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Killer Toxin-Secreting Double-Stranded RNA Mycoviruses in the Yeasts *Hanseniaspora uvarum* and *Zygosaccharomyces bailii*

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Killer toxin-secreting strains of the yeasts *Hanseniaspora uvarum* and *Zygosaccharomyces bailii* were shown to contain linear double-stranded RNAs (dsRNAs) that persist within the cytoplasm of the infected host cell as encapsidated virus-like particles. In both yeasts, L- and M-dsRNAs were associated with an 85-kDa major capsid protein, whereas the additional Z-dsRNA (2.8 kb), present only in the wild-type *Z. bailii* killer strain, was shown to be encapsidated by a 35-kDa coat protein. Although Northern (RNA) blot hybridizations indicated that L-dsRNA from *Z. bailii* is a L_A species, additional peptide maps of the purified 85-kDa capsid from *Z. bailii* and the 88- and 80-kDa major coat proteins from K_1 and K_{28} killer viruses of *Saccharomyces cerevisiae* revealed distinctly different patterns of peptides. Electron microscopy of purified *Z. bailii* viruses (ZbV) identified icosahedral particles 40 nm in diameter which were undistinguishable from the *S. cerevisiae* killer viruses. We demonstrated that purified ZbVs are sufficient to confer the *Z. bailii* killer phenotype on transfected spheroplasts of a *S. cerevisiae* nonkiller strain and that the resulting transfectants secreted even more killer toxin than the original ZbV donor strain did. Curing experiments with ZbV-transfected *S. cerevisiae* strains indicated that the M-dsRNA satellite from *Z. bailii* contains the genetic information for toxin production, whereas expression of toxin immunity might be dependent on Z-dsRNA, which resembles a new dsRNA replicon in yeasts that is not dependent on an L_A helper virus to be stably maintained and replicated within the cell.

Mycoviruses in yeasts resemble cytoplasmatically inherited double-stranded RNA (dsRNA) viruses with separately encapsidated dsRNA segments that are not necessarily infectious to intact cells. Although most of these mycoviruses—including the well-defined viruses of *Aspergillus* and *Penicillium* spp. and the more recently discovered mycoviruses of *Yarrowia lipolytica*—do not confer an easily recognizable phenotype upon the fungal host, dsRNA viruses in *Saccharomyces cerevisiae* and *Ustilago maydis* are associated with distinct killer phenotypes, i.e., the ability to kill sensitive fungi of the same or related genera (3, 11, 16, 22). In *Saccharomyces* spp. there are three families of dsRNAs—designated L-A, L-BC, and M—which are present in cytoplasmic icosahedral virus-like particles (VLPs) that are, at least for L-A and M, involved in the killer phenomenon of its host. These dsRNA viruses are only vertically transmitted between cells by cytoplasmic mixing during mating or heterokaryon formation, although it has recently been shown that they are also capable of penetrating spheroplasts or lithium acetate-treated or mating yeast cells (10, 30).

The best-understood and well-characterized dsRNA killer viruses of *S. cerevisiae* (ScV-L_A, ScV-M) contain a viral genome consisting of two dsRNAs (L-A and M). L-A (4.6 kb) encodes the 76-kDa major coat protein (VL_{1A}-P1) in which the two dsRNA segments are separately encapsidated and the 171-kDa minor capsid-polymerase (*cap-pol*) fusion protein responsible for transcription and packaging of the viral RNAs (7, 8, 14, 15). M contains the genetic information for the killer toxin and the specific and as yet unidentified immunity component which renders the killer cells immune to their own toxin (2, 5). Three killer phenotypes (K_1, K_2, and K_{28}) are currently recognized; they are clearly distinguished by their lack of cross-immunity, their toxin properties, and their lethal mechanisms; killer toxins K_1 and K_2 bind to cell wall β-1,6-D-glucans and disrupt membrane functions in the sensitive target cell, whereas killer toxin K_{28} binds to α-1,3-linked mannotriose side chains of a cell wall mannoprotein and subsequently causes early inhibition of DNA synthesis (4, 38). Genetic analysis of the dsRNA-based killer system in *Saccharomyces* spp. revealed an array of complex interactions between the viral and host genomes, including host-encoded functions involved in maintenance (e.g., *MAK* genes) or expression (e.g., *KEX* genes) of the killer virus, as well as viral genes (e.g., [HOK], [NEX], [EXL]) responsible for dsRNA stability or incompatibility (37, 38, 44, 45).

Soon after the discovery of the viral killer systems in *S. cerevisiae* and *U. maydis*, killer strains were also found in the genera *Debaryomyces*, *Pichia*, *Kluyveromyces*, *Hansenula*, *Candida*, *Cryptococcus*, *Hanseniaspora*, and *Zygosaccharomyces* (21, 23–26, 34, 46). Whereas the genetic basis of the *Kluyveromyces lactis* killer phenotype was shown to be associated with the presence of two dsDNA plasmids which could be transformed and stably expressed in several different yeast genera (17, 18, 33), the molecular basis in all other killer yeasts is either dsRNA based, unknown, or thought to be chromosomally encoded.

The more recently discovered killer strains in the yeasts *Hanseniaspora uvarum* and *Zygosaccharomyces bailii* were shown to possess cytoplasmic L (4.5-kb) and M (1.8-kb) dsRNAs which, in *H. uvarum*, could be transferred to nonkiller strains of *S. cerevisiae* by intergeneric protoplast fusion, resulting in heterokaryons that expressed a stable killer phenotype (23, 25, 47). Additional curing experiments indicated that the secreted killer toxins of both yeasts are genetically encoded by the corresponding M-dsRNAs. Since this was the first observation of a dsRNA-based killer system in yeasts other than

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TABLE 1. Yeast strains used in this study

Strain[a]	Genotype or description	Killer phenotype[b] and dsRNAs[c]	Reference(s)
H. uvarum			
470	Wild type	K^+; L, M_{Hu}	25, 44
471	Wild type	K^+; L, M_{Hu}	25, 44
472	Wild type	K^+; L, M_{Hu}	25, 44
S. cerevisiae			
K7	**a** *arg9*	K_1^+; L_A, M_1	30
K7-S1	**a** *arg9*	K^-; L_A	30, 31
MS100	**a** *leu2 arg9*	K_{28}^+; L_A, M_{28}	30, 31
MS300b	α *leu2 ura3-52 ski2-2*	K_{28}^+; L_A, L_{BC}, M_{28}	30
2267	**a** *leu2 ura3 mkt1 ski2-11*	K_2^+; L_A, M_2	30
1385	α *lys1 ura1*	K_2^+; L_A, L_B, M_2	30, 31
1686	α *arg1 thr1*	K^-; L_C	30, 31
1938	**a** *leu2 arg9*	K^-; L-0	30, 31
GG100-14D	α *ura3-52 his3 trp1 pho3 pho5*	K^-; L_A, L_{BC}	30, 31
MS412a	α *ura3-52 his3 trp1 pho3 pho5*	K^+; L_A, L_{BC}, M_{Zb}	This work
MS412b	α *ura3-52 his3 trp1 pho3 pho5*	K^+; L_A, L_{BC}, M_{Zb}	This work
Z. bailii			
412	Wild type	K^+; L, Z, M_{Zb}	23
FN201	412 cured with cycloheximide	K^-; L, Z	This work
FN207	412 cured with cycloheximide	K^-; Z	This work
FN212	412 cured with cycloheximide	K^-; L	This work
FN213	412 cured with cycloheximide	K^-; L, Z	This work

[a] Strains 2267, 1385, 1686, and 1938 were originally provided by Reed B. Wickner.
[b] The killer phenotype refers to the specific immunity class determined by the M-dsRNA species present.
[c] The L_A species listed are derived from either K_1, K_2, or K_{28} killers. M_{Zb} and M_{Hu} represent natural M-dsRNA species present in killer strains of the yeasts *Z. bailii* and *H. uvarum*, respectively.

Saccharomyces spp., it was the purpose of this study to determine whether these dsRNA "plasmids" are present in cytoplasmic VLPs responsible for the corresponding killer phenotype and capable of infecting nonkiller strains of *S. cerevisiae*. To test this hypothesis, we have isolated VLPs from both yeasts, determined their major capsid proteins, analyzed capsid homologies by peptide mapping, and used the purified VLPs from *Z. bailii* to transfer the K^+ phenotype to a standard laboratory nonkiller strain of *S. cerevisiae*.

MATERIALS AND METHODS

Yeast strains and culture media. Genotypes and dsRNA contents of all yeast strains used in this work are listed in Table 1. YEPD, YEPG, methylene blue agar (MBA; pH 3.7), and minimal media were described previously (27, 30, 31). Yeast strains were cured of their killer phenotype by cultivation in YEPD medium in the presence of cycloheximide (13) or at elevated temperature (43).

dsRNA preparation. dsRNAs were prepared from yeasts that had been grown to late logarithmic/early stationary phase at 30°C in YEPD medium. A rapid dsRNA minipreparation procedure was used for the isolation of total RNA (1, 30). When necessary, rRNA and other single-stranded RNAs were subsequently removed by precipitation with 4 M LiCl (35) before gel electrophoresis. Crude or partially purified dsRNAs (1 μg) were analyzed by electrophoresis on 1% (wt/vol) agarose gels and stained with ethidium bromide (0.5 μg/ml).

5′-End labeling of purified dsRNAs. L- and M-dsRNAs from killer strain *Z. bailii* 412 were isolated as described above and purified by electrophoresis on 0.8% (wt/vol) low-melting-temperature agarose gels. For labeling of the uncapped 5′ termini, each dsRNA (1 μg) was dephosphorylated by treatment with calf intestine alkaline phosphatase, extracted with phenol-chloroform (1:1, vol/vol), and, after ethanol precipitation, dissolved in 5 μl of distilled diethylpyrocarbonate (DEPC)-H_2O. To each sample, 2.5 μl of 10× kinase buffer (0.5 M Tris-HCl [pH 9.0], 50 mM $MgCl_2$, 1 mM EDTA, 10 mM spermine), 100 pmol of [^{32}P]ATP (5,000 Ci/mmol; Amersham), and distilled DEPC-H_2O were added to give a final volume of 24 μl. The reaction was initiated by the addition of 5 U of T4 polynucleotide kinase (5 U/μl; Bethesda Research Laboratories) and incubated for 30 min at 37°C. For termination, 25 μl of ammonium acetate (4 M) and 5 μg of tRNA carrier were added and the labeled dsRNA was ethanol precipitated. Unincorporated nucleotides were removed by resuspending the dried dsRNA pellets in 0.5 M sodium acetate (pH 5.5) and reprecipitating them with ethanol.

Northern blot hybridizations. RNA-RNA hybridizations were performed essentially as described previously (30, 36). Equal quantities of denatured dsRNA (1 μg) were fractionated by electrophoresis through 1.1% agarose–formaldehyde gels and transferred to positively charged nylon membranes (Boehringer) by capillary blotting overnight in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]). The prehybridized membrane was hybridized at 65°C for 18 h with the 5′-end-labeled dsRNA in a solution containing 50% formamide, 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 5× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), and 200 μg of tRNA carrier per ml essentially as described previously (41). Following hybridization, the membrane was washed twice under stringent conditions with 0.1× SSPE–0.1% SDS for 15 min each at 65°C, dried, and exposed to Kodak XAR-5 film at −70°C with an intensifying screen (Dupont).

Purification of VLPs and transfection of yeast spheroplasts. For maximal VLP production, the appropriate killer yeasts were grown in YEPG medium (YEP supplemented with 0.5%

glucose and 4% glycerol) for 4 days at 30°C, harvested by centrifugation, washed with SEKS buffer (1 M sorbitol, 0.1 M EDTA, 0.1 M Na_2SO_3, 0.8 M KCl [pH 7.5]), and resuspended in the same buffer (10 ml/g of wet cells). The cells were treated with Zymolyase-20T (WAK-Chemie; 100 μg/g of wet cells) and incubated at 30°C for 0.5 to 1.0 h. Disruption of the cells and isolation and purification of dsRNA-containing VLPs were performed essentially as described previously (30). Purified and pelleted VLPs were resuspended in PKE buffer (30 mM Na_2HPO_4, 150 mM KCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [pH 7.6]) and either used immediately for infection experiments or stored at −70°C. VLP infection of yeast spheroplasts was done as described by El-Sherbeini and Bostian (10). Briefly, spheroplasts (10^8 cells) of the indicated yeast strains were transfected with 20 μg of purified VLPs (determined as micrograms of capsid protein per milliliter by the micro-bicinchoninic acid [BCA] method) in the presence of 1 μg of plasmid YEp352 (19) to allow selection of transformants in the presence of 10 μg of calf thymus DNA. Selection of Ura^+ transformants was done by plating onto uracil-deficient minimal medium (SC-Ura). After 3 to 5 days at 30°C, prototrophic transformants were picked, cloned, and screened for virus uptake by testing for their killer phenotype.

Capsid proteins and peptide mapping. The major VLP capsid proteins were extracted by precipitation with trichloracetic acid (10%) and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 10% polyacrylamide gels essentially as described previously (12, 30). Peptide mapping of the purified coat proteins was done by digestion with *Staphylococcus aureus* V8 protease (100 μg/ml) for various intervals as described by Cleveland et al. (6). The generated peptide patterns were analyzed by SDS-PAGE and silver staining.

Electron microscopy. For electron microscopy, purified VLPs were negatively stained with 1% (wt/vol) phosphotungstic acid (pH 7.1) on Formvar and carbon-coated 200 mesh copper grids and examined in a Siemens 100 microscope at 80 kV with nominal magnifications of 20,000 to 50,000.

Killer assay. Killer strains and VLP-infected yeast strains were tested for their killer phenotype by being streaked onto MBA plates (pH 4.0) seeded with 10^5 cells of the sensitive strain *S. cerevisiae* 1938; the plates were incubated for 72 h at 20°C. A zone of growth inhibition surrounding the streak indicates toxin production (29).

RESULTS

dsRNA patterns of representative killer strains of *S. cerevisiae*, *H. uvarum*, and *Z. bailii* and their cured derivatives. The patterns of cytoplasmic dsRNAs isolated from some of the strains used in this work (Table 1) are illustrated in Fig. 1A, an agarose gel with approximately equal amounts of RNA loaded in each lane. All of the L-dsRNAs (4.5 kb) had similar mobilities and therefore could not be distinguished from the L-A species of the representative *S. cerevisiae* K_1, K_2, and K_{28} killer strains. Killer strains *H. uvarum* 470 and 471, as well as *Z. bailii* 412, contained L-dsRNAs in roughly the same amounts and a toxin-coding M-dsRNA (1.0, 1.4, and 1.8 kb, respectively) at significantly lower levels. In addition to L and M, *Z. bailii* 412 contained a third RNA species, of 2.8 kb, which was previously shown (23) to be dsRNA in nature since it was resistant to pancreatic RNase A treatment under high-salt conditions (0.8 M NaCl)—conditions sufficient to fully degrade single-stranded RNA (ssRNA). According to the suggested nomenclature (23), this new species of dsRNA will henceforth be designated Z-dsRNA (for *Zygosaccharomyces* dsRNA).

Both cycloheximide-cured derivatives of *H. uvarum* and *Z. bailii* had lost their M-dsRNA (Fig. 1B), demonstrating that, as in *S. cerevisiae*, the killer phenotype is associated with the presence of the corresponding M species. For unknown reasons, the minor amount of M-dsRNA present in the cured nonkiller strain FN213 (Fig. 1B, lane 11) represents a quite common observation with cycloheximide-treated strains of *Z. bailii*; this phenomenon has also been reported for some cured *S. cerevisiae* strains (37). In contrast to cured strains of *S. cerevisiae*, cured clones of *Z. bailii* 412, retaining either L- plus Z-dsRNA or Z-dsRNA only, remained fully immune to the wild-type toxin, as did L-dsRNA-containing strains, which had also lost Z-dsRNA (Fig. 1B). Additional toxin-binding studies revealed that both killer and cured nonkiller strains of *Z. bailii* 412 were perfectly capable of binding exogenously applied killer toxin to the yeast cell wall (data not shown), indicating that toxin immunity is not caused by a lack of primary toxin receptors. Furthermore, spheroplasts of all cured nonkiller derivatives of *Z. bailii* 412 remained likewise immune to the wild-type toxin KT412, suggesting that, unlike in *S. cerevisiae* killer strains, the natural M species of *Z. bailii* is either not involved in or not necessary for the expression of toxin immunity.

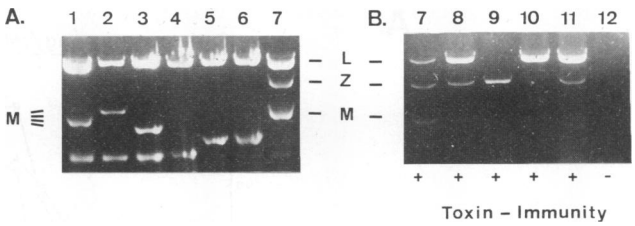

FIG. 1. dsRNA patterns of killer (A) and cured (B) yeast strains. dsRNAs were isolated from the indicated strains (see Table 1) as described in Materials and Methods. Positions of L- and M-dsRNAs, as well as killer toxin immunity of cured nonkiller strains, are indicated. Lanes: 1, *S. cerevisiae* K7; 2, *S. cerevisiae* MS300b; 3, *S. cerevisiae* 2267; 4 to 6, *H. uvarum* 470, 471, and 472, respectively; 7, *Z. bailii* 412; 8 to 11, *Z. bailii* FN201, FN207, FN212, and FN213, respectively; 12, *S. cerevisiae* 1938.

Identification of dsRNA containing VLPs in killer strains of *Z. bailii* and *H. uvarum*. By using procedures that have been previously described for the isolation of K_1, K_2, and K_{28} VLPs from *S. cerevisiae* (10, 30), we were able to identify dsRNA "killer viruses" with very similar properties in the yeasts *Z. bailii* and *H. uvarum*. As shown in Fig. 2B, analysis of sucrose gradient fractions for dsRNA content revealed the presence of L- and M-dsRNA in *H. uvarum* and the presence of L-, Z-, and M-dsRNA in *Z. bailii*. The sedimentation rates of these VLPs were similar to those of purified K_{28} VLPs from the representative *S. cerevisiae* killer strain MS300b (Fig. 2A). VLPs prepared from killer strain *Z. bailii* 412 and purified on sucrose gradients resulted in three peaks, with sedimentation rates being L > M > Z (Fig. 2B). SDS-PAGE of portions from the same gradient showed two major proteins, one of about 85 kDa banding at around fraction 10 and another protein of about 35 kDa which was visible mainly in fraction 12 (Fig. 2C). To determine which protein was associated with each dsRNA, we used virus preparations essentially free of Z-dsRNA and highly enriched for L- and M-dsRNA and VLPs mainly enriched for Z-dsRNA which contained only minor amounts of L- and M-dsRNA. As shown in Fig. 3, L and M VLPs were always associated with the 85-kDa protein, whereas virus preparations highly enriched for the 2.8-kb Z-dsRNA gave a strong signal for the 35-kDa protein, showing only minor amounts of the

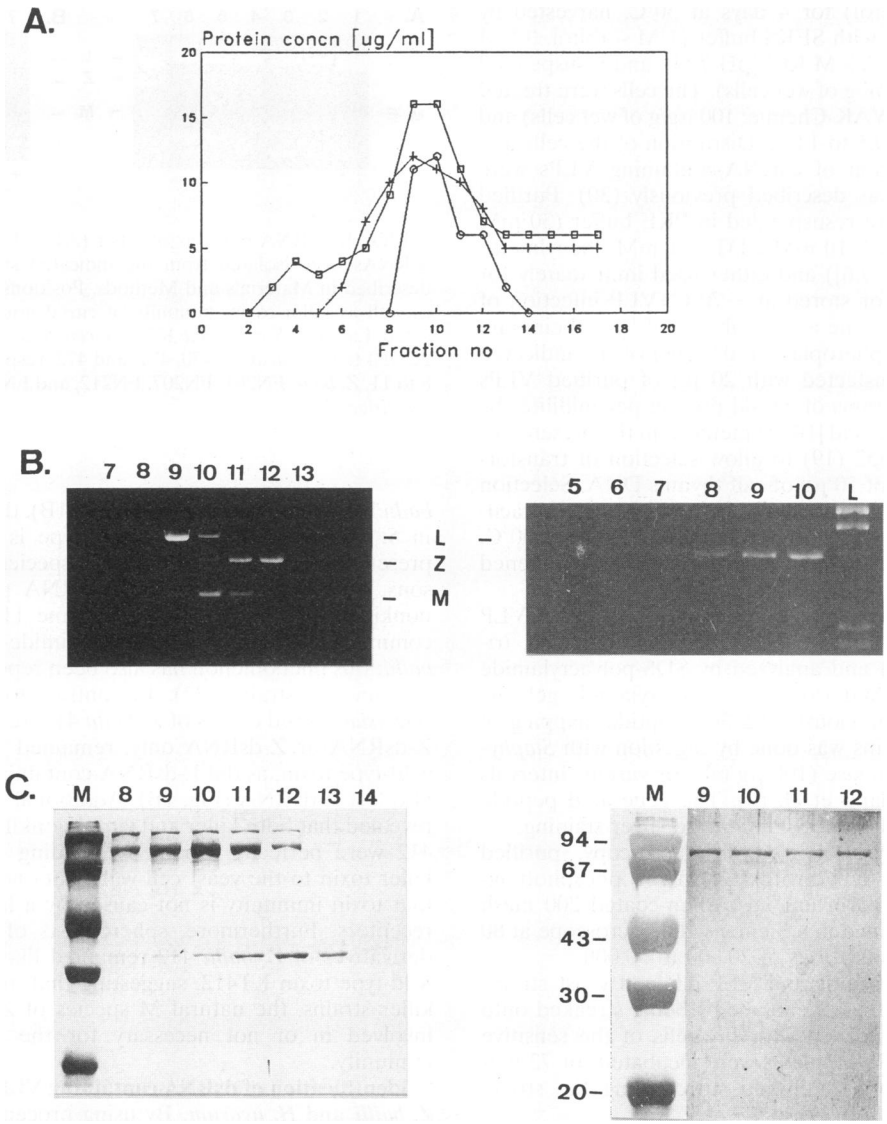

FIG. 2. Protein concentration (A), dsRNA pattern (B), and major capsid proteins (C) of VLPs isolated from *Z. bailii* 412 (left panels) and *H. uvarum* 470 (right panels) after purification by sucrose gradient equilibrium sedimentation. (A) Portions of each fraction of the gradients of *Z. bailii* (□), *H. uvarum* (○), and of the reference killer strain *S. cerevisiae* MS300b (+) were analyzed for their protein concentration by the micro BCA method. (B) Portions of each fraction were extracted with 1 volume of phenol, directly taken from the aqueous phase, and used for agarose gel electrophoresis. (C) Viral capsid proteins present in aliquots of each fraction (10 to 20 μl) were precipitated by the addition of 0.5 ml of trichloroacetic acid (10% [wt/vol]), washed with 1 ml of 0.1% trichloroacetic acid, dissolved in gel-loading buffer (containing 2.5% SDS and 5% β-mercaptoethanol), and used for SDS-PAGE. Fraction numbers, *Hin*dIII-digested lambda DNA standard (L), and sizes (in kilodaltons) of molecular mass standards (M) are indicated. In the figure, the left corresponds to the denser fractions of the bottom of the gradient and the right corresponds to the lighter fractions.

contaminating 85-kDa polypeptide. These data suggest that L- and M-dsRNAs, on the one hand, and Z-dsRNA, on the other hand, are encapsidated by 85- and 35-kDa capsid proteins, respectively. Analysis of the purified proteins isolated from L and M VLPs of *H. uvarum* killer strain 470 revealed only one single protein band with an apparent molecular mass of 85 kDa, showing the same mobility on SDS gels as the 85-kDa major capsid protein of L and M VLPs from *Z. bailii* 412 (Fig. 2C). However, both 85-kDa proteins could be clearly distinguished from the purified 88- and 80-kDa capsid proteins of representative K_1 and K_{28} VLPs of *S. cerevisiae* (Fig. 4). Since VLP preparations of cured derivatives and/or natural nonkiller strains of *H. uvarum* and *Z. bailii* contained only the 85-kDa capsid protein characteristic of L VLPs (data not shown), it can be assumed that in both yeasts, the corresponding M-dsRNAs are encapsidated by the 85-kDa protein, which therefore is likely to be provided by L-dsRNA. The purified polypeptides were further compared with each other and with coat proteins obtained from K_1 and K_{28} VLPs of representative *S. cerevisiae* killer strains. Digestions were performed in parallel for various times with a constant amount of *S. aureus* V8 protease. The partial hydrolysis fragments were then resolved by electrophoresis on a 12.5% polyacrylamide–SDS slab gel. The 85-kDa capsid protein of *Z. bailii* revealed a pattern of peptides

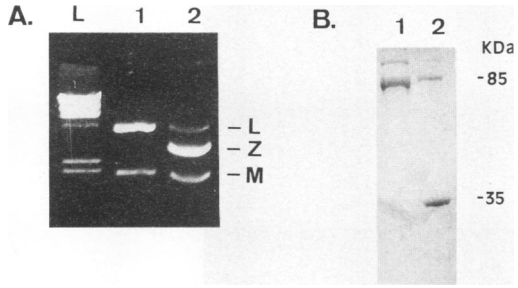

FIG. 3. dsRNA patterns (A) and major coat proteins (B) of purified VLPs from *Z. bailii* 412 highly enriched for either L- and M-dsRNA (lanes 1) or Z-dsRNA (lanes 2). Agarose gel electrophoresis and SDS-PAGE were done as described in the legend to Fig. 2.

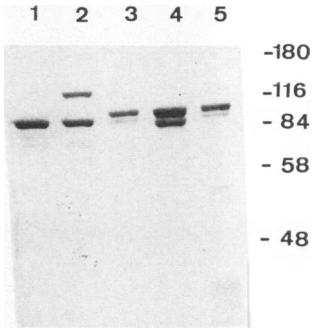

FIG. 4. SDS-PAGE of purified capsid proteins. Capsid proteins of purified VLPs from the killer yeasts *H. uvarum* 470 (lane 1) and *Z. bailii* 412 (lane 2) and from *S. cerevisiae* K7 (lane 3), 2267 (lane 4), and MS100 (lane 5) were fractionated by SDS-PAGE and detected by staining with Coomassie brilliant blue. Note that VLPs from *Z. bailii* 412 (lane 2) represent purified L and M viruses not containing any detectable amounts of ZbV-Z. Molecular mass markers are indicated on the right in kilodaltons.

distinctly different from that obtained after protease digestion of the 88-kDa coat protein of K_1 VLP or the 80-kDa capsid protein of K_{28} VLP, suggesting considerable sequence divergence at the level of the capsid-encoding L-dsRNAs. Proof that these 85-kDa proteins of L and M VLPs from *Z. bailii* and *H. uvarum* are encoded by their corresponding L-dsRNAs will require analysis of the in vitro translation products of denatured L-dsRNA; such analysis is planned for future experiments.

Portions of dsRNA containing sucrose gradient fractions (Fig. 2) were pooled, pelleted, negatively stained, and examined under an electron microscope (Fig. 5). Spherical virions with an estimated diameter of about 38 to 40 nm were seen in preparations of *Z. bailii* and *S. cerevisiae* (K_1) but not in VLP preparations of *H. uvarum* killer strains. The hardly detectable amounts of M-dsRNA in *H. uvarum* (Fig. 2B) and the failure to clearly identify spherical VLPs by electron microscopy are probably due to the extremely low levels of virions present in this yeast; also, they were visible only in preparations derived from cells that had been grown on the nonfermentable carbon source glycerol.

Infection of *S. cerevisiae* with the ZbV killer virus of *Z. bailii*. Transfection experiments involved a mixture of purified L, Z, and M VLPs from *Z. bailii* that was used to infect spheroplasts of the nonkiller, L-A- and L-BC-containing recipient strain *S. cerevisiae* GG100-14D, a strain which was previously shown to be suitable for successful uptake of K_1, K_2, and K_{28} killer viruses (10, 30). Spheroplasts of this strain, cotransformed with the *URA3* plasmid YEp352, could be transfected by VLPs from *Z. bailii* (ZbV) with a very low efficiency of only 4% by using essentially the procedure previously described (10, 30). Two representative transfectants, MS412a and MS412b, showed characteristic *Z. bailii* killing zones on sensitive strain 1938 (Fig. 6A) and portrayed the typical *Z. bailii* killer phenotype with its distinctive immunity pattern. The killing zones produced by the transfectant clones were detectably larger than the rather weak zones produced by the VLP donor *Z. bailii* 412 (Fig. 6A). Infected clones that had been selected for further analysis were cultivated in YEPD medium for about 10 generations and showed a stable killer phenotype, indicating stable inheritance of the killer ZbV. As shown in Fig. 6B (lanes 3 and 4), transfected clones MS412a and MS412b contained L-, Z- and M-dsRNA in amounts roughly twofold larger than those in the wild-type *Z. bailii* 412.

Northern blot analysis of L- and M-dsRNA from *Z. bailii*. Proof that M-dsRNA from *Z. bailii* in the transfected strain MS412 is actually maintained by L-A-HN originally derived from the *S. cerevisiae* host strain comes from Northern blot analysis of different L species probed with a ^{32}P-labeled L-dsRNA from *Z. bailii*. In these experiments, L-dsRNA from *Z. bailii* was used as a probe for hybridization with denatured L-A, L-B, L-C, L-dsRNA from *Z. bailii* killer and nonkiller derivatives and with the L species from ZbV transfectants. The resulting Northern blot (Fig. 7) showed that the labeled probe hybridized strongly with denatured L-dsRNA from killer and cured strains of *Z. bailii* (lanes 1 and 2), weakly with L-A (lane 4) and with a mixture of L-A and L-B (lane 5), but not at all with L-C (lane 6). Since L-B and L-C are known to show about 60% cross-hybridization (32), it can be concluded that L from *Z. bailii*—which did not hybridize with L-C (not even under less stringent hybridization conditions)—most probably lacks L-BC and, as expected, shares partial homology to L-A. The lack of cross-hybridization to L-A seen in Fig. 7, lane 3, is probably due to the small amount of denatured RNA loaded in that lane, as was shown by ethidium bromide staining of the corresponding agarose gel. The transfectant strain MS412 apparently failed to retain L from *Z. bailii* (Fig. 7, lane 3), illustrating the competence of L-A for maintenance of M-dsRNA from *Z. bailii*. When the same nonkiller host strain (*S. cerevisiae* GG100-14D) was infected by ZbV particles highly enriched for L- and M-dsRNA, not containing any detectable amounts of Z-dsRNA (Fig. 3A, lane 1), the resulting transfectants were likewise killers. Furthermore, Northern analysis which was performed immediately after the killer clones had been isolated revealed that ZbV-L was originally taken up by the host but was not stably maintained, explaining the previously observed lack of ZbV-L in transfectants that had been cultivated in YEPD medium for about 100 generations prior to Northern analysis. Therefore, it is justified to conclude that ZbV-M, even in the absence of the L-dsRNA species from *Z. bailii*, is sufficient for the expression of a complete ZbV killer phenotype in an L-A strain of *S. cerevisiae*.

To characterize the toxin-encoding M-dsRNA from the wild-type killer strain *Z. bailii* 412, we used a ^{32}P-labeled M-dsRNA as a probe for Northern blot analysis of denatured M_1-, M_2-, and M_{28}-dsRNA (Fig. 8). With this probe, a very strong signal was obtained with the M species of the wild-type *Z. bailii* 412 (lane 1) and with the M species of the ZbV transfectant *S. cerevisiae* MS412 (lane 2) but no signal could be detected with either M_1 (1.8 kb; lane 3), M_2 (1.5 kb; lane 4), or M_{28} (1.9 kb; lane 5).

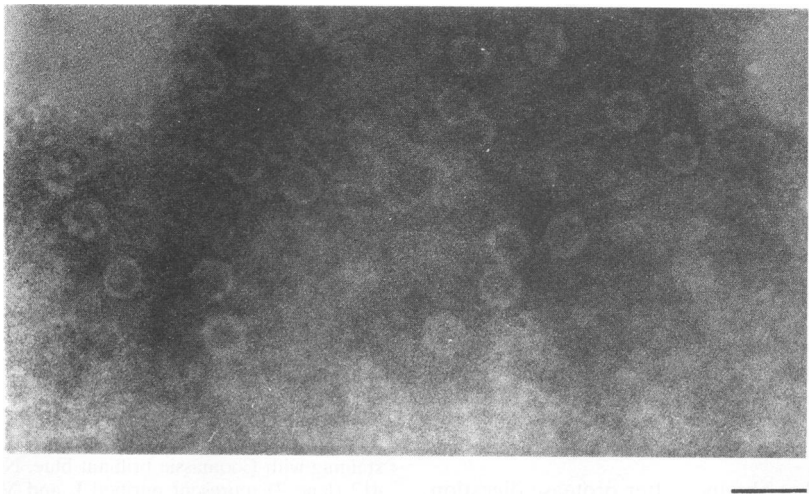

FIG. 5. Electron micrograph of negatively stained VLPs from killer strain *Z. bailii* 412. Bar, 90 nm.

DISCUSSION

The killer phenotype of *Z. bailii* is particularly interesting because of its broad killing activity, exhibiting lethality upon many different yeasts including *S. cerevisiae*, *K. lactis*, *Sporothrix* spp., most strains of *Z. bailii*, *Candida glabrata*, and the pathogenic yeast *Candida albicans* (28). Detailed analysis of the killer toxin and its dsRNA determinant has been hampered by the recalcitrance of the wild-type killer strain *Z. bailii* 412 to genetic analysis. Therefore, identification of encapsidated, cytoplasmic dsRNAs in this yeast that are present as VLPs and the successful transfer of the *Zygosaccharomyces* killer phenotype to standard *S. cerevisiae* laboratory strains by VLP transfection will obviously facilitate a more detailed genetic and biochemical analysis of nuclear mutations which normally affect the maintenance and expression of M-dsRNAs in cells expressing the ZbV killer phenotype.

By using essentially the same transfection procedure as previously described (11, 30), spheroplasts of the nonkiller strain *S. cerevisiae* GG100-14D were infected with the purified viruses isolated from *Z. bailii* (ZbV). The efficiency of this extracellular VLP transmission was only about 4%, very similar to the low infection rate obtained with the K_2 killer virus of *S. cerevisiae* (10). The resulting ZbV transfectant strain, *S. cerevisiae* MS412, took up all three dsRNA viruses (ZbV-L, -Z, and -M) and was a stable killer that produced even more toxin than did the original VLP donor strain, indicating that the purified ZbV killer viruses are sufficient to transfer the complete *Z. bailii* killer phenotype. However, ZbV-L and ZbV-Z were not stably maintained in the transfected host, since we could show that both viruses were lost after the transfectants, which still retained M, had been cultivated for more than 100 generations. Since we could also confirm that loss of this phenotype is correlated with a concomitant loss of M-dsRNA during curing (23), it is highly probable that the *Zygosaccharomyces* killer phenotype is encoded by M, which does not share any significant homology to M_1, M_2, or M_{28}, as was shown by the lack of cross-hybridization in Northern blots (Fig. 8).

A similar analysis of the L species from *Z. bailii* 412 revealed that it belongs to the L-A family, which clearly failed to cross-hybridize with L-C, which itself is known to share about 60% cross-hybridization with L-B (32). The weak hybridization of L_{Zb} with the L-A species from *S. cerevisiae* correlates well with the observed differences in gel mobility and peptide maps of the corresponding 85- and 88-kDa major VLP capsid proteins, which, at least for the major coat protein (VL_{1A}-P1) of ScV-L_A, are genetically encoded by the appropriate L-A

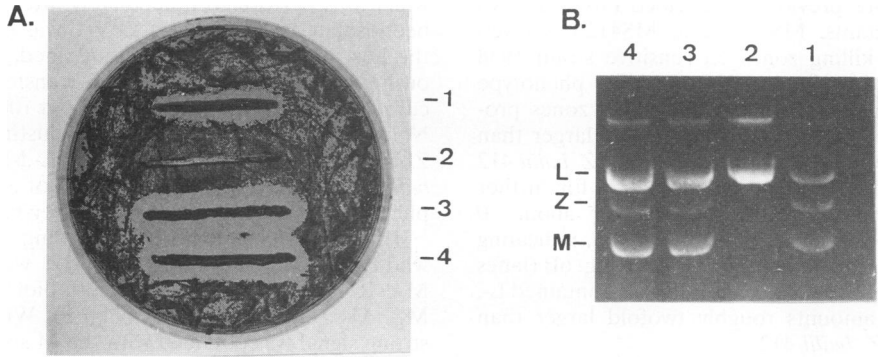

FIG. 6. Killer phenotype (A) and dsRNA pattern (B) of yeast clones infected with the killer ZbV. 1, *Z. bailii* 412 (wild-type killer); 2, *S. cerevisiae* GG100-14D (nonkiller host strain); 3 and 4, ZbV transfectants *S. cerevisiae* MS412a and MS412b, respectively. The indicated strains were tested for their killer phenotype on MBA plates (pH 4.0) seeded with an overlay of the sensitive strain 1938.

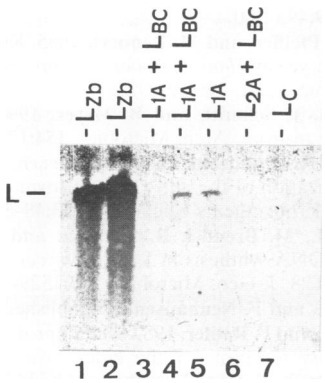

FIG. 7. Northern blot analysis of L-dsRNAs. Denatured dsRNAs from *Z. bailii* 412 (lane 1), *Z. bailii* FN201 (lane 2), *S. cerevisiae* MS412 (lane 3), *S. cerevisiae* GG100-14D (lane 4), *S. cerevisiae* K7-S1 (lane 5), *S. cerevisiae* 1385 (lane 6), and *S. cerevisiae* 1686 (lane 7) were subjected to electrophoresis on a 1.1% agarose–formaldehyde gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled L-dsRNA probe from *Z. bailii* 412. The L-dsRNAs present in each strain are indicated (L_{Zb} represents the L species derived from *Z. bailii* 412).

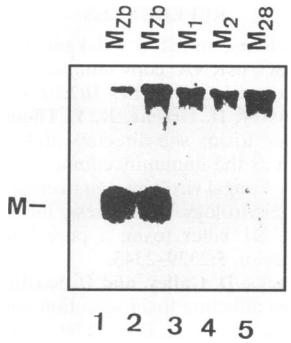

FIG. 8. Northern blot analysis of M-dsRNAs. Denatured total dsRNAs from *Z. bailii* 412 (lane 1), *S. cerevisiae* MS412 (lane 2), *S. cerevisiae* K7 (lane 3), *S. cerevisiae* 1385 (lane 4), and *S. cerevisiae* MS300b (lane 5) were fractionated as described in the legend to Fig. 7 and probed with a ^{32}P-labeled M-dsRNA from *Z. bailii* 412 (M_{Zb}). The M-dsRNAs present in each strain are indicated. (Note that the upper signal visible throughout the lanes represents nonspecific binding artifacts in each well.)

species (Fig. 4). As a direct consequence of that, the transfected, toxin-encoding M-dsRNA from *Z. bailii* can be maintained, encapsidated, and replicated by the L-A-H-encoded *cap* and *cap-pol* gene products, since we could show that in the infected clone *S. cerevisiae* MS412 (after loss of L from *Z. bailii*), the M satellite was encapsidated by the 88-kDa coat protein derived from ScV-L_A (28). In contrast to the wild-type killer strain *Z. bailii* 412, which was curable only by strong and repeated treatment with cycloheximide (in concentrations of up to 10 μg/ml), ZbV-infected clones of *S. cerevisiae* (MS412a, b) could be cured with high frequency by cultivation at elevated temperature (37°C). It has recently been shown (42) that M_1-dsRNA can be heat cured only in strains containing L-A-HN but not in strains carrying L-A-H. H stands for [HOK], which is the property of L-A competent for maintenance of M_1 in *ski* and *SKI* backgrounds, while N ([NEX]) prevents exclusion of M_1 by L-A-E, which is a natural variant of L-A carrying [EXL] that is competent for maintenance of M_1 only in a *ski* background. The products of the chromosomal *SKI* genes negatively control dsRNA replication such that *ski* mutants show a superkiller phenotype with an increased M-dsRNA copy number (38, 45). In contrast to L-A-HN, curing of M_1 in strains carrying L-A-H is usually not successful by simple cultivation at elevated temperature (42), suggesting that M-dsRNA in killer strains of *Z. bailii*, which were curable only by treatment with cycloheximide, might be naturally supported by an L-A-H species lacking [NEX], although we have not tested this directly.

Electron micrographs of purified *Z. bailii* viruses (ZbV-L, -M, and -Z) showed similar-sized particles (40 nm in diameter) which were not distinguishable from ScV-L_A and ScV-M_1 derived from *S. cerevisiae* killer strains.

In contrast to cured killer strains of *S. cerevisiae*, which become toxin sensitive after having lost their toxin- and immunity-encoding M-dsRNA, cured nonkiller derivatives of *Z. bailii* (having both L and Z or L or Z only) remained fully immune, although they were perfectly able to bind exogenously applied killer toxin at the cell wall level (9), thus indicating that immunity is not caused by a lack of primary toxin-binding sites. So far, the molecular basis of toxin immunity in *Z. bailii* remains unknown, but our guess is that it might be due to a lack of secondary membrane receptors, an idea which is supported by the observation that spheroplasts of cured nonkiller strains of *Z. bailii* remained immune against killer toxin KT412, just like the wild-type killer *Z. bailii* 412 (28). Since ZbV-transfected killer strains of *S. cerevisiae* become sensitive to their own toxin once they have lost the 2.8-kb Z-dsRNA, it can be speculated that, at least in the "artificial" *S. cerevisiae* system, this new type of an dsRNA virus (ZbV-Z) might be involved in the expression of killer toxin immunity. To test this hypothesis, we are currently trying to reinfect these suicidal killer strains with purified Z-dsRNA viruses (ZbV-Z), which should enable cells to survive even in the presence of exogenously applied killer toxin.

In contrast to the three known toxin-encoding M-dsRNA viruses of *S. cerevisiae*, which all depend on the existence of an L-A helper virus (ScV-L_A) to be stably maintained and replicated within the yeast cell, Z-dsRNA from *Z. bailii* does not resemble such an L_A satellite since we were able to isolate nonkiller strains of *Z. bailii* which had lost L and M but nevertheless retained Z at normal wild-type levels (Fig. 1B, lane 9). With the help of that strain (FN207) we were able to isolate highly purified ZbV-Z particles which contained only the 35-kDa major coat protein, not even showing traces of the 85-kDa protein present in ZbV-L and ZbV-M. Thus, in analogy to the well-defined ScV-L_A helper virus of *S. cerevisiae*, Z-dsRNA from *Z. bailii* resembles a new type of an autonomously replicating dsRNA, which is most likely to encode its own 35-kDa major capsid protein and probably its own RNA-dependent RNA polymerase necessary for replication and packaging of the viral genome. For further characterization of Z, we intend to analyze its in vitro translation product(s) and to clone a cDNA copy. The complete sequence of Z should enable us to answer the question whether its predicted RNA-dependent RNA polymerase is expressed in vivo as a *cap-pol* fusion protein resulting from a −1 ribosomal frameshifting event, just as was shown for the L_A-encoded RNA-dependent RNA polymerase (8, 20, 39, 40).

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