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Identification of an ARS element and development of a high efficiency transformation system for *Pichia guilliermondii*

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Abstract An **A**utonomously **R**eplicating **S**equence element adjacent to the *RIB1* gene encoding GTP cyclohydrolase II of the yeast *Pichia guilliermondii* was identified by transformation experiments. Detailed sequence analysis unveiled two potential ARS elements located 5′ and 3′ of the *RIB1* open reading frame. The chromosomal fragment containing the ARS-like sequence 3′ to the *RIB1* structural gene, called PgARS, conferred high transformation frequencies of 10^4–10^5 transformants/μg of DNA to a pUC19-derived plasmid in *P. guilliermondii*. The PgARS element also conferred autonomous replication to hybrid plasmids in this host. Based on this element a series of *Escherichia coli* shuttle vectors for efficient transformation of the flavinogenic yeast *P. guilliermondii* was developed.

Key words Autonomously replicating sequence · Flavinogenic yeast · GTP cyclohydrolase II · Transformation system

This manuscript is devoted to the memory of Georgiy M. Shavlovsky

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Introduction

The flavinogenic yeast *Pichia guilliermondii* is of increasing scientific and industrial interest. In addition to a large variety of carbohydrates this microorganism can utilize hydrocarbons as a sole source of carbon and energy (Shchelokova et al. 1974). The ability to synthesize riboflavin (vitamin B_2) in large quantities under conditions of iron starvation (Shavlovsky and Logvinenko 1988 a, b) has attracted industrial interest. Flavin biosynthesis is under the control of negative, Rib80p and Rib81p, and positive, Rib83p and Rib84p, transcription factors, and iron (Shavlovsky and Logvinenko 1988 a, b). Despite progress in developing efficient transformation systems for other "non-conventional" yeast species, like *Kluyveromyces lactis* (Das and Hollenberg 1982; Bianchi et al. 1987), *Kluyveromyces fragilis* (Das et al. 1984), *Pichia pastoris* (Cregg et al. 1985), *Pichia stipitis* (Yang et al. 1994), *Hansenula polymorpha* (Gleeson et al. 1986; Roggenkamp et al. 1986; Tikhomirova et al. 1986), *Candida maltosa* (Takagi et al. 1986; Sasnauskas et al. 1992), *Candida albicans* (Kurtz et al. 1987) and *Yamadazyma* (*Pichia*) *ohmeri* (Piredda and Gaillardin 1994), so far no ARS elements have been identified in *P. guilliermondii* and transformation was restricted to the use of *Saccharomyces cerevisiae* vectors (Kunze et al. 1985 a, b). The construction of high efficiency transformation systems is thus necessary to explore the regulatory mechanisms involved in flavinogenesis and to further exploit the technological applications of this host strain.

The *P. guilliermondii* genes *RIB7*, coding for riboflavin synthase (Logvinenko et al. 1993), and *RIB1*, coding for GTP cyclohydrolase II (Zakal'sky et al. 1990), have been cloned and sequenced (Y. Boretsky, unpublished; Liauta-Teglivets et al. 1995). 5′ and 3′ to the *RIB1* open reading frame ARS-like elements with homology to the ARS consensus sequence, ACS, as defined for *S. cerevisiae* (Cregg et al. 1985; Rowley et al.

1994) were identified. Here we report on the analysis of an ARS element 3′ to the *RIB1* ORF, called PgARS, that confers high transformation efficiency to a pUC19-derived plasmid containing *RIB1* or *RIB7* structural genes in *P. guilliermondii*.

Materials and methods

Strains, media and culture conditions. *P. guilliermondii* riboflavin-deficient mutants *rib1-21* and *rib7-162* with defective GTP cyclohydrolase II and riboflavin synthase, respectively (Shavlovsky et al. 1979), were used in yeast transformations. *Escherichia coli* riboflavin-deficient strains *ribA802-81* and *ribB802-45* [F^-e14^- ($McrA^-$) *lacY1 supE44 galK2 galT22 rfbD1 metB1 mcrB1 hsdS3* ($r_k^- m_k^-$)] with defective GTP cyclohydrolase II and riboflavin synthase, respectively, were used to select and propagate various plasmids that contain *RIB1* or *RIB7* genes of *P. guilliermondii* (Tesliar and Shavlovsky 1983). Standard LB and YPD (1% yeast extract, 2% bacto pepton and 2% dextrose) media were used to propagate bacterial and yeast cells (Ausubel et al. 1990). Transformed *E. coli* strains were maintained on media containing 100 mg/l of ampicillin. Riboflavin-deficient strains of bacteria and yeasts were grown on media containing riboflavin at a concentration of 50 mg/l and 200 mg/l. *P. guilliermondii* cells were cultivated at 29 °C. Transformed yeast and bacterial strains were maintained on riboflavin-deficient media.

Cloning and transformation procedures. *E. coli* transformations were performed according to the method of Cohen (Maniatis et al. 1982). DNA manipulations were carried out as described by Maniatis et al. (1982). *P. guilliermondii* was transformed by the lithium chloride method (Gleeson et al. 1986) or by using a modified spheroplast method as described for *P. pastoris* (Cregg et al. 1985). 1 M sorbitol was replaced by 1 M sucrose in all buffers (SED, SCE, CaS). Transformed spheroplasts were diluted in 1 M sucrose and plated directly on YPD plates containing 2% agar and 1 M sucrose, without using overlay agar. Colonies grew within 4–7 days. Plasmid constructs used in this study are listed in Fig. 1 a and b. Plasmid p19R1 contains a 2.1-kb *Sal*I fragment of the chromosomal DNA fragment carrying the *RIB1* gene (GTP cyclohydrolase II) cloned into the *Sal*I site of pUC19 (Yanish-Perron et al. 1985). Plasmid p19R1Xh contains a 1.68-kb *Sal*I-*Xho*I fragment of the *RIB1* region, and plasmid p19R7 contains a 1.4-kb *Hin*dIII-*Eco*RI fragment of the *RIB7* gene (riboflavin synthase), inserted into the respective sites of pUC19. Plasmids p19R7R1-1 and p19R7R1-5 are derivatives of p19R7 containing a 2.1-kb fragment of *RIB1* cloned into the *Eco*RI site of p19R7 in opposite orientations. Plasmids p19R7-34 and p19R7-38 contain a 0.76-kb *Hin*cII fragment of the 5′ region, and plasmids p19R7-51 and p19R7-55 contain a 1.33-kb *Pst*I fragment of the *RIB1* gene in both orientations, cloned into the *Eco*RI site of p19R7 (Fig. 1 b). Plasmids p19R7-3 and p19R7-7 contain a 0.82-kb *Hin*cII fragment of the 3′ region of the *RIB1* gene, in both orientations, cloned into the *Eco*RI site of plasmid p19R7 (Fig. 1 b). Isolation of plasmid DNA from *P. guilliermondii* transformants was carried out by the "10-min. method" (Hoffman and Winston 1987). Stability of the *P. guilliermondii* transformants was analysed as described previously (Cregg et al. 1985). Southern blot analysis was performed on undigested chromosomal DNA using a 0.76-kb *Hin*cII fragment of the *RIB1* gene and a 1.4-kb *Hin*dIII-*Eco*RI fragment of the *RIB7* gene as hybridization probes. DNA fragments were labelled using a non-radioactive digoxigenin-probe labelling system (Boehringer Mannheim).

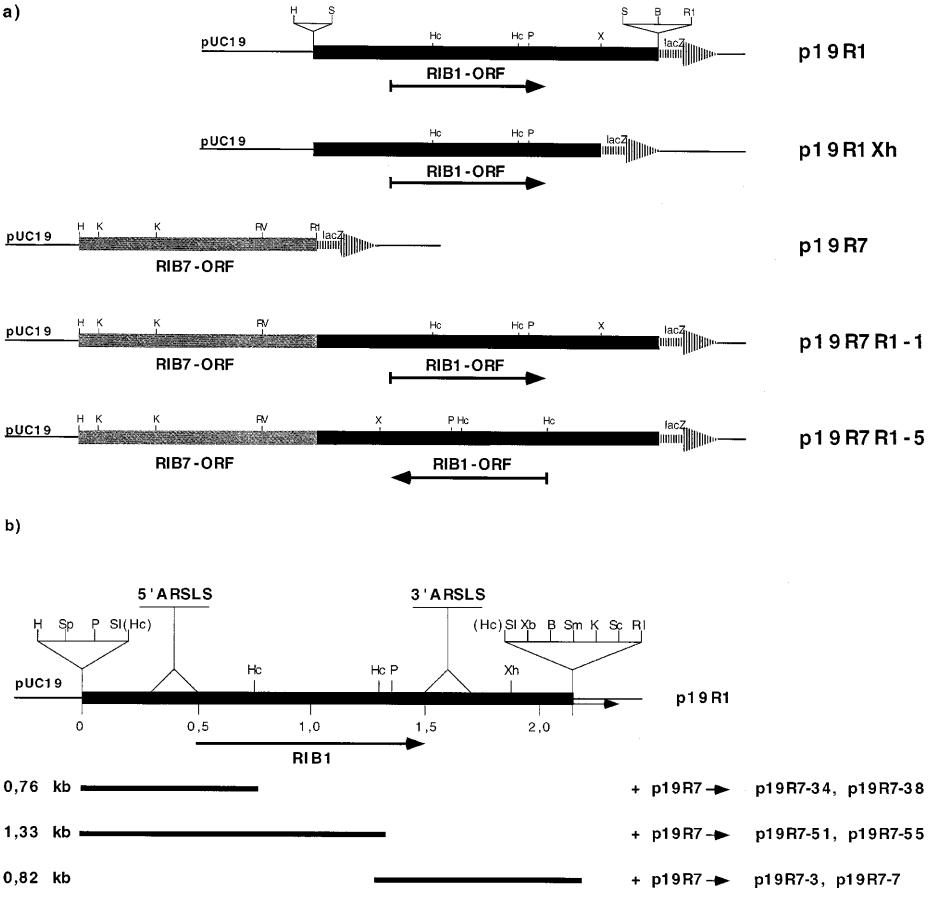

Fig. 1 a, b Clones used in this study. **a** plasmid p19R1 harbors a 2.1-kb *P. guilliermondii* genomic DNA fragment carrying the *RIB1* gene. Plasmid p19R1Xh is a *ΔXhoI*/*Sal*I p19R1. Plasmid p19R7 harbors the *RIB7* gene within a 1.4-kb *P. guilliermondii* genomic DNA fragment. The *RIB1* insert of p19R1 was cloned into the *Eco*RI site of plasmid p19R7 in opposite orientations. **b** *RIB1* gene fragments cloned into the *Eco*R1 site of plasmid p19R7. *Thin lines* depict vector sequence, *triangles* are the multiple cloning sites, *thick lines* represent the cloned yeast genomic DNA fragments

Results

Identification of a *P. guilliermondii* ARS element

The efficiency of transformation of *P. guilliermondii rib1* mutant cells with plasmid p19R1 containing the *RIB1* gene (Fig. 1 a) was about 150 times higher compared to *P. guilliermondii rib7* mutants transformed with plasmid p19R7 harboring the *RIB7* gene (Table 1), using the lithium chloride method. The spheroplast method resulted in a transformation efficiency of 1.5×10^4 transformants/μg of DNA for *rib1* mutants transformed with plasmid p19R1, whereas *rib7* mutants transformed with plasmid p19R7 yielded only 50 transformants/μg of DNA. Both mutants, *rib1* and *rib7*, are isogenic derivatives of the L2 *P. guilliermondii* genetic line, differing only in the *RIB1* and *RIB7* loci. Both p19R1 and p19R7 plasmids used for transformation are based on the bacterial vector pUC19 (Yanish-Perron et al. 1985). Thus, the high transformation efficiency of the plasmid complementing the *rib1* mutation suggests the presence of an ARS element on the genomic fragment harboring the *RIB1* gene.

To analyze this phenomenon in greater detail, a number of p19R7-derived plasmids, as outlined in Fig. 1 a and b, were constructed. Transformation efficiencies, using both lithium chloride and spheroplast transformation procedures, are summarized in Table 1. The 1.68-kb *RIB1* gene fragment (p19R1Xh) confers similar transformation efficiencies as the original *RIB1* construct (p19R1). Insertion of the *RIB1* gene fragment in both orientations into plasmid p19R7 harboring the *RIB7* gene (plasmids p19R7R1-1 and p19R7R1-5) resulted in a 950–1500-fold increase of the transformation efficiency of the *rib7* mutant (Table 1), further supporting the presence of an autonomously replicating sequence (ARS) adjacent to the *RIB1* gene. The presence of an ARS sequence on a plasmid is known to increase the transformation efficiencies of yeast cells 100–10 000-fold (Beggs 1978; Struhl et al. 1979; Cregg et al. 1985).

Table 1 Transformation frequency of *P. guilliermondii* with plasmids that contain either *RIB1*, *RIB7* or both *RIB1* and *RIB7* genes of this yeast species

Strain	Plasmid	Transformation frequency, transf./μg DNA	
		Spheroplast method	Lithium chloride method
rib1-21	p19R1	1.5×10^4	1.5×10^2
rib1-21	p19R7R1-1	5×10^4	–
rib1-21	p19R7	0	0
rib7-162	p19R7	5×10^1	1.2
rib7-162	p19R7R1-1	4.7×10^4	1.2×10^2
rib7-162	p19R7R1-5	7.5×10^4	–
rib7-162	p19R1	0	0

"–": not determined

Characterization of an ARS element adjacent to the *RIB1* gene

Sequence analysis of the *RIB1* gene revealed the presence of two ARS-like sequences 5′ and 3′ to the open reading frame, 5′ARSLS and 3′ARSLS (Fig. 2). Both areas are rich in A and T (71–73% over 120–130 bp), which is significantly higher than the average A + T content of 55–65% reported for chromosomal DNA of *Pichia* species (Storck and Alexopoulos 1970). Furthermore, the ARS-like sequences contain regions similar to the ARS consensus sequence, ACS, defined for *S. cerevisiae* (Rowley et al. 1994). Like PARS sequences in *P. pastoris* (Cregg et al. 1985) the *P. guilliermondii* ARS-like sequences contain numerous short repeats. No comparable regions were identified within, or adjacent to, the *RIB7* gene (Boretsky, unpublished).

To localize and further characterize the putative *P. guilliermondii* ARS, numerous plasmids were constructed that contain the 5′ARSLS or 3′ARSLS inserted into the reporter plasmid p19R7 harboring the *RIB7* gene. Transformation of *rib7* mutants and restoration of riboflavin prototrophy was more than 1000-fold higher with plasmids p19R7-3 and p19R7-7, harboring the 3′ARSLS in both orientations. By contrast, the transformation efficiency of *rib7* mutants transformed with plasmids p19R7-34, p19R7-38, p19R7-51 and p19R7-55 containing the 5′ARSLS was as low as with the control plasmid p19R7 without an insert (Table 2). These data suggest that only the 3′ARSLS confers high transformation efficiencies and that the 5′ARSLS does not function as an autonomously replicating sequence on a plasmid in this yeast species.

Mitotic stability of transformed plasmids

rib7 mutants transformed with plasmids harboring the *RIB7* gene and various 5′ and 3′ ARSLS-containing inserts (see Table 2) were grown for ten generations under non-selective conditions in the presence of 200 mg/l of riboflavin. Transformants harboring plasmids carrying the 5′ARSLS retained the Rib^+ phenotype even after ten generations under non-selective conditions, which might be due to integration of the plasmids into the chromosome. By contrast, 70–90% of the transformants harboring the 3′ARSLS on the plasmid lost their Rib^+ phenotype (Table 2), which is consistent with the observation that plasmids carrying chromosomal ARSs are rather unstable under non-selective growth conditions (Glover 1988). The fact that a residual 10–30% of transformants maintained the plasmid might be the result of the formation of multimeric plasmids, which was described previously for *H. polymorpha* and *C. albicans* (Tikhomirova et al. 1986; Kurtz et al. 1987). Thus, high frequency plasmid-loss suggests the presence of an ARS adjacent to the cloned *RIB1* gene.

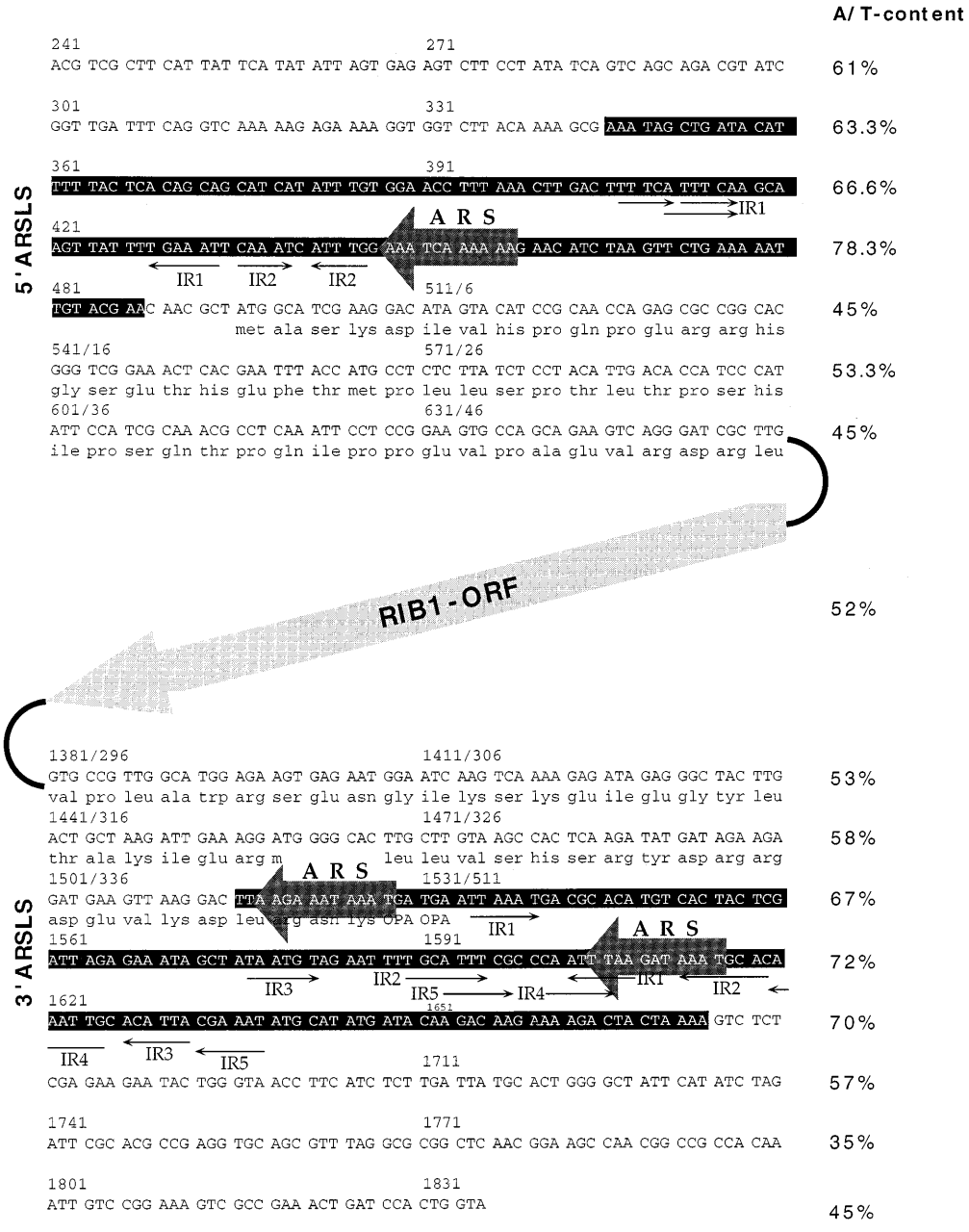

Fig. 2 DNA sequence and deduced amino acid sequence of 5′ and 3′ end regions of the *P. guilliermondii RIB1* ORF (EMBL, accession number Z49093). 5′ ARS-like (*5′ARSLS*) and 3′ ARS-like (*3′ARSLS*) sequences are shown inversed. Sequences homologous to the *S. cerevisiae* ARS consensus sequence (ACS) are shown by *thick dark arrows*. Inverted repeats (IRs) are shown by *thin arrows with their respective numbers*. The adenine plus thymine base pair content (A/T-content) is shown in percent

Table 2 Transformation of *P. guilliermondii* with plasmids that contain 5′ or 3′ ARS-like sequences

Strain	Plasmid	Type of *RIB1* ARSLS cloned	Transformation frequency, transf./μg DNA (spheroplast method)	Loss of RIB$^+$ phenotype after ten generations in nonsel. medium (%)
rib7-162	p19R7	Absent	5.5×10^1	0
rib7-162	p19R7-34	5′ARSLS	4.4×10^1	0
rib7-162	p19R7-38	5′ARSLS	9.7×10^1	0
rib7-162	p19R7-51	5′ARSLS	8.8×10^1	0
rib7-162	p19R7-55	5′ARSLS	5.9×10^1	0
rib7-162	p19R7-3	3′ARSLS	7.5×10^4	88
rib7-162	p19R7-7	3′ARSLS	9.5×10^4	70

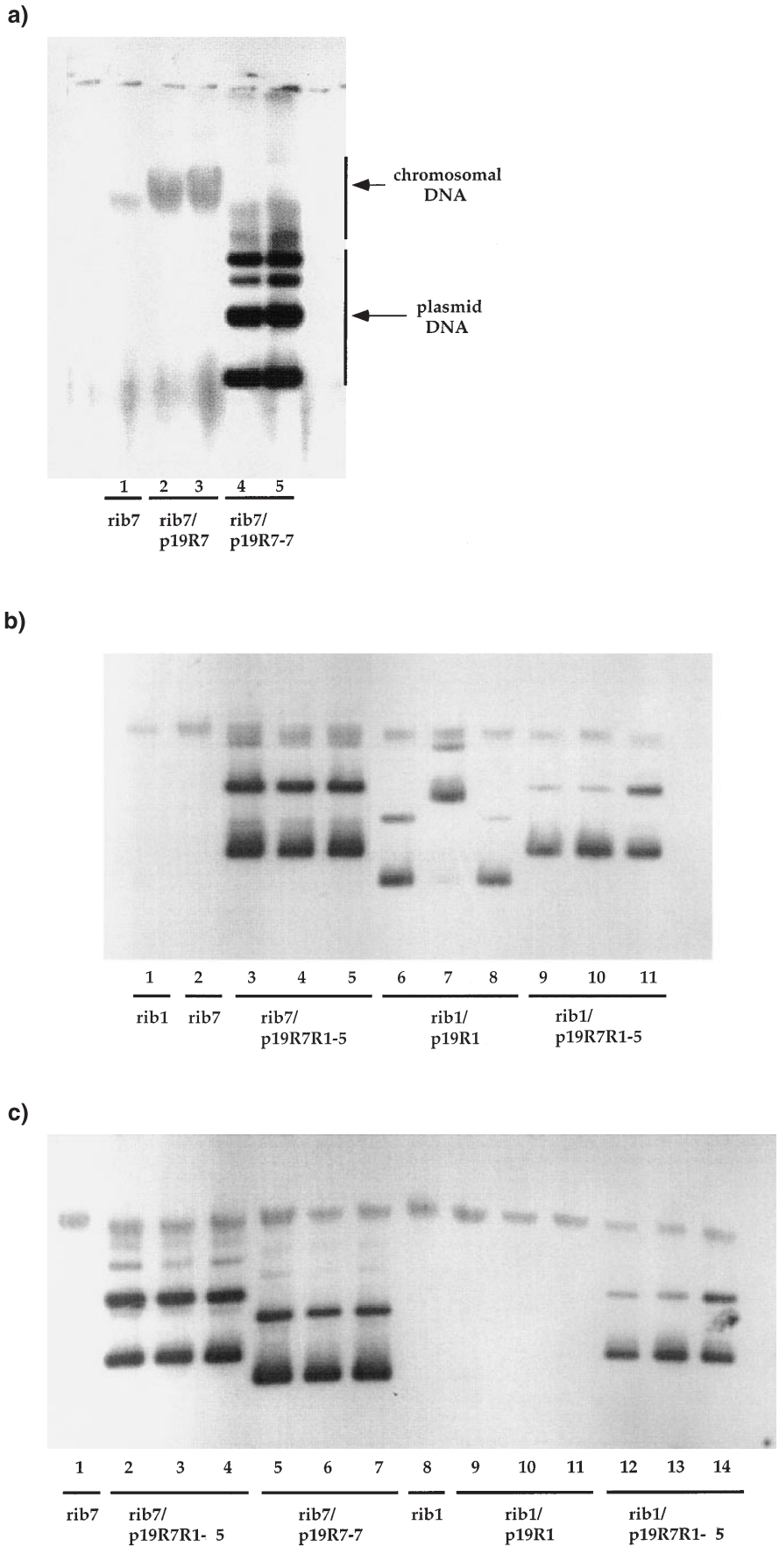

Fig. 3 a–c Southern blot analysis of total DNA of yeast transformants. Mutant and transformed strains from which total DNA was prepared are shown under the corresponding lanes. Undigested total DNA was separated on 0.8% agarose gels, transferred to positively charged nylon membranes and hybridized with a digoxigenin-labelled **a** a 1.4-kb *Hin*dIII-*Eco*RI fragment *R1B7* gene-specific probe, **b** a 0.76-kb *Hin*cII fragment *RIB1* gene-specific probe and **c** a 1.4-kb *Hin*dIII-*Eco*RI fragment *RIB7* gene-specific probe

Autonomous replication of plasmids containing the 3′ *ARS*

Plasmids p19R7-3, p19R7-7, p19R7R1-1 and p19R7R1-5 (Fig. 1 a and b) were isolated from *P. guilliermondii* transformants and transformed into *E. coli*. Plasmid DNA was recovered in a good yield from yeast transformants, further supporting the notion of the episomal character of the plasmids. Restriction analysis of plasmids isolated from *E. coli* transformants did not indicate any alterations, e.g. by recombination of the plasmids, in *P. guilliermondii*. Furthermore, these re-isolated plasmids conferred the same high frequency of transformation when re-transformed into *Pichia* as the "original" clone. Taken together these data strongly suggest that the plasmids are indeed replicating autonomously in *P. guilliermondii*, and that a sequence 3′ to the *RIB1* gene functions as an ARS.

Additional support for the role of an ARS adjacent to the *RIB1* gene was obtained from Southern blot analysis of DNA isolated from transformed cells (Kurtz et al. 1987). Undigested total DNA isolated from yeast transformants was separated on 0.8% agarose gels, transferred to positively charged nylon membranes and hybridized with digoxigenin-labelled *RIB1* and *RIB7* probes. Yeast transformants harboring plasmids p19R1 (*RIB1* gene) and p19R7-7 (*RIB7* gene + 3′ARS) and p19R7R1-5 (*RIB7* gene and *RIB1* gene including the 3′ARS, in opposite orientation), display additional hybridization bands suggestive of additional copies of *RIB1* or *RIB7* DNA (Fig. 3). In contrast, total DNA purified from yeast transformed with parental plasmid p19R7, lacking the *RIB1*-adjacent ARS, produced a single broad band of hybridization at the level of chromosomal DNA. Although this analysis does not allow a quantification of plasmid copy numbers, it further supports the notion that insertion of the 3′ARS into plasmid p19R7 provides it with an autonomous status.

Discussion

A high transformation frequency and an autonomous state of plasmids in yeast cells are provided by ARS elements (Struhl et al. 1979). In general, ARSs are species-specific and usually do not show inter-species functionality. For instance, the *S. cerevisiae* ARS1 is not active in *Schizosaccharomyces pombe* (Beach and Nurse 1981), *K. lactis* (Das and Hollenberg 1982), *K. fragilis* (Das et al. 1984), *P. pastoris* (Cregg et al. 1985), or *C. maltosa* (Takagi et al. 1986). The *S. cerevisiae* 2-μm ori is active to some extent in *K. lactis* (Das and Hollenberg 1982) and *P. pastoris* (Cregg et al. 1985), but is not functional in *K. fragilis* (Das et al. 1984) and *C. maltosa* (Takagi et al. 1986). However, ARS elements from different species may have common features, like a high A + T content or the existence of direct and inverted repeats, and show some homology at the DNA sequence level (Cregg et al. 1985).

We have identified a *P. guilliermondii* ARS, called PgARS, which is located at the 3′ end of the *RIB1* open reading frame. It has ARS-features characteristic for other yeast species, such as a high A + T content (71% over a range of 130 bp), inverted repeats, and sequences homologous to an essential ARS consensus sequence of *S. cerevisiae* (Storck and Alexopoulos 1970; Cregg et al. 1985; Rowley et al. 1994). The function of the second ARS-like region, which is localized upstream of the *RIB1* open reading frame, is unknown. This sequence also has some features of the functional PgARS 3′ to the *RIB1* ORF, but the inverted repeats are short and include non-complementary bases. Most likely, some important but as-yet unidentified sequences that may be required for ARS functionality in *P. guilliermondii* are missing in the predicted 5′ ARS element. Alternatively, as the 5′ element is located within the core promoter region of the *RIB1* gene, the ARS function might be blocked by the transcription machinery.

The PgARS element was used to develop a high-efficiency transformation system for *P. guilliermondii*. We have shown that its presence on recombinant plasmids resulted in a high transformation frequency and an autonomous replication in the yeast cells. A host-vector transformation system, with *rib1* or *rib7* mutants as hosts and pUC19-based plasmids containing the *RIB1* or *RIB7* genes, respectively, as selection markers as well as the ARS element, provides an efficient tool for the development of molecular biology in *P. guilliermondii* and expands the technological applications for this industrially important yeast species.

References

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) (1990) Current protocols in molecular biology, vols 1 and 2. Greene Publishing Association at Wiley – Interscience, New York

Beach D, Nurse P (1981) High-frequency transformation of the fission yeast *Schizosaccharomyces pombe*. Nature 290:140–142

Beggs JD (1978) Transformation of yeast by a replicating hybrid plasmid. Nature 275:104–109

Bianchi MM, Falcone C, Jie ChX, Weslowski-Louvel M, Frontali L, Fukuhara H (1987) Transformation of the yeast *Kluyveromyces lactis* by new vectors derived from the 1.6 μm circular plasmid pKD1. Curr Genet 12:185–192

Cregg JM, Barringer KJ, Hessler AY, Madden KR (1985) *Pichia pastoris* as a host system for transformations. Mol Cell Biol 5:3376–3385

Das S, Hollenberg CP (1982) A high-frequency transformation system for the yeast *Kluyveromyces lactis*. Curr Genet 6:123–128

Das S, Kellermann E, Hollenberg CP (1984) Transformation of *Kluyveromyces fragilis*. J Bacteriol 158:1165–1167

Gleeson MA, Ortori GS, Sudbery PE (1986) Transformation of the methylotrophic yeast *Hansenula polymorpha*. J Gen Microbiol 132:3459–3465

Glover DM (ed) (1988) DNA cloning, vols 1 and 2. A practical approach. Mir, Moscow

Hoffman CS, Winston F (1987) A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene 57:267–272

Kunze G, Petzoldt C, Bode R, Samsonova I, Bottcher F, Birnbaum D (1985 a) Transformation of the industrially important yeasts

Candida maltosa and *Pichia guilliermondii*. J Basic Microbiol 25:141–144

Kunze G, Petzoldt C, Bode R, Samsonova I, Hecker M, Birnbaum D (1985 b) Transformation of *Candida maltosa* and *Pichia guilliermondii* by plasmid containing *Saccharomyces cerevisiae* ARG4 DNA. Curr Genet 9:205–209

Kurtz MB, Cortelyou MW, Miller SM, Lai M, Kirsch DR (1987) Development of autonomously replicating plasmids for *Candida albicans*. Mol Cell Biol 7:209–217

Liauta-Teglivets O, Hasslacher M, Boretsky YuR, Kohlwein SD, Shavlovsky GM (1995) Molecular cloning of the GTP-cyclohydrolase structural gene *RIB1* of *Pichia guilliermondii* involved in riboflavin biosynthesis. Yeast 11:945–952

Logvinenko EM, Stasiv YuZ, Zlochevskiy ML, Voronovsky AYa, Beburov MYu, Shavlovsky GM (1993) Cloning of the *RIB7* gene encoding the riboflavin synthase of the yeast *Pichia guilliermondii* (in Russian) Genetika 29:922–927

Maniatis T, Fritsch E, Sambrook J (eds) (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Piredda S, Gaillardine C (1994) Development of a transformation system for the yeast *Yamadazyma* (*Pichia*) *ohmeri*. Yeast 10:1601–1612

Roggenkamp R, Hansen H, Eckart M, Janowicz Z, Hollenberg CP (1986) Transformation of the methylotrophic yeast *Hansenula polymorpha* by autonomous replication and integration vectors. Mol Gen Genet 202:302–308

Rowley A, Dowell SJ, Diffley JFX (1994) Recent developments in the initiation of chromosomal DNA replication: a complex picture emerges. Biochim Biophys Acta 1217:239–256

Sasnauskas K, Jomantiene R, Lebediene E, Lebedys J, Januska A, Janulaitis A (1992) Molecular cloning and analysis of an autonomous replicating sequence of *Candida maltosa*. Yeast 8:253–259

Shavlovsky GM, Logvinenko EM (1988 a) Supersynthesis of flavins in microorganisms and its molecular mechanisms (in Russian). Prikl Biokhim Mikrobiol 24:435–447

Shavlovsky GM, Logvinenko EM (1988 b) The role of iron in the regulation of protein synthesis in microorganisms (in Russian). Uspechi Mikrobiologii 22:108–133

Shavlovsky GM, Sibirny AA, Kshanovska BV, Koltun LV, Logvinenko EM (1979) Genetic clasification of *Pichia guilliermondii* riboflavin-deficient mutants (in Russian). Genetika 15:1561–1568

Shchelokova IF, Zharova VP, Kvasnikov EI (1974) Obtaining hybrids in haploid strains of *Pichia guilliermondii Wickerham* assimilating oil hydrocarbons (in Ukrainian). Mikrobiol Zh 34:275–278

Storck R, Alexopoulos CJ (1970) Deoxyribonucleic acid of fungi. Bacteriol Rev 34:126–154

Struhl K, Stinchcomb DT, Scherer S, Davis RW (1979) High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc Natl Acad Sci USA 76:1035–1039

Takagi M, Kawai S, Chang MCh, Shibuya I, Yano K (1986) Construction of a host-vector system in *Candida maltosa* by using an ARS site isolated from its genome. J Bacteriol 167:551–555

Tesliar GE, Shavlovsky GM (1983) Localization of the genes coding for GTP cyclohydrolase II and riboflavin synthase on the chromosome of *Escherichia coli* K-12 (in Russian). Tsitol Genet 17:54–56

Tikhomirova LP, Ikonomova RN, Kuznetsova EN (1986) Evidence for autonomous replication and stabilization of recombinant plasmids in the transformants of yeast *Hansenula polymorpha*. Curr Genet 10:741–747

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119

Yang VW, Marks JA, Davis BP, Jeffries TW (1994) High-efficiency transformation of *Pichia stipitis* based on its *URA3* gene and a homologous autonomous replicating sequence, ARS2. Appl Environ Microbiol 60:4245–4254

Zakal'sky AE, Zlochevskiy ML, Stasiv Yu, Logvinenko EM, Beburov M, Shavlovsky GM (1990) Cloning of the *RIB1* gene coding for the enzyme of the first stage of flavinogenesis in the yeast *Pichia guilliermondii*, GTP-cyclohydrolase, in *Escherichia coli* cells. (in Russian). Genetika 26:614–620