Expression and identification of immunity determinants on linear DNA killer plasmids pGKL1 and pGKL2 in *Kluyveromyces lactis*

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ABSTRACT

The linear dsDNA plasmids, pGKL1 (8.9 kb) and pGKL2 (13.4 kb) discovered in *Kluyveromyces lactis*, confer killer and immunity characteristics upon various yeast strains. We have devised an immunity assay and have been able to show the expression of an immunity phenotype in the *K. lactis* transformants harbouring conventional circular plasmids which contain DNA fragments of pGKL1. Using this expression system, the immunity determinant on pGKL1 was identified as ORF5. In addition, the presence of pGKL2 was proved to be essential for the expression of the immunity phenotype. This is the first demonstration of this new pGKL2 function, as distinct from its known functions for the replication and maintenance of pGKL1 in yeast cells.

INTRODUCTION

The killer system under the control of dsRNA plasmids M and L is well known and has been extensively characterized, both genetically and biochemically (1-4). We have studied a novel killer system associated with the presence of two linear dsDNA plasmids, pGKL1 (8.9 kb) and pGKL2 (13.4 kb) (5). Both these linear plasmids were discovered originally in *Kluyveromyces lactis* (5) and can be transferred to *Saccharomyces cerevisiae* (6, 7) and other yeast strains (8). Cells harbouring the two plasmids exhibited killer characteristics arising from the secretion of a killer toxin into the culture medium and, also, a killer-resistant phenotype (immunity⁺) (5, 9). The DNA sequence of pGKL1 has been independently reported by ourselves (11) and by others (12, 13). pGKL1 contains five open reading frames (ORFs), ORF1 through ORF5, which were predicted to encode proteins of molecular weights 68 600, 45 000, 128 700, 28 700 and 50 800, respectively (11). The analyses of the deletion plasmids

of pGKL1 suggested ORF3 on pGKL1 to be responsible for the expression of the killer phenotype, and ORF4 or ORF5 for that of the immunity phenotype (11, 14). The function of pGKL2 remains mainly unresolved. Cells harbouring only pGKL2, or those lacking both pGKL1 and pGKL2 were isolated upon the disappearance of the killer and immunity phenotypes (14); however, cells harbouring only pGKL1 have not so far been isolated, suggesting that pGKL2 contains genes essential for the maintenance of linear plasmids in the host cells. We have reported the characterisation of the killer toxin (9, 10) and are interested in the development of expression systems for killer and immunity phenotypes in yeast cells, in order to resolve the molecular mechanism of the killer-immunity system encoded on pGKL plasmids. In the present paper, we have described the subcloning of ORF4 and/or ORF5 on the circular Escherichia coli-yeast shuttle vector and the expression of the immunity phenotype in K. lactis transformants. We have determined ORF5 to be the immunity determinant on pGKL1 and, in addition, the presence of the pGKL2 plasmid to be required for the expression of the immunity phenotype.

MATERIALS AND METHODS

Strains, plasmids, medium and chemicals

K. lactis NK40 (pGKL1⁻, pGKL2⁺) and NK1 (pGKL1⁻, pGKL2⁻), both derived from K. lactis 2105-1D (pGKL1⁺, pGKL2⁺), were used as the hosts of transformation (14). E. coli strains CS412 (15) and SM31 (16) were used for plasmid construction and amplification. The E. coli-yeast shuttle vector pREI015, containing replication origins from pBR322 and from 2 μ m plasmid, and also containing the aminoglycoside 3'-phosphotransferase gene from Tn903 (kanamycin^R, G418^R) (17), was used as the cloning vector. Transformants harbouring pREI015 derivatives were selected, using 50 μ g/ml kanamycin for all E. coli cultures; and 50 μ g/ml G418 (liquid culture) or 200 μ g/ml G418 (agar plates) for K. lactis. YPD medium (1% yeast extract, 2% peptone, 2% glucose) for yeast and LB medium (1% tryptone, 0.5% yeast extract, 0.1 mM MgCl₂ and 0.5% NaCl) for E. coli were used throughout the experiments. Enzymes were purchased from Toyobo (Japan), except for Zymolyase 60 000 which was obtained from

Seikagaku Kogyo (Japan), and all were used as recommended by the producer. G418 sulfate (Geneticin) was from GIBCO. All other chemicals used were of the highest grade commercially available.

Plasmid construction

Plasmid construction was carried out as follows (see Fig. 1.)

pMT12 (containing ORF4 and ORF5)

The entire sequence of pGKL1, with an EcoRI linker at each end, was ligated to the EcoRI-PvuII large fragment of pBR322 in which the PvuII site had been converted to an EcoRI site using an EcoRI linker, to construct pKEN516. The BamHI-EcoRI fragment (4.5 kb) of pKEN516, containing ORF4 and ORF5, was subcloned into the EcoRI-BamHI sites of pUC13, to make pMT11. The inserted fragment was cut out of pMT11 using EcoRI and SalI and was subcloned into EcoRI-SalI-digested pREI015. The plasmid thus obtained, designated pMT12, contains a 2 μ m replication origin, G418-resistant marker and ORF4 and ORF5 from pGKL1.

pNW007 and pNW003 (containing ORF5)

The XbaI fragment (3.0 kb) of pKEN516 containing ORF5 was subcloned into the XbaI site of pUC13 and the SalI-EcoRI fragment of this plasmid, pNW006, was ligated into SalI-EcoRI-digested pREI015 to construct pNW007. The PstI-HindIII fragment (1.4 kb) containing ORF5 was subcloned into the PstI-HindIII sites of pUC13 to make pNW001, and the DNA fragment containing ORF5 was cut out by means of BamHI and BglII partial digestion. This 1.4 kb fragment was inserted at the BglII site of the expression vector, pRYG1, which contains the promoter cassette of the phosphoglycerate kinase gene constructed by Mellor *et al.* (18).

pNW005 (containing ORF4)

The XmnI-EcoRI fragment (1.2 kb) of pKEN516 containing ORF4 was ligated into SmaI-EcoRI-digested pUC13 to construct pNW002, and the SalI-EcoRI fragment of this plasmid was introduced into SalI-EcoRI-digested pREI015.

Yeast transformation and analyses of plasmids

Yeast transformation was carried out by the Li⁺-salt method (19). Before plating out the transformants onto selective plates (YPD containing 200 μ g/ml of G418), the cells were incubated at 30°C for 16 h in YPD medium to allow expression of the G418-resistant phenotype. The transformants grown on the selective

plates were transferred to YPD liquid medium containing 50 $\mu\text{g/ml}$ of G418 to check for the immunity phenotype and the integrity of the plasmids which they retained. Plasmid DNA prepared from yeast (20) was analyzed after transforming *E. coli* once more in order to amplify the plasmid DNA. To analyze the linear plasmids pGKL1 and pGKL2, DNA fractions (20) were digested with proteinase K (10 $\mu\text{g}/50 \mu\text{l}$) to remove terminal and bound proteins before application to agarose gels.

Assay for immunity to killer toxin

The immunity phenotype of yeast transformants was assayed as follows. 100 μl of YPD medium (pH 5.3) in microtiter wells (Falcon 3070) containing various amounts of purified killer toxin (0-40 ng/100 μl) were inoculated with about 500 cells. The cells were incubated at 30°C without shaking for 3-4 days and the cell growth was determined by measuring the turbidity of the culture diluted ten-fold at OD₆₀₀.

Pure killer toxin was prepared from *S. cerevisiae* F102-2, containing pGKL1 and pGKL2, essentially according to Sugisaki *et al.* (10). The amount of killer toxin was measured by comparison with the colour intensity of Coomassie brilliant blue staining of purified killer toxin (97 kd) on the SDS-PAGE (polyacrylamide gel electrophoresis) gel (12% acrylamide).

RESULTS

Expression of immunity phenotype and identification of its determinant

Killer and immunity determinants have been reported to be encoded on the pGKL1 plasmid (14). Furthermore, the gene for the immunity⁺ phenotype was postulated to be ORF4 or ORF5 on pGKL1, since the deletion plasmid (pGKL1S) lacking ORF3 conferred killer⁻ but immunity⁺ phenotypes to the host cells, and other deletion

Fig. 1. Schematic illustration of plasmid construction. Circular plasmids containing ORF4 and/or ORF5 of pGKL1 were constructed (see Materials and Methods, for detail). DNA: , from pGKL1; , from 2 μm plasmid; , gene for aminoglycoside-3'phosphotransferase from Tn903; , from plasmid pMA91 (18); —, from pBR322. Restriction enzymes used are B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; X, *Xba*I; Xm, *Xmn*I.

Fig. 2. SDS-gel electrophoresis pattern of purified pGKL killer toxin. Purified killer toxin was analyzed by SDS-polyacrylamide gel electrophoresis (12% acrylamide, ref. 29). A sample was solubilized in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of β -mercaptoethanol with (lanes 1 and 3) and without (lanes 2 and 4) boiling for 2 mins. The molecular weight markers used were phosphorylase B (97 kd), bovine serum albumin (66 kd), ovalbumin (45 kd) and carbonic anhydrase (31 kd)(lane 5).

plasmids (F1 and F2), lacking ORF3, ORF4 and ORF5, conferred killer⁻ and immunity⁻ phenotypes (11, 14). The most direct way to identify the gene responsible for immunity is by the expression of the immunity⁺ phenotype upon the introduction of DNA fragments covering ORF4 or ORF5 into the killer-sensitive cells. We have postulated that the genes for the killer and immunity phenotypes could be expressed on the circular plasmid, although the genes coded by the pGKL1 have not so far been expressed after insertion into the circular plasmid. We carried out the subcloning of ORF4 or ORF5 into the *E. coli*-yeast shuttle vector, pREI015, which can replicate autonomously in the absence of pGKL2 in *K. lactis*. Hollenberg *et al.* reported that the 2 μ m origin from *S. cerevisiae* could function as a replication origin for plasmids in *K. lactis* (21). The aminoglycoside 3'-phosphotransferase from Tn903 was reported to be expressed in *K. lactis* so allowing the selection of transformants to G418 (22). To establish a quantitative assay for immunity, the killer toxin was purified from the culture supernatant of *S. cerevisiae* F102-2. The purified killer toxin consists of three subunits, 97 kd, 31 kd and 28 kd, determined by SDS-PAGE (Fig. 2). The two smaller subunits seem to contain an inter-subunit S-S linkage, because their dissociation requires β -mercaptoethanol even in the presence of SDS under boiling conditions (Fig. 2, lanes 1 and 3). Recently, the killer toxin was purified from *K. lactis* (23) and the molecular nature of the toxin preparations purified from *K. lactis* and *S. cerevisiae* was found to be exactly the same (23, Fujimura *et al.*, unpublished results).

Under our assay conditions (see Materials and Methods), *K. lactis* NK40, NK1, and *K. lactis* harbouring pREI052, showed no growth in the presence of 4 ng killer toxin/100 μ l medium (Fig. 3). *K. lactis* IFO1267 and *S. cerevisiae* F102-2 (killer⁺, immunity⁺), each containing pGKL1 and pGKL2 plasmids, grew normally in the presence of 40 ng/100 μ l killer toxin (Fig. 3(A)). Plasmid pMT12, containing ORF4 and ORF5, was constructed (see Fig. 1) and transformed into NK40 cells containing pGKL2 to test the expression of the immunity⁺ phenotype. As shown in Fig. 3(A), the transformant NK40(pMT12) can grow in the presence of more than 4 ng/100 μ l of killer toxin, indicating that the

immunity⁺ phenotype is expressed. When NK40(pMT12) was grown in YPD medium overnight without selective pressure, the majority of cells became sensitive to the killer toxin and G418 with the loss of plasmid pMT12 simultaneously (Fig. 3(A)). These data

Fig. 3. Immunity assay for the *K. lactis* transformants harbouring plasmids derived from pGKL1. (A) NK40(pMT12), (B) NK40(pNW007), (C) NK40(pNW005) and (D) NK40(pREI015), o-o, transformants; ●-●, transformants, the circular plasmids of which have been cured; Δ--Δ, host cells; --- in (A) shows *K. lactis* IFO1267 and *S. cerevisiae* F102-2.

Fig. 4. Immunity assay for transformants lacking pGKL2.
 (A) ■-■, NK40(pMT12), pGKL2⁺; ●-●, NK40(pMT12)-40 and ○-○, NK40(pMT12)-36, both pGKL2⁻; Δ-Δ, NK40
 (B) ■-■, NK40(pNW003), pGKL2⁺; ○-○, NK40(pNW003)-39, pGKL2⁻; Δ-Δ, NK40.
 (C) ○-○, NK1(pNW007); Δ-Δ, NK1.

indicated that the immunity determinant was encoded on ORF4 or ORF5. To establish which gene encodes the immunity determinant, ORF4 and ORF5 were subcloned separately and transformed to NK40. NK40(pNW007), containing ORF5, showed the immunity⁺ phenotype to the same extent as NK40(pMT12) (Fig. 3(B)). On the other hand, NK40(pNW005), containing ORF4, was sensitive to the killer toxin to the same extent as the host cells. Further, we constructed a deletion plasmid of pMT12, designated pMT12DB, having a deletion of BglIII-BglIII (0.8 kb) in ORF5 on pMT12. NK40 containing pMT12DB no longer showed the immunity⁺ phenotype (data not shown). These data clearly indicated that the immunity determinant on the pGKL1 plasmid is encoded in ORF5.

Fig. 5. Agarose gel analyses for linear pGKL2 plasmid. (A) lane 1, molecular weight marker (DNA HindIII digest); lane 2, NK40(pMT12), immunity⁺; lane 3, NK40(pMT12)-40, immunity⁻; lane 4, NK40(pMT12)-36, immunity⁻. (B) lane 1, molecular weight marker; lanes 2, 3, 5 and 6, NK40 (pNW003), all four are immunity⁺ independent colonies; lane 4, NK40 (pNW003)-39, immunity⁻; lane 7, NK40 as a control.

Expression of immunity phenotype requires the presence of pGKL2

In the course of the immunity assay for NK40(pMT12), we isolated some spontaneous G418^R, but immunity⁻, transformants. Plasmids were prepared from both immunity⁺ and immunity⁻ yeast lysates. One portion of the plasmid preparation was analysed using 0.7% agarose gel electrophoresis to test for the presence of the linear pGKL2 plasmid, and another portion was transformed into *E. coli* to test for the presence of the circular plasmid, pMT12. Surprisingly, all the immunity⁻ transformants had lost the pGKL2 plasmid (Figs. 4(A) and 5(A)) even while containing the intact circular plasmid, pMT12 (data not shown). We have constructed another plasmid, pNW003, which contains the ORF5 DNA

fragment of pGKL1. The spontaneously obtained G418^R, but immunity⁻, transformants of NK40(pNW003) again lost the pGKL2 plasmid (Figs. 4(B) and 5(B)). These data suggest that the expression of the immunity phenotype requires the presence of pGKL2 plasmid in the cells. To confirm this hypothesis, K. lactis, strain NK1, which is an isogenic strain of NK40 but lacks both the pGKL1 and pGKL2 plasmids, was transformed with pNW007, and the transformant NK1(pNW007) was tested to establish its immunity phenotype. As shown in Fig. 4(C), NK1(pNW007) displays an immunity⁻ phenotype, confirming that pGKL2 is required for the expression of the immunity phenotype. This is the first demonstration of a pGKL2 function distinct from that of the maintenance of linear plasmids.

DISCUSSION

The killer system associated with pGKL plasmids has unique properties: (i) pGKL plasmids have a linear structure with terminal proteins bound at their 5'-termini (24), (ii) the subcellular localization of pGKL plasmids has been reported as being cytoplasmic (7). In order to identify the genes for killer toxins and immunity protein(s), the subcloning and expression of genes coded on pGKL plasmids is essential. Extensive efforts to construct derivative plasmids of pGKL as linear structures, for example, introducing a marker gene, the subcloning of an ORF region or making a deletion, have so far been unsuccessful. This is because the replication mechanism of linear plasmids is totally unknown and no suitable conditions for manipulating linear DNA plasmids with terminal protein in vitro have been established. Instead of using linear plasmids for the subcloning of the gene encoded on pGKL1, we used here a circular E. coli-yeast shuttle vector for which replication in yeast was driven by the replication origin of the 2 μ m plasmid. We have been able to demonstrate the expression of the immunity⁺ phenotype in K. lactis transformants harbouring circular plasmids which contain the DNA fragment of the ORF5 region. This is the first demonstration that a gene, originally coded on a linear pGKL plasmid, can be expressed in an autonomous replicative circular plasmid, which is usually localized in nucleus. The extent of

expression of the immunity phenotype in our system is still lower than that in the original linear pGKL-system (Fig. 3(A)). This may be due to a decrease in the stability and copy number of the plasmids, or by a decrease in the efficiency of transcription on circular plasmids.

Using our expression system, we were able to find a distinct role for the pGKL2 plasmid, that being its essential requirement for the expression of the immunity⁺ phenotype. So far, however, this has not been examined, because the maintenance of linear pGKL1 itself is dependent upon the presence of pGKL2. Here we used the 2 μ m-origin plasmid for the expression of the pGKL1 DNA fragment, which is able to replicate autonomously in the absence of pGKL2. The actual function of pGKL2 in the expression of immunity is, at present, unknown. The plasmid pGKL2 may encode the gene for (i) a pGKL-specific RNA polymerase, (ii) a regulatory protein which activates the transcription of ORF5, (iii) a protein which activates the ORF5 coded protein to function in immunity or (iv) a protein which directs the ORF5 protein to a location in an appropriate subcellular compartment. The development of an expression system for the killer phenotype and the examination of the effect of pGKL2 on it, would be a further interesting approach in seeking to clarify the above questions.

In the yeast M1-killer system, the killer toxin and immunity domain is synthesized as a 35 kd precursor protein from a single gene, and this precursor molecule itself is reported to confer immunity to the host cells by competing with the mature toxin at a membrane receptor (3). The molecular mechanism of pGKL-immunity is at present totally unknown. The hydropathic analysis of primary protein structure by Kyte and Doolittle (25) suggests that the ORF5 protein is most likely to be a soluble cytoplasmic or a nuclear protein. There is no typical signal sequence-like structure at its NH₂-terminus (11).

The identification and characterization of the immunity protein is the most urgent topic for future experiments and, since the amount of immunity protein expressed in yeast cells was too low to be identified, even when it was encoded on the multi-copy plasmids, the preparation of an antibody to ORF5 protein,

using recombinant DNA techniques such as protein A fusion (26) in E. coli, will be the next step in its identification.

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