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Linear Protein-Primed Replicating Plasmids in Eukaryotic Microbes

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Abstract Linear plasmids of eukaryotic microbes are contemporary manifestations of ancient viruses, which have adjusted to two cellular compartments during evolution, i.e., to the mitochondrium or to the cytoplasm. In either case, infectious viral functions do not (any longer) exist. Mitochondrial as well as cytoplasmic elements display a minimized gene equipment and an archetypical mode of replication. Plasmids of filamentous fungi are selfish DNA elements routinely residing in the mitochondria, in which they underwent coevolution with their hosts. In addition to the archetypical viral B-type DNA polymerase, they typically exclusively encode a viral RNA polymerase. Usually, there are neither positive nor negative impacts on their hosts. In a few instances, however, symptoms redolent of a molecular disease, such as the accumulation of defective mitochondria and early onset of senescence, manifest in plasmid-harboring strains. Cytoplasmic localization, which applies for almost all yeast linear plasmids known so far, evidently enforced a more complex enzyme repertoire (of viral origin) to accomplish autonomous extranuclear and extramitochondrial replication and transcription, such as a helicase,

ssDNA binding proteins, and a capping enzyme. Accompanying cytoplasmic plasmids relying functionally on an autonomous element are rather frequent; some encode protein toxins, which benefits the respective host while competing with other yeasts (killer phenotype). Such toxins assure autoselection of the plasmid system as well. Two distinct toxic principles are known up to the present: one was shown to be a tRNase, whereas the other clearly involves a DNA-damaging mode of action.

1 Introduction

Linear protein-primed replicating plasmids frequently occur in two kingdoms of eukarya, i.e., in planta and fungi. The S plasmids from *Zea mays* were the first discovered linear double-stranded DNA (dsDNA) elements (Pring et al. 1977); soon after, similar genetic traits were detected in the yeast *Kluyveromyces lactis* and in the filamentous ascomycete *Ascobolus immersus* (Gunge et al. 1981; Francou 1981). Though linear plasmids were initially considered to represent rare exceptions from the commonly accepted rule defining plasmids as extrachromosomal covalently closed circular replicons, a great and still growing number of linear elements have since been discovered. To date, they are recognized as rather frequently occurring, extrachromosomal genetic elements with growing evidence for a viral evolutionary origin. Here, we focus on protein-primed replicating linear plasmids in eukaryotic microorganisms, i.e., filamentous fungi and yeasts. Reviews addressing linear plasmids from filamentous fungi and yeasts separately have been published over the years (Meinhardt et al. 1990; Stark et al. 1990; Griffiths 1995; Gunge 1995; Meinhardt and Schaffrath 2001; Gunge and Tokunaga 2004; Jeske et al. 2006a). However, recently established techniques, such as allelic replacement, gene shuffling, site-specific mutagenesis, and—more importantly—the extended molecular characterization of a number of linear plasmids to the sequence level, have greatly increased our knowledge at the molecular genetic level.

2 Common Structural Features

Regardless of the cell type harboring them, linear replicons encounter the problem of avoiding shortening of the termini during each replication round. In eukaryotic chromosomes the telomerase, a ribonucleoprotein with reverse transcriptase activity, adds telomeric repeats copied from an internal RNA template onto the ends of the chromosomes (Greider and Blackburn 1985, 1989). Another strategy to maintain size is brought about by covalently closed DNA ends, as in some prokaryotic and eukaryotic linear DNA elements (see Chapters in this volume by Kobryn (2007), Hertwig (2007), Galligan and Kennell (2007), Hashiba and Nagasaka (2007)).

The majority of fungal and yeast linear plasmids employ a replication mode using protein priming, thereby facilitating full replication of termini. In addition to the protein primers, which remain covalently bound (terminal proteins, TPs) to the 5′ ends of the newly synthesized DNA molecule, all these elements have in common terminal inverted repeat (TIR) sequences resembling both linear genomes of bacteriophages and adenoviruses, suggesting a common mode of replication (Paillard et al. 1985; Kempken et al. 1989; Meinhardt et al. 1990). Indeed, the protein-primed replication mechanism is well studied in adenoviruses and bacteriophages, serving as a model for linear plasmid replication (reviewed by Challberg and Kelly 1982; Salas 1991; Meijer et al. 2001). For *Bacillus* phage phi29, a serine residue of the TP acts as the replication primer by facilitating desoxynucleotidylation of the free hydroxyl group. Along with the TP, the phage-encoded DNA polymerase forms an initiation complex at the second nucleotide of the TIR (Kamtekar et al. 2006). Subsequently, the TP-DNA polymerase complex slides back to the first nucleotide, starting elongation of replication and dissociation of the TP–polymerase complex (Mendez et al. 1992, 1997; Kamtekar et al. 2006). There are no internal origins of replication and the highly processive DNA polymerase is able to replicate the entire phi29 genome (Kamtekar et al. 2004). Though replication initiation may occur simultaneously at both ends, yielding two fully replicated copies, single-strand (ss) replication intermediates occur as well (Fig. 1). By hybridization of the inversely oriented TIR sequences such ssDNAs form so-called panhandle structures, providing a dsDNA end suitable for initiation of a novel replication cycle, eventually resulting in a fully replicated dsDNA copy of the genome (Fig. 1; Challberg and Kelly 1992).

Such a replication mechanism realized in adenoviruses, bacteriophages, and linear plasmids led to the suggestion that not only do the bacteriophages and adenoviruses share a common ancestor, but also linear plasmids may have evolved from an archetypical protein-primed replicating genome some billion years ago (Davison et al. 2003). Interestingly, in both adenoviruses and bacteriophages, the replication module consists of the DNA polymerase and a separate TP encoding gene, which are, however, directly neighbored; in all linear plasmids from fungi and yeasts, a single open reading frame (ORF) encodes both the TP and the DNA polymerase (Meinhardt and Rohe 1993).

Thus, in addition to linearity, and the presence of TPs and TIRs, such a (fusion) TP-DNA polymerase gene represents a common feature likely to be found in all eukaryotic linear plasmids employing protein-primed replication. However, linear plasmids often exist as pairs or triplets, and there are several instances in which only one of the respective plasmids encodes a TP-DNA polymerase, which probably manages replication of the other linear plasmids (lacking such a gene) as well (see below; Kempken et al. 1989; Rohe et al. 1991; Klassen et al. 2001, 2002). Eukaryotic linear plasmids generally also encode their own RNA polymerases for transcription of plasmid-based

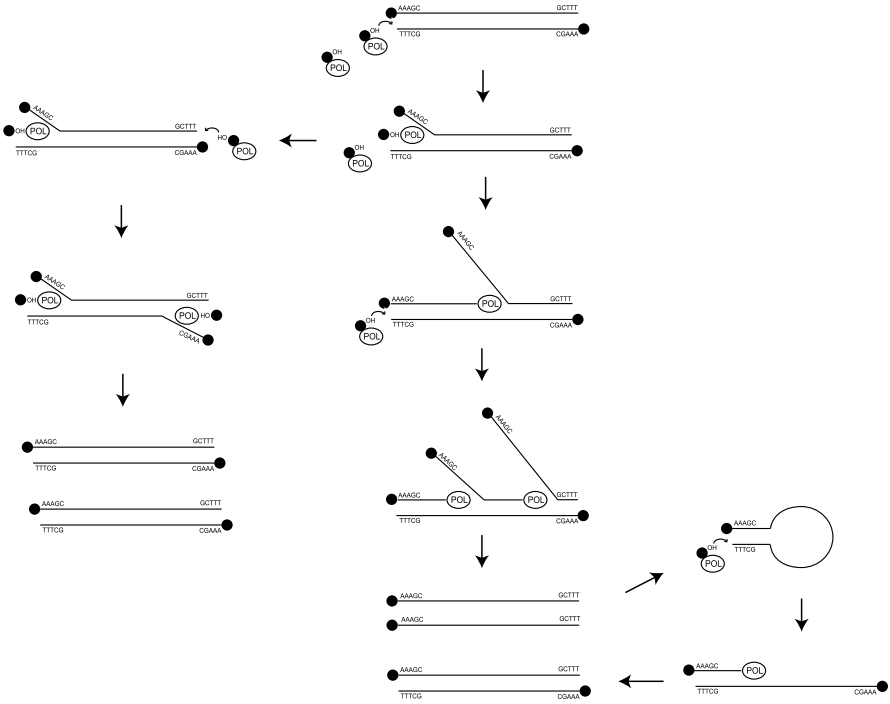


Fig. 1 Protein-primed strand displacement replication. The linear element is schematically represented by *two lines* with TIR sequences (AAAGC/GCTTT). *Filled circles* indicate plasmid-bound TPs; POL represents the TP-DNA polymerase fusion protein; the hydroxyl moiety (OH) of an amino acid acts as the primer

genes (see below), providing another clue for viral ancestry. As for the DNA polymerase, the RNA polymerase encoding gene is not necessarily found on each plasmid but is present at least once in each plasmid system (Kempken et al. 1992).

3 Linear Plasmids in Filamentous Fungi

Among the filamentous fungi, a great number of plasmids have been described for both ascomycetes and basidiomycetes; almost all of them reside in mitochondria (reviewed by Griffiths 1995). The only known exceptions are the linear plasmids from *Alternaria alternata*, which are apparently not localized in mitochondria (Shepherd 1992). Though there are circular plasmids, such as Mauriceville, Fiji, and LaBelle plasmids in *Neurospora* (Griffiths 1995), the great majority are linear molecules with TPs and TIRs. Frequencies of linear plasmids have been determined for a number of fungi belonging to

different genera, such as *Neurospora*, *Claviceps*, *Fusarium*, *Blumeria*, *Epichloë*, and *Podospora* (Arganoza et al. 1994; Samac and Leong 1988; Morgen et al. 1991; van der Gaag et al. 1998; Giese et al. 1990; Tudzynski et al. 1983; Tudzynski and Esser 1986; Düvell et al. 1988). Overall frequencies were estimated to match approximately 8–16% (van der Gaag et al. 1998); however, when the geographic distribution was taken into consideration, it became evident that linear plasmids may be endemic to specific geographic regions, in which they represent the norm rather than an exception. The kalilo plasmid may serve

Table 1 Mitochondrial linear plasmids of filamentous fungi for which complete nucleotide sequences are available (GenBank accession numbers in parentheses). Sizes of plasmids and TIRs are indicated. Coding capacity specifies genes identified; accession numbers for deduced polypeptides are indicated along with their sizes in amino acids (aa). DP, RP: DNA polymerase and RNA polymerase genes; unknown: gene of unknown function

Species	Plasmid (acc. no.)	Size/ TIRs (bp)	Coding capacity (size, acc. no.)	Refs.
Pleurotus ostreatus	pMLP1 (AF126285)	9879 381	unknown (239 aa; AAD39925) RP (903 aa; AAD39926) DP (1346 aa; AAD39927)	Kim et al. (2000)
Neurospora crassa	maranhar (X55361)	7052 349	RP (896 aa; CAA39045) DP (1021 aa; CAA39046)	Court and Bertrand (1992)
Neurospora intermedia	harbin-3 (NC_000843)	7050 350	RP (896 aa; AAD31445) DP (1035 aa; AAD31446)	Xu et al. (1999)
	kalilo (X52106)	8642 1365	RP (811 aa; CAA36326) DP (893 aa; CAA36327)	Chan et al. (1991b)
Gelasinospora sp.	Gel-kal (L40494)	8231 1137	RP (987 aa; AAB41447) DP (831 aa; AAB41448)	Yuewang et al. (1996)
Blumeria graminis	pBgh (AY189817)	7965 610	RP (973 aa; AAO37818) DP (1062 aa; AAO37819)	Giese et al. (2003)
Morchella conica	pMC3-2 (X63909)	6044 720	unknown (306 aa; CAB52198) DP (901 aa; CAA45364)	Rohe et al. (1991)
Podospora anserina	pAL2-1 (X60707)	8395 975	RP (948 aa; CAA43116) DP (1197 aa; CAA43117)	Hermanns and Osiewacz (1992)
Claviceps purpurea	pClKl (X15648)	6752 327	RP (970 aa; Nt. 112-3021) DP (1097 aa; Nt. 6641-3351) 4 minor ORFs	Oeser and Tudzynski (1989)
	pClT5 (X68490)	7113 574	RP (947 aa; Nt. 356-3199) DP (1050 aa; Nt. 6758-3606) 5 minor ORFs	Oeser et al. (1993)
Ascobolus immersus	pAI1 (X15982)	5142 535	DP (1202 aa; CAA34106) 4 minor ORFs	Kempken et al. (1989)

as an example, as it was found routinely in isolates from Hawaii (Yang and Griffiths 1993; Maas et al. 2005), but less frequently in Asia, Africa, Central America, and South Pacific islands (Arganoza et al. 1994). In *Claviceps purpurea* and *Blumeria graminis*, linear plasmids occur frequently irrespective of their geographic origins (Tudzynski and Esser 1986; Düvell et al. 1988; Giese et al. 1990, 2003), being indicative of a widespread distribution in these fungi.

Partial or entire nucleotide sequences are available for a considerable number of linear plasmids from both filamentous ascomycetes and basidiomycetes (Table 1). Sizes vary from 5142 bp (pAI1 of *Ascobolus immersus*) to 9879 bp (pMLP1 of *Pleurotus ostreatus*); all of them possess TIRs ranging in size from 327 to 1365 bp (Table 1).

Most known linear plasmids from filamentous fungi display a rather common genetic organization as for the maranhar plasmid from *Neurospora crassa* (Fig. 2), which contains two ORFs in an inverse orientation encoding the TP-DNA polymerase and the RNA polymerase, respectively. However, differently organized plasmids exist as well, such as pAI1 of *A. immersus* and pMC3-2 of the morel, which encode the typical TP-DNA polymerase fusion gene but lack an RNA polymerase gene (Kempken et al. 1989; Rohe et al. 1991; Fig. 2). In both instances the plasmids concomitantly exist along with other elements, suggesting the RNA polymerase to be encoded by an accompanying plasmid.

Such a scenario has indeed been proven for the linear plasmids discovered first, i.e., the S plasmids S1 and S2 from maize (Levings and Sederoff 1983; Paillard et al. 1985); S1 encodes the TP and DNA polymerase, while the RNA polymerase gene is located on S2.

3.1 The DNA Polymerase

DNA polymerases encoded by fungal linear plasmids are of the viral B type, like enzymes from adenoviruses and phages which display a replication mode employing protein primers (Ito and Braithwaite 1991; Kempken et al. 1992; Rohe et al. 1992). As already mentioned, however, linear plasmid encoded DNA polymerases carry the TPs as an N-terminal extension. Though suggested earlier (Oeser and Tudzynski 1989; Chan et al. 1991; Meinhardt and Rohe 1993), this structure was experimentally proven rather late for pGKL2 of *Kluyveromyces lactis* and later for pMLP1 of *Pleurotus ostreatus* (Takeda et al. 1996; Kim et al. 2000). Except for a weakly conserved motif (SYKN), there is only, if at all, a hardly detectable similarity among TP regions of distantly related plasmid DNA polymerases and the respective proteins from bacteriophages and adenoviruses, suggesting extended structural diversities. It remains totally obscure at present if, how, and where the TP is split off from the polymerase during replication. For the *Neurospora* plasmid kalilo, the molecular mass of the TP was determined to be approximately 120 kDa

(Vierula et al. 1990). It was suggested that it actually may represent the full-size TP-DNA polymerase fusion protein (Chan et al. 1991). On the contrary, however, for pMLP1 of *Pleurotus ostreatus*, as well as another linear plasmid, pMLP2, it was shown that TPs comprise only 70 and 72 kDa, respectively; consistently, DNA polymerase domains were immunologically not detectable, providing evidence for in vivo processing of the protein (Kim et al. 2000). Also, the molecular mass (approx. 36 kDa) of the TP of pGKL2 (Stam et al. 1986; Takeda et al. 1996) rules out a full-size TP-DNA protein. Replication initiation has been studied in detail for phage phi29; it was proven that the initiation complex consisting of TP and DNA polymerase starts DNA synthesis at the ends of the template. Dissociation of the above complex occurs shortly after elongation has begun, i.e., after the synthesis of approximately six nucleotides (Mendez et al. 1997; Kamtekar et al. 2006).

The highly processive phi29 DNA polymerase replicates the entire phage genome unassisted by a helicase or sliding clamp. Such unique features rely on the terminal protein region 2 (TPR2), which is similarly inserted in all DNA polymerases using protein primers. The TPR2 domain forms a doughnutlike structure, which enables high processivity and facilitates separation of template and nontemplate strands before entry into the polymerase active site (Kamtekar et al. 2004; Rodriguez et al. 2005). TPR2-like domains in linear plasmid encoded DNA polymerases (Dufour et al. 2000, 2003; Rodriguez et al. 2005) are assumed to compare functionally to the phi29 enzyme (Jeske et al. 2006a).

3.2 The RNA Polymerase

RNA polymerases encoded by mitochondrial linear plasmids from both plants and fungi are of the bacteriophage T7 type (Kuzmin et al. 1988; Oeser 1988; Oeser and Tudzynski 1989; Kempken et al. 1992; Cermakian et al. 1997). The latter does not employ a protein-primed replication mode; indeed, it is only distantly related to protein-primed replicating bacteriophages. The T7 RNA polymerase constitutes an archetypical single subunit enzyme which is not only encoded by T7-like phages and linear plasmids, but also is the common RNA polymerase in (plasmid free) mitochondria throughout the eukarya (Masters et al. 1987; Tiranti et al. 1997; Weihe et al. 1997; Cermakian et al. 1996). It has been suggested that the mitochondrial RNA polymerase, acquired from a T7 progenitor early in eukaryotic evolution, replaced the eubacterial multisubunit RNA polymerase that was originally present in the bacterial progenitor of the mitochondrium (Shutt and Gray 2006). Thus, while the DNA polymerase encoded by mitochondrial linear plasmids is clearly of viral origin (similar to adenoviral and phi29-like enzymes), it remains obscure whether the RNA polymerase encoding gene was acquired directly from a viral progenitor or from the eukaryotic host. Interestingly, phi29-like phages

do not possess their own RNA polymerases but recruit host encoded enzymes (reviewed by Meijer et al. 2001). It remains to be elucidated why T7-like RNA polymerases are required for maintenance of linear plasmids residing in mitochondria, although there is a T7-like polymerase anyway.

Though T7-like RNA polymerase genes are routinely found in known mitochondrial linear plasmids, it is not to be excluded that plasmid systems devoid of such genes may exist. Mitochondrial linear plasmids are known which encode a TP-DNA polymerase only (e.g., pAI1 and pMC2-3); however, sequencing of the entire set of plasmids present in such strains is necessary to resolve whether there is a T7-like RNA polymerase gene.

3.3
Additional ORFs

ORFs encoding predicted polypeptides differing articulately from DNA and/or RNA polymerases exist in a number of mitochondrial linear plasmids. Rather frequently, such reading frames overlap the DNA or RNA polymerase genes, as for pClK1, pClT5, and pAL-1 (Oeser and Tudzynski 1989; Oeser et al. 1993; Kempken et al. 1989).

In pMLP1 of *Pleurotus ostreatus* and pMC3-2 of *Morchella conica* large ORFs are located upstream of the DNA or RNA polymerase encoding genes (Rohe et al. 1991; Kim et al. 2000; Fig. 2). As the predicted protein of the respective pMLP1 ORF is remarkably basic, it was presumed to function as a TIR binding protein, possibly involved in replication initiation (Kim et al. 2000). However, such information is not conserved among mitochondrial linear plasmids which rules out its general requirement. A function is not known or attributable to any of the known additional reading frames; there is no obvious similarity either to functionally assigned genes or predicted polypeptides, or to each other. Hence, mitochondrial linear plasmids generally encode exclusively TPs, DNA polymerase, and RNA polymerase, and seldomly nonessential other proteins.

3.4
Phenotypes Associated With Fungal Linear Plasmids

Linear plasmids were detected in a number of plant pathogenic filamentous fungi; however, they apparently do not contribute to a pathogen's detrimental properties but rather persist as neutral passengers (Griffiths 1995).

In *Neurospora*, insertion of the linear plasmids kalilo and maranhar into the host's mitochondrial DNA routinely results in onset of senescence due to mitochondrial malfunction, ultimately leading to death (reviewed by Griffiths 1992; Bertrand 2000). Kalilo and maranhar insertion causes large inverted repetitions of mitochondrial DNA adjacent to the insertion site (Bertrand et al. 1985, 1986; Court et al. 1991). As a specific (defective) mitochondrial

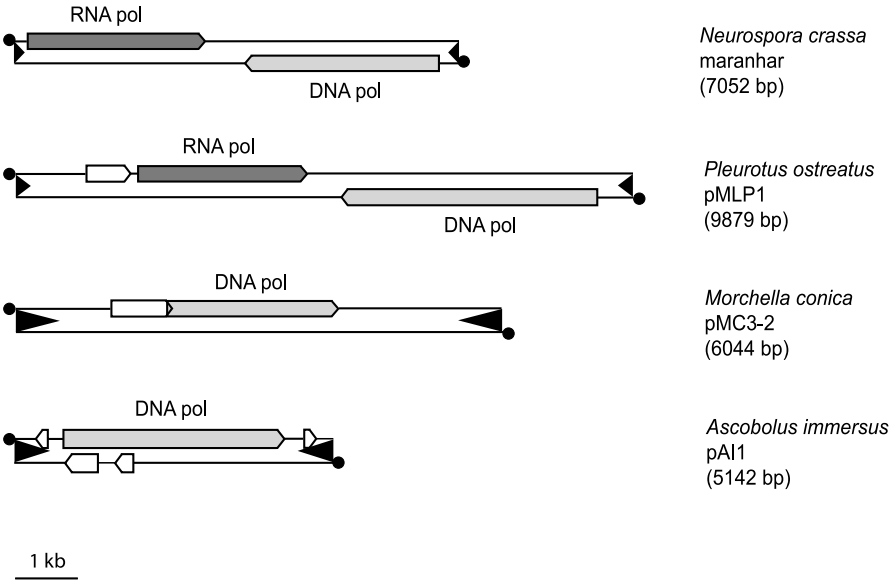


Fig. 2 Schematic representation of the structure and coding capacity of mitochondrial linear plasmids of filamentous fungi (a selection). *Arrows* indicate ORFs and their transcriptional direction; TPs are depicted as *filled circles*; TIRs correspond to *filled triangles*

DNA variant with inserted plasmids (IS-kalDNA or IS-marDNA) dominates in a particular senescent culture, it has apparently originated from a single insertion event (Myers et al. 1989). Supposedly, functionally impaired IS-kalDNA or IS-marDNA carrying mitochondria trigger mitochondrial replication to compensate for the defect and thus gradually replace wild-type mitochondria dividing at normal rates (Myers et al. 1989; Griffiths 1995).

Integration of a linear plasmid (pAL2-1) into the mitochondrial DNA has also been reported for *Podospora anserina* (AL2); however, in marked contrast to *Neurospora*, insertion of pAL2-1 resulted in a prolonged life span of the regularly aging fungus (Hermanns et al. 1994, 1995). Since other isolates of *P. anserina* carrying pAL2-1 homologous elements senesce normally (van der Gaag et al. 1998), such a phenomenon was apparently strain specific. Moreover, when *P. anserina* served as a model for studying the lifetime prolonging effect of calorie restriction (Maas et al. 2004), it was disclosed that life span (under calorie restriction) is indeed shortened by pAL2-1; hence, as for kalilo and maranhar, pAL2-1 was considered to induce an early onset of senescence (Maas et al. 2004, 2005).

In summary and by way of conclusion, most fungal linear plasmids appear to be cryptic elements, and occasionally they are associated with a molecular disease leading to massive accumulation of defective mitochondria which is—by the way—accompanied by a vast propagation of the inserted plasmid

copy. Thus, fungal linear plasmids represent selfish or parasitic DNAs with no discernible positive effects on their hosts.

4
Linear Plasmids in Yeasts

Yeast linear plasmids, originally detected in *K. lactis* (Gunge et al. 1981), are known to occur in a panoply of ascomycetous species belonging to different genera (such as *Pichia*, *Candida*, *Debaryomyces*, *Saccharomycopsis*, *Schwanniomyces*, *Botryoascus*) and also in the basidiomycetous representative *Trichosporon* (see Table 2; Kitada and Hishinuma 1987; Ligon et al. 1989; Worsham and Bolen 1990; Hayman and Bolen 1991; Bolen et al. 1992; Cong et al. 1994; Fukuhara 1995; Chen et al. 2000). A systematic screening among isolates deposited in the CBS-type culture collection revealed linear plasmids in 1.8% of the strains analyzed (Fukuhara 1995). At first sight—compared to filamentous fungi—few specimens seemed to harbor such genetic elements; however, plasmid occurrence in isolates of the same species is remarkably frequent, e.g., 16% for the pGKL system in *K. lactis* and 54% for pTP1 in *Trichosporon pullulans* (see also Table 2). The latter is so far the only basidiomycetous yeast species known to harbor linear plasmids and it remains to be elucidated whether this is an exception.

A striking difference between linear plasmids of filamentous fungi and yeasts concerns their localization (Table 2; Gunge et al. 1982; Ligon et al. 1989; Cong et al. 1994; Fukuhara 1995). There are only two mitochondrial yeast linear plasmids known (pPH1 and pPK1 from *Pichia heedi* and *P. kluyveri*; Blaissoneau et al. 1999), whereas almost all elements in filamentous fungi are mitochondrially associated (see above; Griffiths 1995). Based on the sequence data available for a number of cytoplasmic yeast linear plasmids and the mitochondrial pPH1, three types can be distinguished (Fig. 2): (1) mitochondrial elements, which resemble linear plasmids of filamentous fungi—indeed, genes encoding TP-DNA and RNA polymerase of pPH1 are arranged as for maranhar; (2) cytoplasmic autonomous elements, which are relatively large linear plasmids spanning at least 12 kb; in addition to TP-DNA and RNA polymerase other functions, apparently required for cytoplasmic inheritance, are encoded; and (3) cytoplasmic nonautonomous elements; smaller linear plasmids (i.e., < 12 kb), depending strictly on an aforementioned autonomous element; nonessential functions, such as a killer protein may be encoded.

4.1
Autonomous Elements

Extranuclear linear plasmids residing in the cytoplasm constitute the prevailing version of accessory genetic elements in yeast; routinely, they exist

Table 2 Yeast linear plasmids. Mitochondrial (pPH1, pPK1) and cytoplasmic linear plasmids of ascomycetes and basidiomycetes (pTP1) are given along with their sizes (in kb) and coding capacity. DP: DNA polymerase; RP: RNA polymerase; T: toxin; I: immunity; CB: chitin binding protein; CE: capping enzyme; SSB: single-strand binding protein; TRF: terminal recognition factor. Plasmid presence is derived from data accessible from the CBS (http://www.cbs.knaw.nl/) and/or original publications on the respective plasmid system (see also Cong et al. 1994; Fukuhara 1995)

Species	Plasmid system (kb)	Coding capacity	No. of strains analyzed (plasmids present)
Ascomycetes			
Pichia heedi	pPH1 (7.1)	DP/RP	3 (2)
Pichia kluyveri	pPK1 (7.2)	DP/RP	16 (3)
Kluyveromyces lactis	pGKL1 (8.9)	DP/T/I	31 (5)
	pGKL2 (13.5)	DP/RP/CE/SSB/TRF	
Debaryomyces hansenii	pDH1A (8.2)	DP/CB[a]	25 (1)
	pDH1B (14.4)	DP/RP[b]	
Debaryomyces hansenii TK	pDHL1 (8.4)	DP/CB[c]	1 (1)
	pDHL2 (9.2)		
	pDHL3 (15)		
Pichia acaciae	pPac1-2 (6.8)	T/I	2 (1)
	pPac1-1 (13.6)	DP/RP/CE/SSB/TRF	
Debaryomyces robertsiae	pWR1A (8.1)	T/I	2 (1)
	pWR1B (14.6)	DP/RP/CE/SSB/TRF	
Pichia pastoris	pPP1A (9.5)		4 (2)
	pPP1B (13)		
Candida tartarivorans	pCX1A (8)		1 (1)
	pCX1B (13)		
Pichia nakazawae	pPN1A (7)		2 (1)
	pPN1B (8)		
	pPN1C (15)		
Pichia inositovora	pPin1-3 (9.7)	DP/T	1 (1)
	pPin1-2 (13)		
	pPin1-1 (18)		
Debaryomyces etchellsii	pPE1A (6.7)	CB	4 (3)
	pPE1B (12.8)	DP/RP/CE/SSB/TRF	
Schwanniomyces occidentalis	pSoc1-1 (8.1)		2 (1)
	pSoc1-2 (13.4)		
Saccharomyces kluyveri	pSKL (14)	DP/RP/CE/SSB/TRF	5 (1)
Saccharomycopsis malanga	pSM2A (8.7)		2 (1)
	pSM2B (15)		
Saccharomycopsis crataegensis	pScr1-3 (5)		3 (2)
	pScr1-2 (7)		
	pScr1-1 (15)		
Botryoascus cladosporoides	pBC1A (7)		1 (1)
	pBC1B (14)		
Debaryomyces polymorphus	pDP1 (18)		1 (1)
Basidiomycetes			
Trichosporon pullulans	pTP1 (16)		13 (7)

[a] based on heterologous hybridization with pGKL1 derived probes
[b] based on heterologous hybridization with pGKL2 derived probes
[c] based on partial DNA sequences

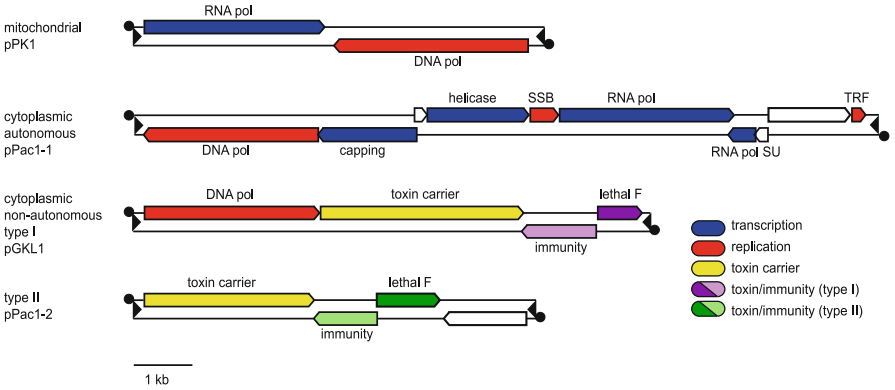


Fig. 3 Schematic representation of yeast linear plasmids: mitochondrial, cytoplasmic autonomous/nonautonomous, and toxin type I/II encoding. Symbols as in Fig. 2. Known or assumed functions of predicted proteins are given. Colors correspond to functional categories as indicated

as pairs or triplets, one of which is invariably self-sufficient with respect to replication and maintenance. Only in a few cases (e.g., *Saccharomyces kluyveri* pSKL, *T. pullulans* pTP1, and *Debaryomyces polymorphus* pDP1), was an autonomous element discovered without any attendance. Sequencing autonomous linear plasmids from *K. lactis*, *S. kluyveri*, *D. etchellsii*, and *P. acaciae* (Hishinuma et al. 1984; Tommasino et al. 1988; Hishinuma and Hirai 1991; Larsen and Meinhardt 2000; Klassen et al. 2001; Jeske and Meinhardt 2006) revealed a strictly conserved genetic organization, irrespective of the taxonomic or geographic origin. Exemplified for pPac1-1 in Fig. 3, such an illustration in principle applies for any of the autonomous cytoplasmic plasmids sequenced thus far. pGKL2 and pSKL from *K. lactis* and *S. kluyveri* negligibly deviate as they possess an additional nonessential gene of unknown function (ORF1). Since replication and transcription is confined genetically to the nucleus, autonomous cytoplasmic elements must necessarily provide the enzymatic repertoire ensuring both replication and transcription. With the exception of the above mentioned ORF1 in pGKL2 and pSKL, nonessential additional functions, such as the killer phenotype, are invariably encoded by an accompanying smaller linear plasmid, which, however, strictly depends on an autonomous element in terms of cytoplasmic gene expression and maintenance.

4.1.1 DNA Polymerase, SSB, and Terminal Recognition Factor

Each autonomous linear plasmid harbors a gene encoding a TP-DNA polymerase (Tommasino et al. 1988; Hishinuma and Hirai 1991; Hishinuma et al.

1984; Stark et al. 1984; Sor and Fukuhara 1985; Klassen et al. 2001; Klassen and Meinhardt 2003; Jeske and Meinhardt 2006). Hence, any cytoplasmic plasmid system possesses at least one viral B-type DNA polymerase; however, as for the pGKL system of *K. lactis*, another TP-DNA polymerase locus may be present. Since loss or knockout of either of the two polymerase encoding genes cannot be functionally complemented by the remaining locus, each plasmid evidently replicates via its own DNA polymerase (Kitada and Gunge 1988; Schaffrath et al. 1995). Such specificity is supported by the fact that TPs of pGKL1 and pGKL2 differ in size. Indeed, it was experimentally proven that TPs of pGKL2 are encoded as the N-terminal region of pGKL2 Orf2 (Takeda et al. 1986), agreeing with DNA polymerases specifically replicating the plasmid that encodes them.

In multiple plasmid systems possessing a single TP-DNA polymerase gene only (on the autonomous element), as for pPac1-1, pPac1-2 and pPE1A, pPE1B of *P. acaciae* and *D. etchellsii*, respectively, replication of nonautonomous elements is probably mediated by the same enzyme. Though it is to be expected that autonomous and nonautonomous elements should have identical TPs in these instances, there is no discernible sequence homology with respect to TIR sequences (Klassen et al. 2002, 2004; Jeske and Meinhardt 2006), which are considered to harbor replication origins. Thus, the TPs rather than the TIRs provide the basis for the specificity seen in systems with more than one DNA polymerase.

The viral B-type DNA polymerase–TP fusion protein is apparently sufficient for replication of linear plasmids in mitochondria. However, cytoplasmic replication needs additional plasmid encoded functions, such as the single-strand DNA binding protein (SSB), for stabilization of replication intermediates and the (rather basic) terminal recognition factor (TRF) which specifically binds to TIRs and is presumably involved in replication initiation (McNeel and Tamanoi 1991; Tommasino 1991; Schaffrath and Meacock 2001). Indeed, pGKL2 encoded SSB were shown to interact with ssDNA without sequence preference (Schaffrath and Meacock 2001), whereas binding sites for TRF comprise nucleotides 107–183 in the pGKL1 TIR and 126–179 in the pGKL2 TIR. Though there is no obvious sequence similarity, both TIR regions are rich in long dA–dT stretches presumably folding into similar secondary structures (McNeel and Tamanoi 1991).

Genes encoding TP-DNA polymerase, SSB, and TRF were proven to be essential for cytoplasmic inheritance in the pGKL1,2 system (Schaffrath and Meacock 1995; Schaffrath et al. 1995; Tiggemann and Meinhardt, unpublished results). The enzymatic machinery involved in replication of cytoplasmic linear plasmids clearly resembles the scenario seen in phi29-like bacteriophages, since virus encoded ss- and dsDNA binding proteins are involved in replication initiation in addition to the B-type DNA polymerase and TPs (Meijer et al. 2001).

4.1.2 The Cytoplasmic Trancriptase Complex

A rather unique RNA polymerase, a DExH/D box helicase and an mRNA capping enzyme, represent key elements of the cytoplasmic transcriptional apparatus. Consistent with their pivotal function, each of the above genes were proven essential for plasmid maintenance (Schaffrath et al. 1995, 1997; Larsen et al. 1998).

The RNA polymerase (Fig. 3) appears to consist of two different subunits, encoded by pGKL2 ORF6 and ORF7, and homologous genes in other plasmid systems. The architecture of the enzyme resembles β and β′-subunits of the multisubunit *Escherichia coli* RNA polymerase; all β and several domains of the β′ subunit exist in the predicted pGKL2/Orf6 protein (Wilson and Meacock 1988; Thuriaux and Sentenac 1992; Schaffrath et al. 1995). However, two β′ domains, located usually close to the C terminus, reside in a separately encoded protein (pGKL2 Orf7p or its homologues; Schaffrath et al. 1997; Jeske et al. 2006a), suggesting β′ functions can be assigned to separate subunits, a scenario known for the archeon *Halobacterium halobium* (Leffers et al. 1989). Thus, concerning its domain architecture the linear plasmid encoded RNA polymerase displays a rather unique and simple structure, most closely related to multisubunit RNA polymerases, in marked contrast to the single subunit, T7-like RNA polymerase encoded by mitochondrial linear plasmids.

The predicted protein encoded by pGKL2 ORF4 (and respective homologues in other autonomous linear plasmids) belongs to the DExH/D box family which comprises RNA helicases and NTPases (Tommasino et al. 1988; Stark et al. 1990; Jeske et al. 2006a). Though ORF4 was proven to be essential for plasmid maintenance (Schaffrath et al. 1997), the precise function of the predicted polypeptide remains to be elucidated. There are (at least) 34 DExD/H helicase homologues in *Saccharomyes cerevisiae*, most of which are essential (de la Cruz et al. 1999). Members of this family are instrumental in a number of diverse processes related to RNA metabolism (such as ribosome biogenesis, mRNA splicing, RNA degradation, nuclear export) as well as transcription and translation (de la Cruz et al. 1999; Tanner and Linder 2001). Not all representatives assigned to such a family must necessarily possess helicase activity but may rather supply energy for other processes (by NTP hydrolysis), such as the disruption of ribonucleoprotein complexes (Schwer 2001; Jankowsky and Bowers 2006). Interestingly, vaccinia virus NphI, a DExH/D protein, displays ATPase but lacks helicase activity. The enzyme, stimulated by ssDNA, acts as an energy-coupling factor for transcription elongation and mRNA release upon termination (Deng and Shuman 1998). Be that as it may, though the role of the DExH/D protein encoded by pGKL2 ORF4 systems remains obscure at present, it is likely to be involved in the rather unique cytoplasmic transcription process.

An unambiguously essential component of the cytoplasmic transcription machinery is the mRNA capping enzyme (encoded by pGKL2, ORF3, and homologous genes in other systems) (Larsen et al. 1998; Tiggemann et al. 2001; Klassen et al. 2001; Jeske and Meinhardt 2006). Orf3p displays RNA triphosphatase and guanylyltransferase activities (Tiggemann et al. 2001). During mRNA cap formation, RNA triphosphatase removes the γ-phosphate from the 5′ end of the nascent mRNA, followed by formation of an unusual 5′–5′ linkage between guanosine monophosphate and the processed 5′ transcript end by the guanylyltransferase, in a reaction that involves a covalent intermediate between a lysine residue of the enzyme and GMP. Finally, the cap methyltransferase modifies the guanosine residue at position N7 utilizing *S*-adenosylmethionine (SAM) as the methyl donor (Martin et al. 1975; Shuman and Hurwitz 1981; Cong and Shuman 1993; Shuman and Schwer 1995; Bisaillon and Lemay 1997). In contrast to RNA triphosphatase and guanylyltransferase activities, cap methyltransferase activity was not obtained for the purified Orf3p, though there is a potential SAM binding site (Larsen et al. 1998; Tiggemann et al. 2001). Overall, Orf3p resembles the capping enzyme known from the cytoplasmic vaccinia virus, in which triphosphatase, guanylyltransferase, and methyltransferase domains are located in the polypeptide (vD1) encoded by the vaccinia D1 gene. However, vD1 displays only faint cap methyltransferase activity, which is stimulated tremendously (more than 30-fold) upon heterodimerization with the vaccinia D12 gene product (Cong and Shuman 1992; Higman et al. 1992, 1994; Mao and Shuman 1994). It remains to be elucidated, however, whether the methyltransferase activity of the linear plasmid encoded capping enzyme (Orf3p) requires a stimulatory subunit, too. Concerning domain architecture and sequence similarities, the linear plasmid encoded Orf3p clearly corresponds to capping enzymes of irido- and poxviridae, which differ strikingly from fungal nuclearly encoded capping enzymes as—in the latter—RNA triphosphatase, guanylyltransferase, and cap methyltransferase activities reside in separate polypeptides. The persuasive similarity of the mRNA capping machinery of cytoplasmic linear plasmids to that of cytoplasmic pox viruses again strongly supports the conclusive presumption for viral ancestry.

4.1.3 Cytoplasmic Transcription

Since the cytoplasmic transcriptase complex in general, and the RNA polymerase in particular, convincingly differs from the nuclearly encoded host enzyme, cytoplasmic transcription of linear plasmid based genes was—not astonishingly—shown to be driven by unique promoters clearly differing from nuclear ones (Kämper et al. 1989a,b, 1991; Gunge et al. 2003; Romanos and Boyd 1988; Stark et al. 1990). Based on cytoplasmic in vivo recombination approaches using either yeast nuclear or reporter genes of bacterial

origin fused to pGKL1 and pGKL2 sequences, a 6-bp spanning conserved motif preceding the native ORFs was defined as being necessary and sufficient for cytoplasmic transcription (upstream conserved sequence, UCS: 5′-ATNTGA-3′; Schründer and Meinhardt 1995; Schaffrath et al. 1996; Schickel et al. 1996; Schründer et al. 1996). The UCS motif is typically located approximately 30 bp upstream of the start codon of an ORF; however, distances may vary. Sequences distal of the UCSs were found to be irrelevant, ruling out additional promoter motifs (Schickel et al. 1996).

Consistent with the high degree of conservation of transcriptase complexes, UCS motifs are located upstream of every ORF in any cytoplasmic plasmid system, irrespective of the host (Bolen et al. 1994; Hishinuma and Hirai 1991; Fukuda et al. 1997; Klassen et al. 2001, 2002, 2004; Klassen and Meinhardt 2003; Jeske and Meinhardt 2006). Accordingly, a UCS derived from the *D. etchellsii* plasmid pPE1B was shown to act as a functional promoter in the *K. lactis* pGKL system (Klassen et al. 2001), concomitantly providing further evidence for a functionally conserved cytoplasmic transcriptase.

Transcription initiation, analyzed for a number of ORFs in the pGKL system, occurs at multiple sites, routinely around 8–16 bp downstream of the UCS motif, with a preference for one site (Romanos and Boyd 1988; Jeske et al. 2006b). Since Northern analyses revealed monocistronic transcripts only, defined termination of transcripts was suggested to occur (Schaffrath et al. 1995; Jeske et al. 2006b). To date, however, the termination mechanism remains totally obscure, as for the potential 3′ modification such as polyadenylation.

Though there are similarities between a potential DExH/D box ATPase (Orf4p) encoded by linear plasmids and the viral NphI proteins being instrumental in transcription termination, it has not been proven that this largely uncharacterized enzyme functions similarly in linear plasmid systems.

4.2 The Nonautonomous Elements

Routinely, cytoplasmic linear plasmids occur as systems in which an autonomous element is accompanied by one or more nonautonomous attendants (Table 2), some of which encode their own DNA polymerase (pGKL1, pDHL1, pPin1-3, and pDH1A), while others are lacking such genes (pPE1A, pPac1-2, pWR1A) (Hishinuma et al. 1984; Stark et al. 1984; Sor and Fukuhara 1985; Cong et al. 1994; Fukuda et al. 1997; Klassen et al. 2002, 2004; Klassen and Meinhardt 2003). As already mentioned, the DNA polymerases of the pGKL system specifically replicate their own linear plasmid (Kitada and Gunge 1988; Schaffrath et al. 1995), consistent with different TPs of autonomous and nonautonomous elements. Such a scenario may also be realized in the pDHL, pPin, and pDH1 systems. Irrespective of the DNA polymerases, a nonautonomous plasmid not only depends on the corresponding autonomous element in terms of cytoplasmic replication (SSB, TRF) but also

in transcription (RNA polymerase, helicase, and capping enzyme; see above). An obvious function of nonautonomous elements is evident in the killer systems, as they typically encode a protein toxin as well as the respective immunity function, the latter safeguarding the killer strain against its own toxin.

4.2.1 Killer Plasmids

Linear plasmid encoded toxins were detected in *K. lactis*, *Pichia inositovora*, *P. acaciae*, and *Debaryomyces robertsiae* (synonym *Wingea robertsiae*) (Gunge et al. 1981; Worsham and Bolen 1990; Hayman and Bolen 1991; Klassen and Meinhardt 2002; see Table 2). Genetic and biochemical analyses provided evidence for large (>100 kDa) heteromeric proteins which are encoded by two separate genes (Table 2). The most thoroughly studied toxin is the *K. lactis* zymocin. A detailed picture is available with respect to biogenesis and multistep action on sensitive target cells. Though there is evidence for similar mechanisms being instrumental in target cell entrance, clearly different intracellular toxin targets must be conceded in other instances, eventually leading to classification of two functional subtypes (I and II).

4.2.1.1 The pGKL1 Encoded Zymocin

(a) Biogenesis

The *K. lactis* zymocin is a heterotrimeric (α, β, γ) glycoprotein, the subunits of which display molecular masses of 99, 30, and 28 kDa, respectively (Stark and Boyd 1986). The smallest subunit (γ) is encoded by ORF4 of pGKL1; the two larger ones originate from a single gene product (Orf2p) by posttranslational processing (Hishinuma et al. 1984; Stark et al. 1984, 1990; Sor and Fukuhara 1985; Stark and Boyd 1986; Tokunaga et al. 1987).

Both Orf2p and Orf4p carry typical signal peptides mediating their entry into the secretory pathway. Upon translocation to the endoplasmic reticulum (ER), signal peptides are probably cleaved off (Tokunaga et al. 1990); subsequently, in the Golgi, processing of Orf2p at positions KR_{29} and KR_{894} occurs by means of the Kex1 protease, ultimately giving rise to mature α- and β-subunits (Stark and Boyd 1986; Wésolowski-Louvel et al. 1988; Tanguy-Rougeau et al. 1988; Stark et al. 1990). Secreted holotoxin forms the α,β,γ-complex in which β and γ are covalently linked via a disulfide bond; the α-subunit at least contains one internal disulfide bridge (Stark and Boyd 1986; Stark et al. 1990). Protein glycosylation is restricted to the α-subunit, in which a single N-linked oligosaccharide chain was detected, whereas both β and γ are non-glycosylated (Stark and Boyd 1986). Inhibition of either α-subunit glycosylation by tunicamycin or precursor processing by *kex1* mutation pre-

vents zymocin secretion (Sugisaki et al. 1985; Wésolowski-Louvel et al. 1988; Tanguy-Rougeau et al. 1988). The γ-subunit, though carrying a functional signal peptide shown to efficiently mediate secretion of heterologous proteins, fails to be efficiently secreted in the absence of the αβ-precursor protein (Tokunaga et al. 1988, 1989, 1990, 1991). Thus, zymocin γ secretion apparently requires cosecretion and interaction with the glycosylated α-subunit or the αβ-precursor (Tokunaga et al. 1990). Interestingly, the γ-subunit is efficiently secreted without the need for the αβ-precursor when the heterologous pre–pro sequence of the *S. cerevisiae* α-mating-type factor is placed in between the γ-signal peptide and the mature polypeptide (Tokunaga et al. 1989, 1990, 1992). Since the pre–pro sequence is cleaved by the KEX protease in the Golgi and basal secretion levels of the native γ-subunit are not affected by a *kex* mutation, it has been assumed that both unprocessed pre–pro sequence and αβ-precursor govern γ passage from the ER to the Golgi (Tokunaga et al. 1990; Gunge and Tokunaga 2004). Hence, even though γ secretion requires cosecretion of toxin subunits, the assembly of holotoxin is not essential and interaction between separately encoded subunits (i.e., γ and αβ) most likely occurs prior to cleavage of the αβ-precursor into mature α- and β-subunits (Tokunaga et al. 1990).

The zymocin encoding killer plasmids pGKL1 and pGKL2 have been transferred to other yeast species, such as *Kluyveromyces marxianus* (synonyms *K. fragilis* and *Candida pseudotropicalis*) and *S. cerevisiae* (Gunge and Sakaguchi 1981; Gunge et al. 1982; Sugisaki et al. 1985). Irrespective of their host they confer the ability to secrete zymocin, suggesting toxin biogenesis to be similar. Indeed, *S. cerevisiae* cells harboring pGKL1 and pGKL2 were shown to secrete zymocin consisting of correctly processed subunits. Moreover, mutations in the *KEX2* gene, which is homologous to *K. lactis KEX1*, prevent secretion of both holotoxin and the αβ-precursor (Stark et al. 1990; Tokunaga et al. 1990).

(b) Mode of Action: Access to the Target Cell

Reminiscent of the long-known *S. cerevisiae* K28 toxin as well as of bacterial and plant A/B toxins, the lethal strategy of zymocin involves import of a toxic subunit into target cells. For zymocin, the cell cycle arresting activity resides exclusively in the γ-subunit (Tokunaga et al. 1989; Butler et al. 1991b). Conditional expression of the encoding gene (pGKL1 ORF4) mimics the effect of exogenously applied native zymocin, i.e., it provokes G1 cell cycle arrest, as does exozymocin (White et al. 1989; Tokunaga et al. 1989; Stark et al. 1990; Butler et al. 1991b,c).

Though γ is responsible for the G1 arrest, it requires both of the other subunits (α and β) to gain access to target cells, a process that involves cell wall contact and transmembrane passage (Tokunaga et al. 1989; Stark et al. 1990). For genetic analysis of factors involved in early (binding and uptake) and late events (intracellular action of γ) during zymocin action,

toxin-resistant mutants were isolated and classified according to resistance or sensitivity toward intracellular γ expression (Butler et al. 1991c, 1994; Kawamoto et al. 1993; Kishida et al. 1996; Frohloff et al. 2001). Mutants affected in early steps exclusively can survive exozymocin (class I), whereas loss of essential functions involved in intracellular action of γ renders a mutant resistant to both intracellularly expressed γ and exogenously applied holotoxin (class II).

Cell wall binding is mediated by the zymocin's α-subunit, which contains a cysteine-rich chitin binding domain (PROSITE 00026) typically found in plant chitin binding proteins and chitinases. There is also a characteristic chitinase domain (Pfam PF 00704) of the glycosyl hydrolase family 18 (Stark et al. 1990; Butler et al. 1991a; Jablonowski et al. 2001a). Only quite recently, we additionally identified a LysM signature (Pfam PF 01476) in front of the chitin binding domain. LysM signatures were originally identified in cell wall degrading proteins (lysins) of bacteria and bacteriophages, in which they function as peptidoglycan binding sites (Birkeland 1994; Pontig et al. 1999). Since carbohydrate moieties in both peptidoglycan and chitin are structurally similar, it is tempting to speculate that the LysM signature acts in concert with the chitin binding domain to facilitate the ascertained tight binding of the toxin complex to the target cell's chitin (Jeske et al. 2006a). Consistently, zymocin has both chitin binding and chitinase activity; moreover, several mutations affecting the major chitin synthase (Chs3) of *S. cerevisiae* cause class I zymocin resistance, concomitantly providing convincing evidence for chitin being the zymocin receptor (Takita and Castilho-Valavivius 1993; Butler et al. 1991a; Jablonowski et al. 2001a).

Chronologically following cell wall binding but still preceding target interference, there are two key players known to be involved in γ uptake, i.e., the plasma membrane sphingolipid mannosyl-diinositolphosphoceramide $M(IP)_2C$ (synthesized by the Kti6/Ipt1 protein) and the plasma membrane ATPase Pma1, both conjointly required for toxin action from outside as judged from class I resistance of *ipt1* and *pma1* mutants (Mehlgarten and Schaffrath 2004; Zink et al. 2005). The crucial role of $M(IP)_2C$ for zymocin uptake is further supported by reduced zymocin sensitivity of different sphingolipid mutants displaying shortened $M(IP)_2C$ levels (Zink et al. 2005). In contrast to *chs3* cells, which lack zymocin binding (due to the absence of the primary cell wall receptor, see above), the *ipt1* mutant, though proficient for cell wall binding of the toxin, denies γ import; thus, the Ipt1 synthesized sphingolipid might act as a secondary membrane receptor (Zink et al. 2005). Alternatively, however, a yet unidentified $M(IP)_2C$-dependent protein may serve as such a secondary plasma membrane receptor. Normal glutaredoxin levels are significant for toxin uptake as well, since high copy *GRX3* encoding glutaredoxin provokes class II zymocin resistance (Jablonowski et al. 2001b). Glutaredoxin exceeding normal levels may impact β/γ uncoupling or γ-toxin import (Schaffrath and Meinhardt 2004).

Following membrane passage, γ relies on intracellular activation mediated by the plasma membrane H^+ATPase Pma1 (Mehlgarten and Schaffrath 2004; Zink et al. 2005). Curiously, though they are not deficient in γ uptake, *pma1* mutants display class I zymocin resistance which can be overruled by an excess of H^+ (Mehlgarten and Schaffrath 2004). Hence, γ must exist during the uptake process in a transient dormant form, which is converted by H^+ flux into the active conformation (induced either experimentally by acidification of the cell exterior or by the H^+ATPase) (Mehlgarten and Schaffrath 2004). Interestingly, other protein toxins gaining access to target cells, such as the botulinum or diphtheria toxins, depend on acidification of endocytotic vesicles by plasma membrane H^+ATPases for induction of conformational changes as well, resulting in the insertion of a membrane spanning domain into the endosomal membrane normally facilitating toxin passage into the cytoplasm. It remains to be elucidated whether such a scenario also applies to zymocin action; if so, zymocin containing endocytotic vesicles must be formed independently of the well-established End3/End4-dependent pathway (Raths et al. 1993; Wesp et al. 1997) as zymocin action requires neither of them (Jablonowski et al. 2001a).

Though the exact mechanism facilitating transmembrane passage of the γ-subunit is not entirely understood, the remarkably hydrophobic β-subunit is believed to take part (Stark et al. 1990; Jablonowski et al. 2001a; Mehlgarten and Schaffrath 2004; Zink et al. 2005). Thus, it constitutes the prime candidate among the toxin subunits for mediating transmembrane passage of γ, either directly or with the aid of endocytotic vesicles. In support of this, a similarity (even though weak) of the β-peptide to *E. coli* TolQ has been noted (Stark et al. 1990), the latter being a factor required for transmembrane passage of colicins (Sun and Webster 1987). For understanding early toxin responses, it is of particular importance to know whether zymocin's toxicity involves breakdown of the disulfide bridge connecting β and γ in holotoxin, and to define detailed functions of the sphingolipid $M(IP)_2C$ and Pma1 in binding and mediating passage across the plasma membrane.

(c) Mode of Action: The Intracellular Target

The cell cycle arresting γ-subunit strictly requires the so-called Elongator complex, originally described to be involved in elongation during RNA polymerase II transcription (Otero et al. 1999; Wittschieben et al. 1999; Winkler et al. 2001; Frohloff et al. 2001). In addition, Elongator was considered to be instrumental in rather diverse processes, such as transfer RNA (tRNA) modification and exocytosis (Huang et al. 2005; Rahl et al. 2005). Only quite recently, however, it was shown that Elongator's key function concerns posttranscriptional modification of tRNAs, and concomitantly it was proposed that transcription and exocytosis defects of respective mutants are downstream effects due to defective tRNA modification (Esberg et al. 2006).

The Elongator complex consists of six subunits, each of which is required for the formation of 5-methoxy-carbonylmethyl (mcm^5) and 5-carbamoylmethyl groups (ncm^5) at the wobble nucleoside uridine in certain tRNAs (Huang et al. 2005). Target cells lacking any one of these subunits display class II toxin resistance and, consistent with tRNA modification representing the key function of Elongator, such modified tRNAs in fact constitute the molecular targets of zymocin. In vitro, the γ-subunit cleaves three mcm^5-modified tRNA species ($tRNA^{Glu}_{UUC}$, $tRNA^{Lys}_{UUU}$, and $tRNA^{Gln}_{UUG}$) specifically at the 3′ side of the wobble nucleoside with a strong preference for $tRNA^{Glu}$, generating a 5′-hydroxyl group and a 2′3′ cyclic phosphate end (Lu et al. 2005; Jablonowski et al. 2006). Supporting the conclusion that such modified tRNAs represent the target sites, overexpression of $tRNA^{Glu}_{UUC}$ alone or $tRNA^{Lys}_{UUU}$ and $tRNA^{Gln}_{UUG}$ in combination confers zymocin resistance (Butler et al. 1994; Jablonowski et al. 2006; Lu et al. 2005).

As for Elongator subunits Elp1-6, removal of several Elongator interacting proteins, such as Kti11-13, Sit4, Sap185, and Sap190, confers immunity toward zymocin (Frohloff et al. 2001, 2003; Jablonowski et al. 2001b,c, 2004; Fichtner et al. 2002a,b, 2003; Fichtner and Schaffrath 2002; Mehlgarten and Schaffrath 2003). Since *kti11-13* mutants not only display zymocin resistance but also lack mcm^5 modifications of tRNAs (Huang et al. 2005), the paramount importance of such modification for zymocin action again became evident.

Another zymocin relevant enzyme is Trm9/Kti1, a tRNA methyltransferase probably acting in concert with Elongator to carry out wobble nucleoside modification (Kalhor and Clarke 2003; Jablonowski et al. 2006). While tRNAs of cells mutated in either *ELP1-6* or *KTI11-13* do not possess the entire mcm^5 group, *trm9/kti1* mutants lack the methyl group of mcm^5 only; nevertheless, they display robust toxin resistance (Huang et al. 2005; Lu et al. 2005; Jablonowski et al. 2006). The significance of the methyl group for target recognition by γ is supported by the finding that the zymocin-protective effect of $tRNA^{Glu}$ overexpression is abolished when the methyltransferase Trm9 is present at elevated levels (Jablonowski et al. 2006).

Interestingly, tRNA targets are known from bacterial protein toxins as well, e.g., for colicins E5 and D (Ogawa et al. 1999; Tomita et al. 2000). As for the zymocin, both latter toxins are tRNases cleaving specifically either at the 5′ or the 3′ side of the wobble nucleoside of certain tRNAs and produce a 2′3′ cyclic phosphate end (Ogawa et al. 1999; Tomita et al. 2000). However, irrespective of a similar mode of action of both bacterial toxins, the lack of sequence homology and differences in substrate recognition as well as catalytic mechanisms have led to the conclusion that such toxins have acquired similar functions through convergent evolution (Masaki and Ogawa 2002). The γ-subunit of zymocin, representing a new member of toxic tRNases, does not display significant similarity to either of the others, which may support the opinion that

attacking tRNA constitutes an effective toxic principle that can be realized independently during evolution by making use of unrelated proteins.

(d) Toxin Immunity

Essentially, zymocin producing cells are resistant toward the toxin; such self-immunity strictly relies on the availability of the killer plasmid pair pGKL1 and pGKL2; however, partially cured strains carrying exclusively pGKL2 are susceptible to toxin (Gunge et al. 1981; Niwa et al. 1981). Accordingly, immunity is accomplished by pGKL1. As already outlined, three of the four pGKL1 genes are either involved in replication (ORF1) or encode toxin subunits (ORF2 and ORF4). Since deletion of a pGKL1 region spanning ORF2, ORF3, and ORF4 gave rise to nonimmune nonkillers, whereas deletion solely of ORF2 produced immune nonkillers, pGKL1 ORF3 was held responsible for zymocin immunity (Hishinuma et al. 1984; Sor and Fukuhara 1985; Stark and Boyd 1986; Stark et al. 1990). In fact, expression of ORF3 from an ARS vector in a strain devoid of pGKL1 conferred toxin resistance (Tokunaga et al. 1987). However, the acquired immunity was only partial; high toxin doses ($400\ ng\ ml^{-1}$) still caused noticeable growth inhibition, and were indeed tolerated by cells carrying pGKL1. Moreover, expression of ORF3 from an ARS vector conferred immunity exclusively in the presence of pGKL2 (Tokunaga et al. 1987). Thus, it was suggested that pGKL2 mediates expression of ORF3 from ARS vectors; however, this lags behind pGKL1 based expression (Tokunaga et al. 1987; Stark et al. 1990). Alternatively, pGKL2 might encode an additional factor required for full zymocin immunity. There is an additional nonessential ORF on pGKL2 that does not exist in other yeast autonomous elements (ORF1; see above). Though the predicted polypeptide displays significant similarities to pGKL1 Orf3p this ORF has evidently no function in zymocin immunity, as deletion mutants remained immune (Schaffrath et al. 1992).

The biochemical basis of immunity so far remains unknown; nevertheless, pGKL1 ORF3 not only protect cells from exogenously applied zymocin but also antagonizes intracellularly expressed γ (Tokunaga et al. 1989), indicating that zymocin protection is not realized by exclusion of γ but rather involves intracellular interference with γ function.

For colicin D, which exerts a zymocin-like toxic principle (see above), it was proven that the corresponding immunity protein (ImmD) structurally mimics the respective tRNA substrate and, forming a tight complex with the toxic domain, blocks the tRNase active site (Graille et al. 2004). Whether similarities between zymocin and colicins are restricted to (presumably convergently evolved) toxic principles or extend to like immunity mechanisms needs to be discovered.

4.2.1.2 Other Killer Plasmids and Encoded Toxins

Except for pGKL1, there are three nonautonomous linear plasmids from different yeast species known to encode killer toxins, i.e., pPac1-2 of *P. acaciae*, pPin1-3 of *P. inositovora*, and pWR1A of *D. robertsiae* (Gunge et al. 1981; Worsham and Bolen 1990; Hayman and Bolen 1991; Klassen and Meinhardt 2002). Each of the aforementioned elements encodes a chitin binding protein, structurally akin to the zymocin α-subunit (Klassen and Meinhardt 2002, 2003; Klassen et al. 2004). Additionally, hydrophobic C-terminal regions very similar to the zymocin β-subunit were identified in such deduced chitin binding polypeptides, suggesting chitin binding and subsequent import of a cell cycle arresting or lethal subunit as for zymocin. Consistently, mutations affecting the target cells major chitin synthase (Chs3) conferred toxin resistance to all of the toxins (Klassen and Meinhardt 2003; Klassen et al. 2004). Moreover, reminiscent of the γ-subunit, separate structural genes encoding potentially secreted proteins were identified in either case (Fig. 3).

For the *P. inositovora* plasmid pPin1-3, such protein does indeed compare to zymocin γ and—not astonishingly—as for zymocin, toxicity is impaired in an Elongator (*elp3*) mutant (Klassen and Meinhardt 2003). The functionally related *P. inositovora* toxin together with *K. lactis* zymocin were assigned to a corporate class of linear plasmid encoded toxins (type I), which are characterized by their dependency on cell wall chitin and, more importantly, on a functional Elongator complex (Schaffrath and Meinhardt 2004; Jeske et al. 2006b). Distinct from the *K. lactis* system, however, immunity is not encoded by the linear plasmids in *P. inositovora* (Hayman and Bolen 1991). Though there is a gene similar to the *K. lactis* pGKL1 ORF3 (see above) in the toxin encoding pPin1-3 (Klassen and Meinhardt 2003), its function has not been studied.

The killer plasmids pPac1-2 and pWR1A from *P. acaciae* and *D. robertsiae*, respectively, are structurally closely related, but their resemblance to type I toxin encoding elements (pGKL1 and pPin1-3) stretches only across the ORF encoding the chitin binding polypeptide, whereas the separately encoded and potentially secreted proteins (pPac1-2 Orf2p and pWR1A Orf3p) hardly bear any likeness to zymocin γ and/or the *P. inositovora* equivalent (Klassen et al. 2004). However, as for zymocin γ, intracellular expression of either protein phenocopies the impact of the extracellularly applied toxin on target cells; thus, their function as intracellularly acting toxin subunits immediately comes to mind (Klassen et al. 2004).

In accordance with functions different from zymocin γ, toxins of *P. acaciae* (PaT) and *D. robertsiae* do not require Elongator (Klassen et al. 2004). Hence, tRNAs with an Elongator-dependent mcm^5-modified wobble uridine are unlikely to be their targets (see above). Interestingly, a mutation in the *TRM9* gene (encoding a tRNA methyltransferase known to be involved in completion of the mcm^5 modification) (Kalhor and Clarke 2003; Lu et al.

2005; Jablonowski et al. 2006) confers resistance to the type II toxin of *P. acaciae* to a certain degree (McCracken et al. 1994; our unpublished results). Taking also into consideration that other than for zymocin, overexpression of $tRNA^{Glu}_{UUC}$ has no protective consequences in type II toxicity (our unpublished results), Trm9 may have an additional cellular role besides mcm^5 modification of tRNA.

Type II toxins arrest target cells in the S phase of the cell cycle and activate the intra-S-phase DNA damage checkpoint, a scenario that agrees with a replication inhibitory and/or DNA damaging function (Klassen et al. 2004). The *P. acaciae* toxin (PaT) induces death in a two-step fashion; during the first 3–4 h in toxin, target cells lose their viability to approximately 30% that of control levels. Such a phase is characterized by hyperphosphorylation of the DNA-damage checkpoint kinase Rad53 and mutation induction, indicating DNA damage to be involved in lethality at this stage (Klassen et al. 2004; Klassen and Meinhardt 2005). Our recent work has revealed that DNA damage induced by PaT occurs during the S phase of the cell cycle and probably involves formation of broken replication forks. Congruently, mutants impaired in stalled replication fork stabilization and recovery, as well as in double-strand break (DSB) repair, react extremely sensitively to PaT (our unpublished results).

A period comprising several hours of constant viability in toxin (∼30%) follows until the final decline to less than 1% occurs. Final cell death is characterized by the appearance of typical apoptotic markers, including abnormal nuclear morphology, reactive oxygen species, DNA fragmentation, and phosphatidylserine flipping (Klassen and Meinhardt 2005). Since Rheovirus encoded killer toxins with clearly distinct modes of action (such as K1 and K28 from *S. cerevisiae* and zygocin from *Zygosaccharomyces bailii*) also activate programmed cell death in target cells (Reiter et al. 2005), apoptosis is apparently an aftereffect and not confined to individual toxins.

Interestingly, type I and type II toxins are both influenced by the mating-type status of the target cell. Diploid cells are not only significantly more resistant to both of the toxins compared to either of the haploid parents, but also overexpression of the *MATa* locus in a *MAT*α strain, as well as mutations in genes required for silencing of the cryptic mating-type loci (*SIR* genes), render target cells resistant to toxins of both types (Butler et al. 1994; Klassen et al. 2006). Such an effect is likely due to the repression of haploid-specific genes required for the action of either toxin and, thus, may be due to the cellular uptake mechanism, which is akin in either case (see above; Table 3). Moreover, both zymocin and PaT interfere with the mating competence of target cells, as these are refractory (Klassen et al. 2006). Thus, both killers act efficiently on haploid cells only and prevent them from mating, thereby ensuring most effective target cell killing under unfavorable environmental conditions, and causing sporulation of target cells which compete for limited resources (Klassen et al. 2006).

Table 3 Genes essential for killer toxin action, arranged according to their function in target cells. Toxin resistance and susceptibility of individual mutants to killer toxins from *K. lactis* (Kl), *P. acaciae* (Pa), *P. inositovora* (Pi), and *D. robertsiae* (Wr) are indicated. Pa, Pi, and Wr resistance/sensitivity is not proven for each gene in a given category

Target cell modification (relevant genes)	Toxin relevant process	Toxin resistance	Toxin susceptibility	Refs.
Chitin synthesis defect *CHS3, CHS4, CHS5, CHS6, CHS7*	Cell wall receptor; uptake	Kl/Pa/Pi/Wr		Takita and Castilho-Valavivius 1993; Kawamoto et al. 1993; Butler et al. 1991a; Jablonowski et al. 2001a; Klassen et al. 2004; McCracken et al. 1994
Plasma membrane sphingolipid synthesis defect *IPT1, LAG1, LAC1*	Uptake; membrane receptor (?)	Kl	Pa	Zink et al. 2005; McCracken et al. 1994; unpublished results
Plasma membrane H^+ATPase defect *PMA1, PTK2*	Intracellular activation	Kl/Pa		Mehlgarten and Schaffrath 2004; McCracken et al. 1994; unpublished results
Simultaneous expression of *MAT*a and *MAT*α *SIR1-4; HMR, HML*	Uptake (?)	Kl/Pa		Butler et al. 1994; Klassen et al. 2006
High copy glutaredoxin *GRX3*	Uptake (?)	Kl		Jablonowski et al. 2001b
Elongator subunit defect *ELP1/IKI3, ELP2, ELP3, ELP4, ELP5/IKI1, ELP6*	tRNA modification, transcription, exocytosis	Kl/Pi	Pa/Wr	Yajima et al. 1997; Frohloff et al. 2001; Klassen et al. 2004; McCracken et al. 1994
Elongator relevant factor defect *KTI11, KTI12, ATS1/KTI13, HRR25/KTI14, URM1, UBA4, SIT4, SAP185, SAP190*; overexpression of *SAP155*; *KTI12*	Elongator modification/ interaction	Kl	Pa	Frohloff et al. 2001; Fichtner and Schaffrath 2002; Mehlgarten and Schaffrath 2003; Fichtner et al. 2003; Jablonowski et al. 2001b,c; Klassen et al. 2004; McCracken et al. 1994

Table 3 (continued)

Target cell modification (relevant genes)	Toxin relevant process	Toxin resistance	Toxin susceptibility	Refs.
tRNA overexpresion $tRNA^{Glu}_{UUC}$; $tRNA^{Lys}_{UUU}$ + $tRNA^{Gln}_{UUG}$	Target overexpression	Kl	Pa	Butler et al. 1994; Lu et al. 2005; Jablonowski et al. 2006; unpublished results
Methyltransferase *TRM9/KTI1*	tRNA methylation	Kl/Pa		Lu et al. 2005; Jablonowski et al. 2006; McCracken et al. 1994; unpublished results
Diphthamide synthesis *DPH2, YIL103w*	?	Kl		Fichtner et al. 2003

As for type I, immunity to type II toxins is procured by genes located on the nonautonomous, toxin encoding elements pPac1-2 and pWR1A (Worsham and Bolen 1991; our unpublished results). Predicted immunity factors encoded by pPac1-2 ORF4 and pWR1A ORF5 (our unpublished results) display striking similarities to each other but not to type I immunity encoded by pGKL1 ORF3 (see above). As for the latter, however, PaT immunity acts by intracellular interference with Orf2p function, rather than preventing its uptake (unpublished results).

In accordance with fundamental differences concerning both toxicity and immunity of type I and II toxins, pGKL1 Orf3p protects exclusively from the action of type I toxin, whereas at least partial cross-immunity was detected between *P. acaciae* and *D. robertsiae* toxin/immunity systems (our unpublished results).

Localization of an immunity conferring gene on the toxin encoding plasmid provides an autoselection mechanism for the entire system, as cells of a natural population having occasionally lost such an element are counterselected by toxin-producing neighbor cells. All known linear plasmid encoded killer toxins (except that of *P. inositovora*, see above) eliminate plasmid-free strains of the same species, suggesting that such toxins may indeed have autoselection purposes rather than providing an advantage to the host. Generally speaking, in killer/immunity combinations encoded by yeast linear plasmids there are (at least two) functionally distinct cargo proteins (types I and II), which exist conjointly with a respective compatible immunity factor. The cargo proteins, representing the effective principle of the toxin in either case, rely on a conserved carrier (the chitin binding/hydrophobic protein) which facilitates transport into target cells.

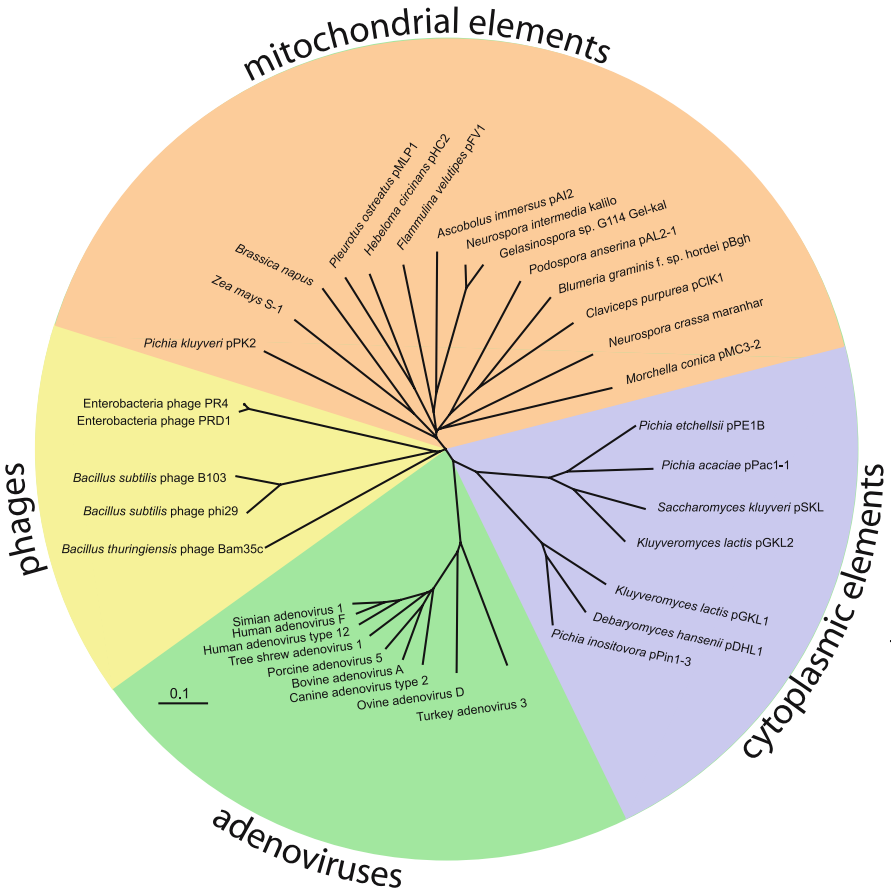
4.2.2 Cryptic Elements

Despite their frequent occurrence, a killer phenotype could not be attributed to most nonautonomous elements (Table 2). Since, to date, no other alternative traits (other than killer toxin production and immunity) could be disclosed, they were considered cryptic elements (Cong et al. 1994; Fukuhara 1995). For some of them, entire or partial nucleotide sequence data are available and potential coding capacities were addressed by heterologous hybridization to pGKL1 derived probes (Cong et al. 1994; Fukuda et al. 1997, 2004; Klassen et al. 2002). Accordingly, cryptic elements may either encode their own DNA polymerase (pDHL1, pDH1A) or are devoid of it (pPE1A); the latter holds true also for the nonautonomous killer elements (pPac1-2, pWR1A). Surprisingly, each of the nonautonomous plasmids contains an ORF encoding a polypeptide resembling the zymocin αβ-precursor, i.e., the conserved toxin carrier complex (see above). A detailed inspection, however, is only possible for pPE1A, since it is the only cryptic linear plasmid entirely sequenced. There is a mutation in the chitinase active site and no hydrophobic region similar to zymocin β (seen in all linear plasmid encoded killer toxins) could be identified. Moreover, an additional secreted protein (like zymocin γ) is not encoded. Thus, pPE1A unlikely encodes a killer toxin, which may have escaped detection. Nevertheless, the zymocin α-like protein encoded by pPE1A ORF2 is secreted and binds to chitin in vitro (Klassen et al. 2002).

In summary, there is convincing evidence for conserved zymocin α-like protein even in cryptic elements, though its function outside of a killer toxin complex remains obscure at present. Stable inheritance of such plasmids in many different yeast species could, however, hardly be understood in cases where there is no autoselection or an alternative positive effect for the host cell.

5 Evolution of Linear Plasmids in Eukaryotes

Eukaryotic linear plasmids, either mitochondrial or cytoplasmic, in principle replicate like genomes of adenoviruses and phi29-like bacteriophages (see above), which has led to the conclusion that they share a common ancestor (Meinhardt et al. 1986, 1990; Kuzmin et al. 1988; Oeser and Tudzynski 1989; Rohe et al. 1992; Kempken et al. 1992). For reconstruction of the phylogeny, both DNA and RNA polymerases have been employed, since the respective genes were found in almost every linear plasmid system. Figure 4 shows an updated phylogenetic tree based on the viral B-type DNA polymerase sequences with basic features agreeing with previous calculations (Rohe et al.



1992; Kempken et al. 1992). Mitochondrial and cytoplasmic linear plasmids are clearly separated, which is indicative of early phylogenetic divergence and adaptation to different cellular compartments. Moreover, cytoplasmic DNA polymerases are more closely related to enzymes from adenoviruses than to those of mitochondrial plasmids. Along with the plant linear plasmids, which also reside in mitochondria, the latter form a separate branch. Adenoviruses as well as the bacteriophage PRD1 (which infects Gram-negative bacteria and employs a phi29-like replication mechanism) were recently shown to display clear similarities concerning virion structure (Benap and Steven 2000; Benson et al. 1999, 2000; San Martin and Burnett 2003). Such findings agree with the assumption that adenoviruses and (PRD1/phi29-like) bacteriophages have a common ancestor which predates the occurrence of eukaryotes (Davison et al. 2003).

The calculated relatedness of cytoplasmic linear plasmids and adenoviruses points to separation from their ancestor prior to the emergence of

◀ **Fig. 4** Phylogenetic tree for protein-primed replicating genetic elements based on B-type DNA polymerases. Besides bacteriophages, present day manifestations in eukaryotes exist in mitochondria, in the cytoplasm, and in the nucleus (adenoviruses). Since mitochondrial elements are clearly separated from the cytoplasmic and nuclear plasmids, they must have diverged early during evolution; divergence of the nuclear and cytoplasmic elements dates back to a later point. Yet later, autonomous and nonautonomous elements split. The bar denotes 10% divergence. GenBank accession numbers of individual sequences: CAC08221.2: *Pichia etchellsii* pPE1A; CAA38621.1: *Saccharomyces kluyveri* pSKL; CAA25568.1: *Kluyveromyces lactis* pGKL1; CAA30603.1: *K. lactis* pGKL2; CAA09497.1: *Debaryomyces hansenii* pDHL1; CAD91889.1: *Pichia inositovora* pPin1-3; CAA72340.1: *Pichia kluyveri* pPK2; BAB13496.1: *Flammulina velutipes* pFV1; CAA36327.1: *Neurospora intermedia* kalilo; NP_053000.1: *Pleurotus ostreatus* pMLP1; CAA43117.2: *Podospora anserina* pAL2-1; NP_862206.1: *Blumeria graminis* f. sp. hordei pBgh; AAB41447.1: *Gelasinospora* sp. G114 Gel-kal; JQ0301: *Claviceps purpurea* pClK1; S26947: *Podospora anserina* pAL2-1; S05362: *Ascobolus immersus* pAI2; S26985: *Neurospora crassa* maranhar; CAA45364.2: *Morchella conica* pMC3-2; BAC16364.1: *Brassica napus* linear plasmid; P10582: *Zea mays* S-1; NP_659515.1: Ovine adenovirus D; AP_000236.1: Porcine adenovirus 5; YP_094032.1: Bovine adenovirus A; AAN84890.1: Tree shrew adenovirus 1; AP_000478.1: Turkey adenovirus 3; AP_000613.1: Canine adenovirus type 2; YP_213966.1: Simian adenovirus 1; AAA42478.1: Human adenovirus type 12; NP_040853.1: Human adenovirus F; P03680: phage phi29; AAP83475.1: phage Bam35c; NP_690635.1: phage B103; AAX45903.1: phage PRD1; AAX45594.1: phage PR4

eukaryotes. In contrast, the distinct separation of mitochondrial elements from both cytoplasmic linear plasmids and adenoviruses suggests that mitochondrial elements diverged later, possibly in the postendosymbiont era. Consistently, plasmid pPK2 of *Pichia kluyveri*, one of the few known mitochondrial linear yeast elements (Blaisonneau et al. 1999), is more closely related to mitochondrial linear plasmids from filamentous fungi and plants than to the cytoplasmic linear plasmids from other *Pichia* species. In general, the relatedness of mitochondrial linear elements reflects the phylogenetic relations of their hosts (Rohe et al. 1992), suggesting them to originate from a single mitochondrial ancestor which may have been acquired as an endosymbiotic (bacteriophage-like) genetic element. In contrast to bacteriophages and adenoviruses, linear plasmids lost almost all viral attributes (such as a capsid or an infectious cycle), presumably as an adaptation to the propagation mode of their hosts, since extranuclear inheritance, either as cytoplasmic or mitochondrial element, does not necessarily include an infectious virion for efficient propagation.

6 Concluding Remarks and Perspectives

A large number of eukaryotic microbes and plants contain linear plasmids which are descendants of ancient viruses. Hence they afford an opportu-

nity to trace evolutionary mapping beginning with preeukaryotic elements to the point of vertebrate adenoviruses. In filamentous fungi such virus-like elements are exclusively confined to mitochondria; here, they commonly exhibit extremely minimalized genomes encoding basically and essentially only a DNA and an RNA polymerase. Mitochondrial linear plasmids exist either as neutral passengers within the organelle or as harmful attendants which—by integrating into the mitochondrial genome—shorten the host's life span. Studying aging in such interactions may facilitate general insights into molecular mechanisms of senescence.

The phylogenetic root of the T7-like RNA polymerase, highly conserved among mitochondrial linear plasmids, still remains obscure even though the organelles possess enzymes of that type anyway. Concerning the enzymatic repertoire needed for cytoplasmic gene expression of yeast linear plasmids, much work is to be done until a comprehensive picture of the rather unique process of cytoplasmic transcription emerges, including termination and posttranscriptional modification.

For killer toxins of either type (I and II), research will have to address early events, such as binding to the cell wall receptor and the probable transport to a yet unknown membrane receptor. Elucidating the definite mode of DNA damage occurring in type II toxin-treated cells constitutes another challenge. Investigating transmembrane passage and intracellular trafficking of the toxic subunits will not only provide basic knowledge for this rather peculiar phenomenon, but will also help to establish similarities and differences to known protein toxins of clearly diverse evolutionary origins.

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