Chapter 17
A Wide-Range Integrative Expression Vector (CoMed) System for Yeasts

Gerhard Steinborn, Gotthard Kunze, and Gerd Gellissen

Contents

Abstract No single yeast-based platform exists which is optimal for every protein. It is advisable to assess several platform candidates in parallel for optimal expression characteristics in a given case. For this approach, a wide-range yeast vector has been established that can be targeted to the various yeast host strains. The vector is built up in a modular way. In its basic form, it contains conserved rDNA-derived segments for targeting. For heterologous gene expression control, it is equipped with a promoter that is functional in all yeast species tested so far. For selection, a range of dominant and auxotrophic selection markers can be employed. Examples are presented applying vector variants with dominant or auxotrophic selection markers to the comparative simultaneous integration and expression of single or multiple foreign genes in a range of yeast platforms.

Keywords Vector, modular way, auxotrophic selection markers, dominant selection markers, yeast platforms

17.1   The Need for a Wide-Range Expression Vector System

In the previous chapters of this book a plethora of yeast species and expression platforms based on these yeasts have been presented. Yeasts include a great diversity of organisms. In general, fungi are excellent hosts for the production of recombinant proteins, as detailed in the previous chapters. They offer a desired ease of genetic manipulation and rapid growth to high cell densities on inexpensive media (Romanos et al., 1992; Heinisch and Hollenberg, 1993; Sudbery, 1996; Gellissen,

2000, 2002). As eukaryotes, they are able to perform multiple posttranslational modifications, thus producing even complex foreign proteins that are often identical or very similar to native products from plant or mammalian sources (Ruetz and Gros, 1994; Gilbert et al., 1994; Wittekindt et al., 1995; Vozza et al., 1996; Gellissen, 2000, 2002; Valenzuela et al., 1982; Sudbery, 1996). Only few examples are available for the production of the same protein in a range of fungal species and it is thus difficult to evaluate the advantages or disadvantages of a platform for a particular product development. Constituting a diverse group yeasts exhibit differences in productivity, processing or glycosylation. Some examples of such differences are briefly listed in the following; for a more detailed description the reader is referred to other chapters of the book describing individual fungal systems. The first yeast expression platform was based on the traditional baker's yeast *S. cerevisiae*. Although successfully applied to the production of pharmaceuticals like insulin or hepatitis B vaccines, some important disadvantages became quickly apparent, which limit its general use in biotechnology. Glycoproteins are often over-glycosylated, and terminal mannose residues in N-linked glycans are added by an α-1,3 bond which is suspected to be allergenic (Jigami and Odani, 1999; Guengerich et al., 2004). Instead, non-allergenic terminal α-1,2 bonds are found to be present in *Hansenula polymorpha* and *Pichia pastoris* (Montesino et al., 1998, 1999; Bretthauer and Castellino, 1999; Guengerich et al., 2004). In *Arxula adeninivorans* patterns of O-glycosylation vary depending on morphological status thereby potentially providing an option to produce a foreign protein with or without O-glycosylation in an identical strain (Wartmann et al., 2002a). The protease content differs among yeasts: in a recent comparative study it was shown that the cytokine IL-6 is correctly processed from a MFα1 leader/IL-6 fusion in *A. adeninivorans*, but that N-terminally truncated cytokines are secreted from *H. polymorpha*, *P. pastoris* and *S. cerevisiae* hosts (Steinborn et al., 2006, 2007). The narrow substrate specificity of *S. cerevisiae* hampers fermentation design (Bruinenberg, 1986; Romanos et al., 1992). Most other biotechnologically applied species can grow on a much wider range of substrate thereby providing a high versatility for the selection of attractive promoter elements and as a consequence enabling various options for fermentation design. In individual cases, hydrophobic proteins may impose problems to a particular host, but not to others. The two methylotrophic species *H. polymorpha* and *P. pastoris* differ in their methanol requirement for the activation of promoters derived from genes of the methanol pathway (Guengerich et al., 2004; Kang and Gellissen, 2005). These few arbitrary examples already illustrate the necessity of carefully considering a range of fungal organisms before deciding on an expression platform. All of them have particular favourable characteristics and specific product examples attest to the advantages of the individual platform. However, all systems have drawbacks and limitations: sometimes attempts to produce a heterologous protein fail completely; in other cases, productivity, secretion or modification and processing are severely impaired as pointed out before, thereby preventing the development of a competitive production process or a marketable product. It is evident that no single yeast system is optimal for all proteins. Hence, predictions for a successful strain and process development

can only be made to a certain extent, and misjudgments cannot be excluded. This in turn means that the initial selection may result in costly time- and resource-consuming failures. It is therefore desirable to assess several selected yeast platforms in parallel for criteria like appropriate protein processing or secretion in a given case. A vector that can be targeted to the various platform candidates greatly facilitates such a comparison.

## 17.2   Design and Essential Elements of a Wide-Range Expression Vector

The design of a vector suited for a wide range of fungal organisms has to meet several prerequisites. Such a plasmid has to contain a targeting element suitable for all test species. The promoter that drives heterologous gene expression has to be functional in all these organisms. The vector/host system has to employ a dominant selection marker or a sequence that can complement the auxotrophy in all selected organisms.

Obvious targeting elements that are highly conserved among the various yeasts are sequences of the rDNA genes encoding ribosomal RNAs. These genes are present in high copy number and are typically clustered as head-to-tail tandem arrays of identical units (rDNA). Transcription occurs in a special compartment in the nucleus, called the nucleolus (Warner et al., 1972). They are readily accessible to all component required for an efficient transcription. The copy number of rDNA repeats in yeasts ranges from 30–50 in *H. polymorpha* (Waschk et al., 2002) to 200 in *S. cerevisiae* (Maleszka and Clark-Walker, 1993).

The rDNA repeats are in most instances organized as arrays of rRNA genes and non-coding intergenic spacer regions as detailed in Fig. 17.1.

Each rRNA gene is transcribed into a single precursor molecule by RNA polymerase I. Subsequently, this precursor is processed to form the 18S, 5.8S and 25S (28S) rRNAs. During this process, the external transcribed spacer (ETS) and the internal transcribed spacers (ITS1/2) are excised. Precursor transcription starts at the leader sequence of the 5′ ETS and stops at the 3′ end of the 25S (28S) rRNA gene. The intergenic non-transcribed spacers (NTS1/2) include promoter, enhancer and suppressor elements to control RNA polymerase I (Pol. I)-directed transcription (Udem and Warner, 1972). In yeasts, the coding region for the 5S subunit transcribed by RNA polymerase III (Pol. III) is either located in the NTS located between successive rRNA precursors, as has been shown for *S. cerevisiae* (Johnston et al., 1997), *Ashbya gossypii* (Wendland et al., 1999) and *H. polymorpha* (Klabunde et al., 2002) or represented elsewhere in the genome as it is the case with *A. adeninivorans* (Steinborn et al., 2005).

The rDNA sequences are highly conserved during evolution. This conservation is restricted to sequences encoding the various rRNA species; the sequences of the non-coding segments can be quite divergent. Therefore, elements derived from coding sequences or elements containing extended segments of conserved sequences
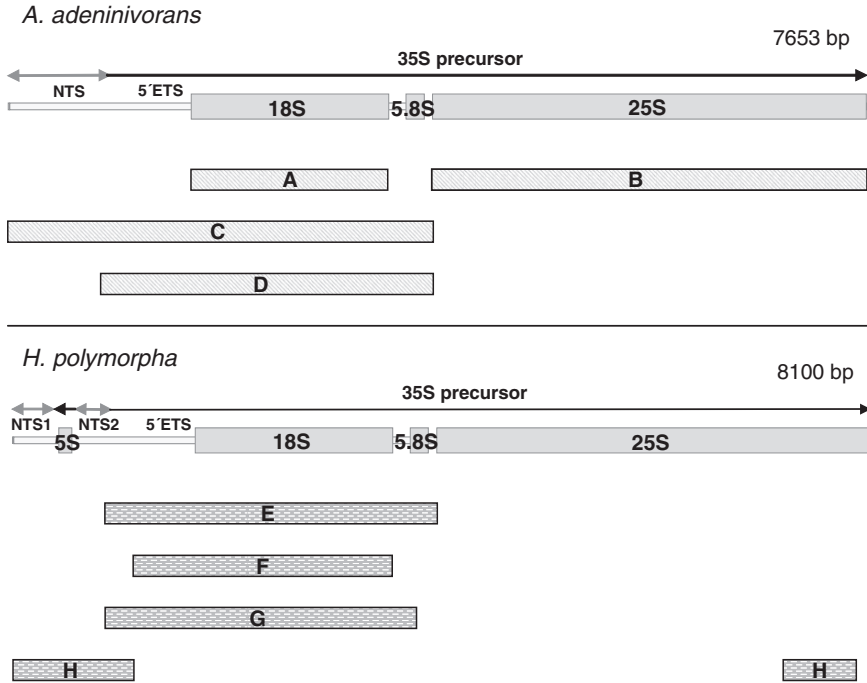
Fig. 17.1 *A. adeninivorans* and *H. polymorpha* rDNA units and the position of derived rDNA-targeting elements. The rDNA unit contains genetic elements in the following order: NTS1 (non-transcribed spacer), 5S rRNA, NTS2, the sequence of 35S precursor with the ETS1 (external transcribed spacer), followed by the sequence of the 18S, 5.8S and 25S rDNA. This gene order has been confirmed for *S. cerevisiae*, *H. polymorpha* and other yeast species, for *A. adeninivorans* the 5 S RNA is excluded from the unit represented elsewhere in the genome. (E-H) represents the position of the targeting segments of *H. polymorpha* and (A-D) that of *A. adeninivorans* assessed for transformation. Inclusion of this element in targeting vectors resulted in low transformation efficiency and unstable transformants in case of (F), in high transformation efficiency and stable transformants in case of (A-E,G,H)

have to be employed in the construction of a vector for wide-range application. Elements exclusively consisting of non-coding sequences are likely to function in a species-specific manner. However, non-conserved sequences that are important for mitotic stability have been described for *S. cerevisiae* (Lopes et al., 1996), and sequences that modulate expressibility have been postulated in *H. polymorpha* (Klabunde et al., 2003). Most of the regulatory elements in the non-coding sequences have been analyzed in *S. cerevisiae*. In light of the low extent of homology and the lack of conclusive experimental data, it can only be assumed that such functional sequences are also present at equivalent locations in the *H. polymorpha* and in other yeast species. rDNA targeting with different sequences has been described for a range of yeast species including *S. cerevisiae* (Lopes et al., 1996), *A. adeninivorans* (Rösel and Kunze, 1996), *H. polymorpha* (Klabunde et al., 2005),

Kluyveromyces lactis (Bergkamp et al., 1992) and *Yarrowia lipolytica* (Le Dall et al., 1994; Madzak et al., 2005). Only recently, rDNA sequences of *A. adeninivorans* and *H. polymorpha* have been defined as targeting elements with appropriate characteristics comprising both coding and non-coding sequences (Steinborn et al., 2005).

For selection, a range of dominant selection markers like the *E. coli*-derived *hph* gene conferring resistance against hygromycin B (Wartmann et al., 2002b; Rösel and Kunze, 1998) can be used. Alternatively, genes of different sources that complement auxotrophies of respective host strains can be chosen. Examples are the *A. adeninivorans*-derived *LEU2* gene or the *H. polymorpha*- or *S. cerevisiae*-derived *URA3* genes (Steinborn et al., 2006). A new attractive element is the *A. adeninivorans*-derived *TRP1* gene (Steinborn et al., 2007). Obviously, use of such complementation markers is restricted to the existing range of respective auxotrophic strains.

For expression control of the heterologous gene, a *TEF1* promoter like that derived from *A. adeninivorans* (Rösel and Kunze, 1996) can be employed when addressing a large number of platforms. Other control elements are likely to elicit appropriate expression levels in a restricted number of yeasts or in a single species only.

The basic design of a wide-range yeast vector with a selection of components is provided in Fig. 17.2 and Table 17.1.

It is built up in a modular way. By easy exchange of modules, such a vector can be converted into a plasmid that is optimal for an individual platform, for instance by inserting an expression cassette with a *MOX* promoter element that elicits efficient gene expression in methylotrophic yeasts only. Variants of this basic vector for the secretory production of certain compounds are under development. In yet another design, it is possible to linearize the plasmids in a way that leaves behind all bacterial DNA sequences.
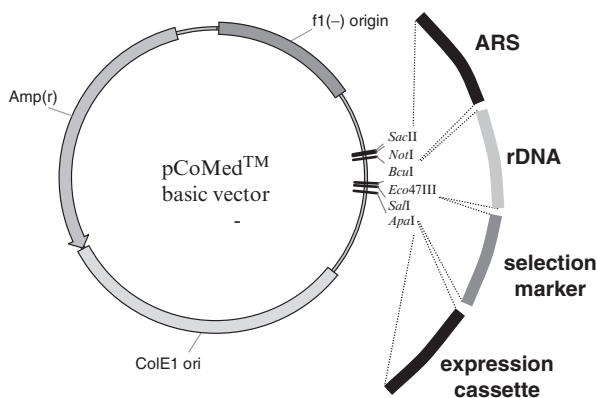
Fig. 17.2 Basic design of the CoMed™ vector. The CoMed™ basic vector contains all *E. coli* elements for propagation in the *E. coli* system and a MCS for integration of ARS, rDNA, selection marker and expression cassette modules. For this purpose, ARS fragments are flanked by *Sac*II and *Bcu*I restriction sites, rDNA regions by *Bcu*I and *Eco*47III restriction sites, selection markers by *Eco*47III and *Sal*I restriction sites and promoter elements by *Sal*I and *Apa*I restriction sites

Table 17.1 ARS, rDNA regions, selection markers and promoter elements of the CoMed vector system

| Region/gene	Donor organism	Reference
∎ ARS		
∎ 2 µm DNA	*S. cerevisiae*	Beggs et al. (1976)
∎ ARS1	*S. cerevisiae*	Gullov and Friis (1985)
∎ HARS	*H. polymorpha*	Kang and Gellissen (2005)
∎ SwARS	*Schw. occidentalis*	Piontek et al. (1998)
∎ rDNA region		
∎ NTS2-ETS-18SrDNA-ITS1	*H. polymorpha*	Ilgen et al. (2005)
∎ 25S rDNA	*A. adeninivorans*	Rösel and Kunze (1998)
∎ 18S rDNA	*A. adeninivorans*	Steinborn et al. (2005)
∎ ITS-5S-ETS-18S-ITS-5,8S-ITS	*A. adeninivorans*	Steinborn et al. (2005)
∎ NTS2-ETS-18SrDNA-ITS1	*A. adeninivorans*	Steinborn et al. (2005)
∎ Selection marker		
∎ *URA3*	*S. cerevisiae*	Rose et al. (1984)
∎ *LEU2*	*S. cerevisiae*	Froman et al. (1984)
∎ *ALEU2m*	*A. adeninivorans*	Wartmann et al. (2003a)
∎ *ATRP1*	*A. adeninivorans*	Steinborn et al. (2006)
∎ *HIS4*	*P. pastoris*	Thill et al. (1990)
∎ Expression cassette (promoter)		
∎ *FMD* promoter	*H. polymorpha*	Gellissen (2000)
∎ *MOX* promoter	*H. polymorpha*	Gellissen (2000)
∎ *TPS1* promoter	*H. polymorpha*	Amuel et al. (2000)
∎ AOX1 promoter	*P. pastoris*	Raschke et al. (1996)
∎ *TEF1* promoter	*A. adeninivorans*	Wartmann et al. (2002b)
∎ *AHSB4m* promoter	*A. adeninivorans*	Wartmann et al. (2003b)
∎ *GAA* promoter	*A. adeninivorans*	Bui et al. (1996)
∎ *ALIP* promoter	*A. adeninivorans*	Böer et al. (2005)
∎ *AINV* promoter	*A. adeninivorans*	Böer et al. (2004a)
∎ *AXDH* promoter	*A. adeninivorans*	Böer et al. (2004b)
∎ *RPS7* promoter	*Y. lipolytica*	Müller et al. (1998)

17.3 Application of the Wide-Range Vector to Protein Production in Various Yeasts

For proof a concept, GFP production was assessed in a range of yeast platforms transforming the different platform candidates in parallel with a single plasmid. For this purpose, a *GFP* reporter gene was employed which was inserted between the constitutive *A. adeninivorans*-derived *TEF1* promoter and the *S. cerevisiae*-derived *PHO5* terminator for expression control. Again, the resulting plasmid (pAL-HPH-TEF-GFP) was successfully used to transform *A. adeninivorans, S. cerevisiae, D. hansenii, D. polymorphus, H. polymorpha* and *P. pastoris* strains. It was found to be integrated in low copy numbers in all transformants.

Transformants were tested for the recombinant product either by Western Blot analysis or by fluorescence microscopy. The amounts varied only slightly among various transformants (Terentiev et al., 2004a; Fig. 17.3). In a second example, an expression/integration vector was constructed for the secretion of the pharmaceutically important cytokine interleukin-6 (IL-6), now combining an rDNA targeting sequence and an *A. adeninivorans*-derived *LEU2* gene (Wartmann et al., 2003a) for selection. For assessment, we inserted an expression cassette harbouring an ORF for a MFa1/IL-6 fusion protein under control of the *TEF1* promoter described before and transformed *leu2* auxotrophic strains of *A. adeninivorans*, *H. polymorpha* and *S. cerevisiae*. Again, mitotically stable strains were generated. Representatives of the three derived strain collections efficiently secreted the recombinant cytokine into the medium. In this case, product differences could be observed when comparing the secretion products of the different yeast species: the *H. polymorpha* and *S. cerevisiae*-derived molecules were found to be of smaller size than that secreted from the *A. adeninivorans* host. A more detailed comparative MS analysis of tryptic peptides revealed an N-terminal truncation at position Arg8 in *H. polymorpha* and *S. cerevisiae*, but a correctly processed mature IL-6 in *A. adeninivorans* (Steinborn et al., 2006, 2007). This is probably due to the lack of a thiol protease in this dimorphic species. The result emphasizes the need of a careful early pre-selection of a platform for the development of a production process.

17.4   Wide-Range rDNA Integration of Multiple Expression Cassettes

Following a previous observation that the integrated heterologous DNA can be present as multiple clusters inserted in the rDNA, it was demonstrated that rDNA plasmids, each equipped with the identical targeting element and the identical selection marker, but bearing different reporter genes could be integrated simultaneously into the rDNA of *H. polymorpha* (Klabunde et al., 2002) Thus, this approach provides an attractive tool for the rapid generation of recombinant strains from a diverged background that simultaneously co-produce several proteins in desired stoichiometric ratios. The following example describes the comparative assessment of different yeasts for the production of polyhydroxyalkanoates (PHA) co-integrating and co-expressing three genes of the PHA synthetic pathway from *Ralstonia eutropha*. For simultaneous assessment, three different yeasts, namely *D. polymorphus*, *D. hansenii* and *A. adeninivorans,* were selected. For introduction of the new metabolic pathway, wide-range expression vectors were equipped with the genes *phb*A*, phb*B and *phb*C of the polyhydroxyalkanoate (PHA) biosynthetic pathway of *Ralstonia eutropha* encoding β-ketothiolase, NADPH-linked acetoacetyl-CoA reductase and PHA synthase under control of the *A. adeninivorans*-derived *TEF1* promoter. Following the previous examples, the vectors were further equipped with an rDNA sequence and the *E. coli*-derived *hph* gene for wide-range integration and selection. Representatives of the three resulting strain collections were found to
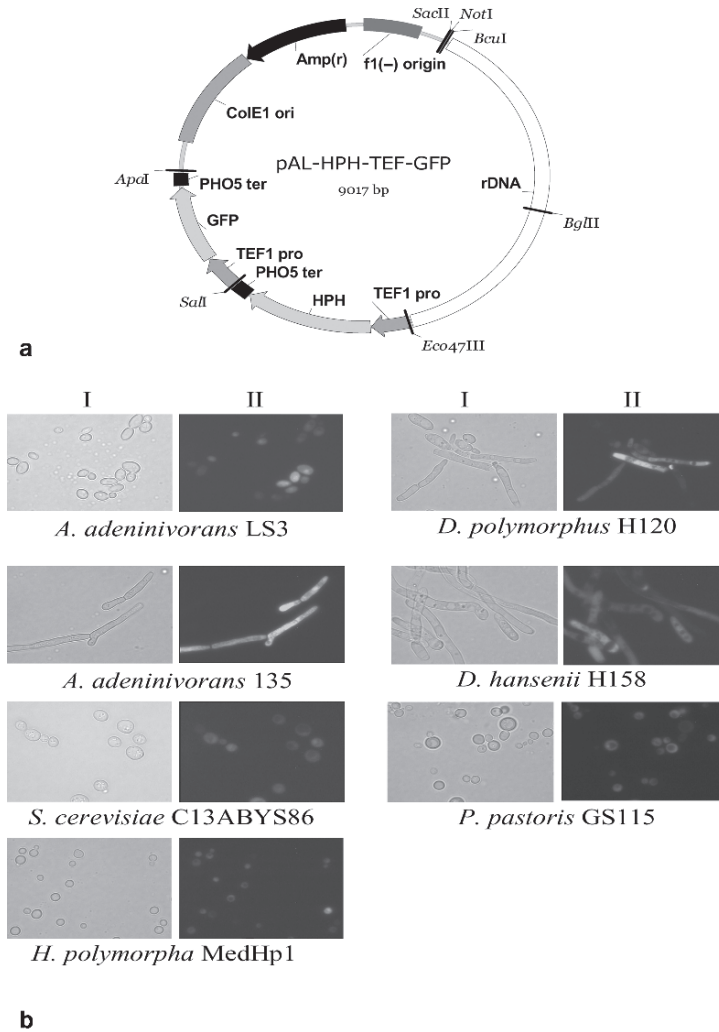
Fig. 17.3 Comparative assessment of GFP production in various yeasts. (A) Physical map of the expression/integration vector pAL-HPH-TEF-GFP used in this study. The vector contains the 25S rDNA sequence of *A. adeninivorans* (rDNA, white box) and an expression cassette for the *E. coli*-derived *hph* gene as selection marker in the order *A. adeninivorans*-derived *TEF1* promoter (TEF1 pro, grey segment), the *hph*-coding sequence (HPH, grey segment), *S. cerevisiae*-derived *PHO5* terminator (PHO5 ter, black bar). The vector further contains a second expression cassette with *TEF1* promoter – *GFP* ORF – *PHO5* terminator elements and an unique *Bgl*II site within the rDNA sequence for linearization. (B) Detection of recombinant GFP-producing yeast cells by fluorescence microscopy. Transformants were cultured for 48 h in YEPD medium at 30 °C and subsequently used for fluorescence analysis. (I) transmission, (II) GFP-fluorescence

contain all three heterologous genes as single copies mitotically stable integrated into the genome. In fed-batch cultivations in minimal medium supplemented with 1% ethanol as carbon source, the recombinant *A. adeninivorans* cells were able to convert efficiently the substrates acetyl-CoA and propionyl-CoA to PHA (2.2% of dry weight). In contrast, this level is relatively low with 4.2×10^{-3}% and 4.8×10^{-3}% of dry weight at the recombinant *D. hansenii* and *D. polymorphus* strains, respectively. Here, further optimization of the cultivation condition should improve this situation (Terentiev et al., 2004b; Steinborn et al., 2006, Fig. 17.4)
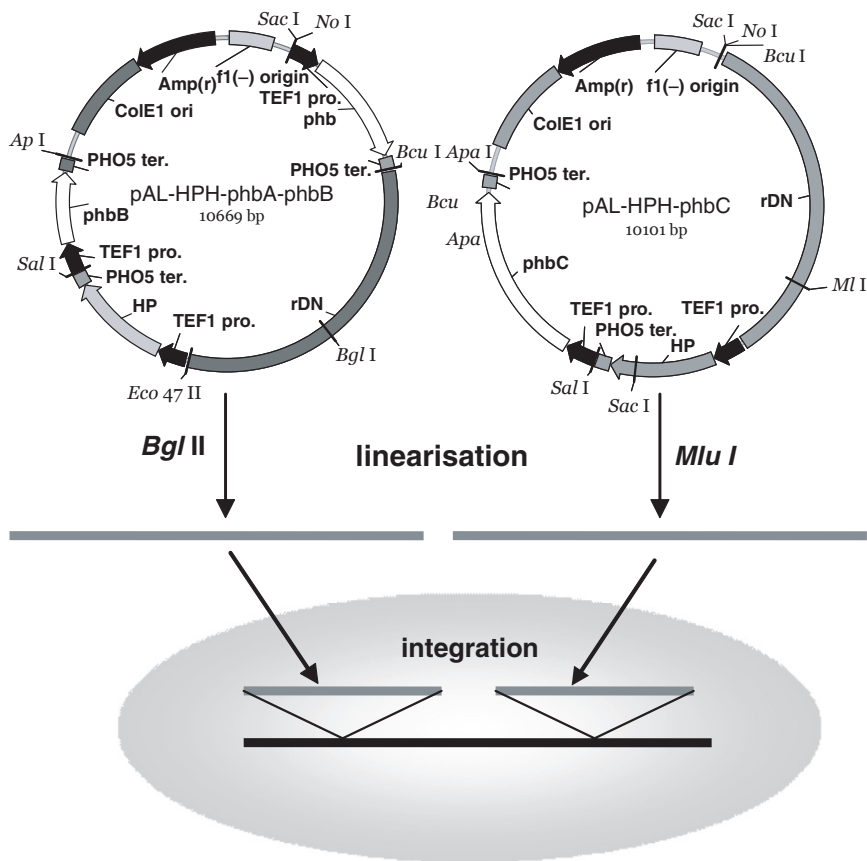
Fig. 17.4 PHA production in *A. adeninivorans, D. hansenii and D. polymorphus*. Transformation procedure based on simultaneous integration of the plasmids pAL-HPH-phbA-phbB and pAL-HPH-phbC into the 25S rDNA of *A. adeninivorans* LS3, *D. hansenii* H158 and *D. polymorphus* H120. The two plasmids pAL-HPH-phbA-phbB and pAL-HPH-phbC containing the expression cassettes with *phbA, phbB* and *phbC* genes are linearised by *Bgl*II or *Mlu*I digestion, respectively. The resulting fragments flanked by 25S rDNA sequences are co-integrated into the 25S rDNA by homologous recombination. Transformants are selected firstly by resistance to hygromycin B. In a second step, after PCR, these transformants were selected containing both plasmids (pAL-HPH-phbA-phbB and pAL-HPH-phbC)

In yet another example, the co-integration approach was applied to the improvement of IFN-γ secretion. IFN-γ was found to poorly secrete as overglycosylated proteins from various hosts (Gellissen et al., 2002). For potential improvements, strains were generated in which the gene for the cytokine was co-integrated and co-expressed together with candidate genes that could potentially influence and improve secretion and glycosylation. Of several candidate genes tried, the *H. polymorpha*-derived *CNE1* gene encoding calnexin was found to improve secretion of the cytokine considerably. The size of the secreted product corresponded to that of core-glycosylated molecules (Steinborn et al., 2006).

17.5 Conclusions and Perspectives

Integration of heterologous DNA into a range of yeast hosts in parallel has been successfully demonstrated for transformation with a single plasmid and for co-transformation with several plasmids. The existing catalogue of elements will be continuously supplemented by new promoter elements and a range of selection markers and the respective auxotrophic hosts. The CoMed vector system is very versatile and easy to handle. This important tool makes possible the simultaneous assessment of a wide range of yeast for a particular product and process development. Having it at hand, a high probability of success for an anticipated development can be envisaged.

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