ice cold and do not vortex the cells to resuspend them (slow pipetting is best).
2. Wash cells a final time in 20 ml of cold 1 M sorbitol, then resuspend in 0.5 ml of cold 1 M sorbitol (final volume including cells will be 1.0 to 1.5 ml).
3. Use these cells directly without freezing to achieve the most transformants.
4. To freeze competent cells, distribute in 40-µl aliquots to sterile 1.5-ml minicentrifuge tubes, and place the tubes in a −70°C freezer.

### Preparation of Electrocompetent Cells and Electrotransformation of H. polymorpha

The procedure for growth and preparation of electrocompetent H. polymorpha cells is taken from Gellissen (59) and is
described below. All solutions should be autoclaved except for the DTT solution, which should be filter sterilized.

1. Inoculate 200 ml of YPD medium with an appropriate volume of fresh preculture and grow until the cells reach an OD_{600} of 0.8 to 1.2.
2. Harvest the cells by centrifugation and resuspend them in 0.2 volume of prewarmed (37°C) 50 mM potassium phosphate buffer (pH 7.5), then add DTT (1 M) to a final concentration of 25 mM.
3. Incubate the cells at 37°C in a water bath (without shaking) for 15 min.
4. Harvest the cells by centrifugation and wash twice: the first in 1 volume and the second in 0.5 volume of STM buffer (270 mM sucrose, 10 mM Tris-HCl, pH 7.5, 1 mM MgCl₂) while being kept at 0°C.
5. Resuspend the final cell pellet in 0.005 volume of STM buffer and dispense the cells in 60-µl aliquots. Freeze the aliquots at -70°C.
6. To transform, thaw aliquots of competent cells in ice, and add plasmid DNA (200 ng to 1 µg). Transfer the cells to a prechilled 2-mm cuvette.
7. After careful drying with a paper towel, place the cuvette in the electroporator and pulse (e.g., 2.0 kV, 25 µF, 200 ohm, for a Bio-Rad Gene Pulser II device). Note: Conditions for electroporation vary considerably depending on the instrument. Be sure to follow the instrument’s instructions.
8. Immediately add 1 ml of YPD medium, transfer the cells to a microtube, and incubate at 37°C for 1 h of recovery.
9. Harvest cells by centrifugation, wash them once in 1 volume and the second in 0.5 volume of STM buffer (270 mM sucrose, 10 mM Tris-HCl, pH 7.5, 1 mM MgCl₂), while being kept at 0°C.
10. Resuspend the final cell pellet in 0.005 volume of STM buffer and dispense the cells in 60-µl aliquots. Freeze the aliquots at -70°C.
11. To transform, thaw aliquots of competent cells in ice, and add plasmid DNA (200 ng to 1 µg). Transfer the cells to a prechilled 2-mm cuvette.
12. After careful drying with a paper towel, place the cuvette in the electroporator and pulse (e.g., 2.0 kV, 25 µF, 200 ohm, for a Bio-Rad Gene Pulser II device). Note: Conditions for electroporation vary considerably depending on the instrument. Be sure to follow the instrument’s instructions.

**Secretion of HSA**

1. Pick a colony of an HSA-secreting strain and inoculate into 50 ml of YPD medium in a sterile Erlenmeyer flask. Incubate with shaking overnight.
2. The next day, shift the cultures by centrifugation. To shift, determine the OD_{600} of each culture and centrifuge 200 OD_{600} units (OD_{600} unit = volume in milliliters × OD_{600} reading) of each culture.
3. After centrifugation, decant the supernatant and suspend each cell pellet in 10 ml of BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate [pH 6.0], 1.34% YNB with ammonium sulfate and without amino acids, 0.5% methanol). Transfer each culture to a small (50-ml) sterile shake flask and place in a 30°C incubator with vigorous shaking. Before starting the induction, harvest 0.5 ml of each culture for a t = 0 sample.
4. Centrifuge the t = 0 samples in a microcentrifuge for 5 min at maximum speed. Transfer the supernatants to a clean tube. Discard the cell pellets but save the supernatants by storing them frozen at -20°C.
5. Add methanol to each culture to a final concentration of 0.5%, every 12 h.
6. Take additional aliquots of HSA-secreting culture (or negative control) at 12, 24, 48, and 64 h and prepare them for storage at -20°C as described above.
7. Thaw and analyze proteins in each HSA sample by SDS-PAGE to evaluate HSA synthesis and secretion over time. For each sample, mix 25 µl of culture medium supernatant with 4 µl of the 6× SDS-PAGE loading. Load 25 to 30 µl on an SDS-polyacrylamide gel. Follow standard procedures for Coomassie staining of an SDS-polyacrylamide gel.

**Expression of β-lac**

1. Pick a colony of each of the two β-lac-producing *P. pastoris* strains and inoculate into 50 ml of YPD medium. In one strain, the gene for *bla* is fused to the inducible

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**Protocol for Small-Scale Expression in *P. pastoris***

This protocol describes how to perform a shake flask study of two *P. pastoris* strains, one secreting a protein, human serum albumin (HSA), and a second expressing an intracellular enzyme, bacterial β-lactamase (β-lac) (see Fig. 3). The HSA gene is expressed from the methanol-inducible AOX1 promoter, whereas the β-lac gene is expressed constitutively from the GAP promoter. (These strains can be obtained from the authors by contacting J.M.C.) For the strains expressing HSA, samples of culture medium are collected and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For strains expressing β-lac, a simple qualitative enzyme assay is employed.

**FIGURE 3** Protocol for assays of protein expression in *P. pastoris*.
alcohol oxidase (AOXI) promoter. In the other, bla expression is regulated by the constitutive glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter.

2. The next day, remove a 1-ml aliquot of each culture to a clean microcentrifuge tube. Determine the absorbance of each culture at OD_{600} and centrifuge 80 OD_{600} units of each. Then resuspend the 80 OD_{600} units in 2 ml of sterile water.

3. For each culture, add 1 ml of cells to each of two flasks containing 150 ml of medium, one with YNB glucose medium and one with YNB methanol medium.

4. Determine the OD_{600} of each culture and harvest 20 OD_{600} units of each by centrifugation. Decant the supernatant medium and save the pellets at ~20°C. These are t = 0 samples.

5. Place the remainder of the two cultures at 30°C with shaking. Add additional methanol to each methanol medium culture (i.e., not the glucose medium cultures), to a final concentration of 0.5%, at 12 h.

6. At 6, 12, and 24 h, determine the OD_{600} of each culture and harvest 20 OD_{600} units from each. Centrifuge each sample, decant the supernatants, and store cell pellets in labeled tubes at ~20°C.

7. Transfer all β-lac 20-OD_{600} cell pellet samples from storage at ~20°C (plus at least one nonexpressing negative control sample) to an ice cold bucket to thaw.

8. Suspend cell pellets in 100 µl of ice-cold breaking buffer (25 mM Tris-HCl, pH 7.5). Add one scoop (~100 µl) of glass beads to each sample.

9. Vortex as vigorously as possible. Multiple samples (up to 12) can be broken simultaneously using a multisample vortex head (Disruptor Genie, Scientific Industries, Inc.) in the cold box by vortexing constantly at 4°C for 10 min.

10. Centrifuge samples for 10 min at full speed in a microcentrifuge at 4°C.

11. Transfer each supernatant to a clean minicentrifuge tube. (Be careful to avoid transferring pellet and cell debris; i.e., transfer only clear cell extract.) Save supernatants (cell-free extracts) in labeled tubes specifying the strain and time point.

12. Determine protein concentration in each sample using the Pierce assay or other method.

13. Carry out assays for β-lac activity in extracts as follows. For each strain and time point, transfer 5 µg of protein to a microcentrifuge tube containing 198 µl of ice-cold breaking buffer. Include a negative control with breaking buffer substituted for extract. Start the assay by adding 2 µl of PADAC substrate solution. Mix and incubate at 30°C for 5 min. β-Lac activity is indicated by a change of color from purple to yellow.

### 21.1 SYNTHETIC DNA

As synthetic DNA continues to drop in price, it is now routinely used to speed up construction of expression cassettes for metabolic engineering. Gene synthesis is useful to eliminate unwanted restriction and alter specific regions of the protein. More importantly, through codon optimization, it can also increase translation rates. Codon optimization was based initially on the observation that organisms use a nonrandom subset of the 61 possible sense codons available in the genetic code. This can result in a bias in their codon usage. Codon usage varies from one species to another. It is consistent within the genes of any given genome, and the codon usage in open reading frames correlates with the most abundant aminoacyl-tRNAs present in the cell (150). It is not clear, however, that altering codon usage alone will result in an optimal translation rate. In addition to codon preference based on tRNA usage, it is also necessary to take into account secondary structures and contextual optimization. For example, it is possible to increase protein synthesis by optimizing the initial codons in an open reading frame, thereby avoiding the formation of secondary structures which may interfere with the ribosomal complex (76, 80, 98). Codon optimization can adjust the overall G+C usage to something more appropriate for the producing organisms, and contextual codon optimization can avoid the sequential use of certain acyl-tRNAs that interfere with one another during translation (70). Optimization of codons based on frequency of acyl-tRNA usage alone does not give consistently better results, so care should be taken in considering the various factors in designing synthetic genes.

While limited sequence optimization—such as the elimination of restriction sites or specific codons—might be done manually, the optimization of open reading frames for various codon usage tables can be aided through the use of software (53) or online tools (180). Reducing ambiguity in the overlaps for optimized gene assembly requires elaborate algorithms and significant computational power (103).

Codon optimization can be highly effective in the expression of bacterial (19, 178) or human proteins in yeasts. It can eliminate cryptic polyadenylation signals in native mRNA, such as AT-rich stretches (76), thereby increasing the efficiency of protein synthesis (162). Codon optimization has been used to increase production of several proteins in *P. pastoris* (75, 76, 118, 170, 179).

Synthetic DNA can be used to increase expression of open reading frames, but it is somewhat more difficult to apply to the construction of eukaryotic promoters since these often have high AT content, along with inverted and direct repeats that can interfere with DNA synthesis. The cost of synthetic DNA is dropping rapidly, and genes up to 1,500 bp in length can be obtained from commercial operations with turnaround times of a few weeks or less.

### 21.1 1. ENHANCING OVEREXPRESSION OR SECRETION

Many factors besides transcription formation determine the efficiency of protein production. One of the most critical is oxygen supply. During heterologous protein production in large-scale bioreactors, regions of the reactor vessel are relatively poorly mixed. Moreover, even if the whole vessel is mixed, packets of fluid are rapidly depleted in oxygen, generating cycles of aeration and oxygen deprivation. This is particularly true in high-density fermentations for the production of enzymes or other heterologous proteins. The oscillation between aeration and oxygen limitation can disrupt cellular metabolism. One interesting approach to overcome this problem is the heterologous expression of a bacterial hemoglobin (Vhb) from the gram-positive bacterium *Vitreoscilla* (159). Trapping oxygen when cells are exposed to aerobic conditions and releasing it when under less aeration can mitigate the effect of poor mixing. Expression of Vhb increased recombinant β-galactosidase production in *P. pastoris* (181), improved cell growth and secretion of extracellular enzymes in *Y. lipolytica* (12), and enhanced ethanol production by *S. cerevisiae* (26), including the production of ethanol from xylose (141).

Excessive oxygen can be toxic to yeasts due to the production of reactive oxygen species such as superoxide...
21.1 2. CHAPERONE, FOLDASE, CELL WALL, AND ER FACTORS

Overexpression of heterologous proteins in a recombinant host can saturate or overload the endoplasmic reticulum (ER) secretory pathway, thereby resulting in misfolding (57, 148, 151). There are two basic modes of eukaryotic protein translocation. In cotranslational translocation, secretion occurs contemporaneously with peptide synthesis. The growing polypeptide chain moves from the ribosomal tunnel into a membrane channel formed by the Sec61 membrane complex, and the whole process is driven by GTP hydrolysis during translocation. In posttranslational translocation, the Sec62 membrane complex forms a pore between the cytosol and the ER membrane. It binds the signal sequence of the preformed protein, which is then drawn through the membrane (132).

Kar2p (the yeast equivalent to the mammalian BiP) is a member of the Hsp70 family of heat shock proteins that serves as a ratchet to draw the preformed protein through the membrane in posttranslational secretion. Further processing such as folding, disulfide cross-linking, and glycosylation then occurs in the lumen of the ER (9, 32, 62).

Several proteins in the ER are involved in this process. Protein disulfide isomerase (PDI) appears to play a role in folding. Overexpression of proteins involved in the ER secretory pathway has been reported to improve secretion in S. cerevisiae. Overexpression of PDI in K. lactis strongly stimulated secretion of highly disulfide-bonded serum albumin (8) and human lysozyme (82). Likewise, overexpression of PDI in P. pastoris was able to increase secretion of a hookworm vaccine protein that contained 20 disulfide linkages (79). Overexpression of the chaperones Kar2, Ssa1, or PDI can improve protein secretion by two- to sevenfold in P. pastoris (188).

A systematic study of yeast genes to enhance protein secretion in S. cerevisiae showed the cell wall protein Ccw12 and the ER resident thiol oxidase, Ero1, as lending the most general enhancement (176). Gasser et al. (58) used a transcriptomics-based approach to examine a P. pastoris strain that would overexpress human trypsinogen (81). Likewise, overexpression of PDI in P. pastoris is preferred for the production of N-glycosylation (130). In K. lactis, overexpression of KISOD1 effectively reduced oxygen stress during heterologous protein production.

21.1 3. HUMANIZED YEAST EXPRESSION SYSTEMS

21.1 3.1. Market

Monoclonal antibodies (MAbs) represent the fastest growing sector of biotherapeutic proteins (107). The global therapeutic MAb market grew to $5.4 billion in 2002, with chimeric MAbs accounting for 43% of sales (133). From 2004 to 2008, growth actually exceeded expectations (128). The global sales of MAbs have been reported as $33 billion in 2008 as compared to $27 billion in 2007 (116). This explosive growth stems from the tremendous ability of MAbs to bind a plethora of antigens ranging from toxins, viruses, and bacteria to cancer cells. The main applications, however, lie in their potential to boost novel treatments of cancer and inflammatory and infectious diseases. In 2004, the global clinical antibody pipeline had 132 products in development (133). As of 2009, more than 500 antibody-based therapeutics were in development, with 200 more in clinical trials (84, 128, 134). These factors suggest that large-scale antibody production in yeasts and fungi might be a major emerging opportunity (see reference 56).

Clinically proven MAbs have been produced in tissue culture (49), mainly Chinese hamster ovary cells, with its incumbent costs (107). This technology results in MAbs similar but not identical to their native counterparts. Large-scale tissue culture (up to 200,000 liters) has an estimated productivity of only 1 to 2 mg liter$^{-1}$ h$^{-1}$, with an estimated annual productivity of 8 to 16 kg/year (177). In 2006, the estimated cost for producing a MAb by mammalian tissue culture was between $300 and $3,000 (US) per g, with the major cost centers coming from low titers and downstream purification (49). While such MAbs are valuable as biotherapeutics, the doses required are typically much larger than for other biologics (89), typically >100 mg, due to their low potency (84). As a result, MAbs are among the most expensive pharmaceuticals available, with annual costs for cancer therapies reaching up to $35,000 US/year per patient (48). Clearly this is an area in need of improved production technology, especially in light of efforts to control medical costs while expanding coverage.

21.1 3.2. Yeast Production Systems

MAbs must be capable of two binding events if they are to be therapeutically effective. In the first event, the variable domain, which determines the antibody specificity, binds to a specific target protein on the cell surface. In the second event, the immune system’s effector cells bind to constant region (Fc) of the antibody and destroy the cell to which the antibody is attached through a process known as antibody-dependent cell cytotoxicity. This cytotoxic process depends on a specific Fc Asn$\rightarrow$N glycosylation and is affected by the glycan composition of the constant region (108, 140). Glycosylation patterns vary with different mammalian cell lines, and the pattern elucidating the most potent antibody-dependent cell cytotoxic response for a given cell type is complex, which leaves open the possibilities of further development and optimization.

MAbs are obtained by (i) immunizing mice with a specific antigen, (ii) harvesting the mouse spleen lymphocytes, (iii) hybridizing the lymphocytes with myeloma cells, (iv) screening the resulting hybridoma cells for antibodies to the desired antigen, and (v) carrying the selected potent clones into tissue culture. Antibody production then depends on the efficiency of the scale-up and downstream processing (84). To produce antibodies in yeast cells, it is further necessary to clone the specific variable sequence into a host for which the glycosylation machinery has been altered to match that of humans.

P. pastoris is preferred for the production of N-glycan-containing biopharmaceuticals since it exhibits a low frequency of serine O mannosylation. Moreover, mannoglycosylation is much less extensive in P. pastoris.
and unlike S. cerevisiae, P. pastoris lacks the transferase necessary to create terminal α-1,3-linked mannosyl residues that can cause strong antigenic responses in humans (42). The glycosylation pathways for yeasts and mammalian systems have recently been reviewed in excellent detail and do not need to be repeated here (83, 138). In brief, deletion of OCH1, which codes for the mannosyltransferase of the cis-Golgi apparatus, eliminates the native hyperglycosylation machinery. Combining the correct Golgi leader heavy- and light-chain domains are fused to a Kar2 signal protocol for production of complex N-glycosylated proteins peptide to ensure processing and secretion through the ER.

The glycosylation pathways for yeasts and mammalian high-mannose-type proteins.

REFERENCES


163. Reference deleted.


21. Protein Expression in Nonconventional Yeasts


Keywords: Protein expression, nonconventional yeasts, gene expression, recombinant DNA, genetic transformation