Secretion of active anti-Ras single-chain Fv antibody by the yeasts *Yarrowia lipolytica* and *Kluyveromyces lactis*

Dominique Swennen,† Marie-Françoise Paul,‡ Laurence Vernis,¶ Jean-Marie Beckerich,§ Alain Fournier and Claude Gaillardin

Author for correspondence: Dominique Swennen. Tel: +33 1 30815444. Fax: +33 1 30815457. e-mail: swennen@grignon.inra.fr

*Yarrowia lipolytica* and *Kluyveromyces lactis* secretion vectors were constructed and assessed for the expression of heterologous proteins. An anti-Ras single-chain antibody fragment (scFv) coding sequence was fused in-frame to different pre- or prepro-regions, or downstream from a reporter secretory gene (*Arxula adeninivorans* glucoamylase), separated by a Kex2 protease (Kex2p)-like processing sequence. Both organisms are able to secrete soluble scFv, with yields depending on the nature of the expression cassette, up to levels ranging from 10 to 20 mg l\(^{-1}\). N-terminal sequence analysis of the purified scFv showed that fusions are correctly processed to the mature scFv by a signal peptidase or a Kex2p-type endoprotease present in *Y. lipolytica* and *K. lactis*. The scFv protein also retains the capacity to bind to a glutathione S-transferase (GST)–Harvey-RasVal\(^{12}\) fusion, indicating that the antibody is functional. These results indicate that the yeasts *Y. lipolytica* and *K. lactis* have potential for industrial production of soluble and active scFv.

**Keywords:** heterologous secretion, glucoamylase

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**INTRODUCTION**

Single-chain antibody fragments (scFv) are recombinant products of great interest for immunodiagnostics and immunotherapy. A number of systems for the production of scFv have been described. Functional scFv have been purified from mammalian cell supernatant cultures and are promising cancer therapy reagents (Nishida *et al.*, 1998; Whittington *et al.*, 1998). Expression of scFv in transgenic plants has been proposed as a way to produce and store pharmaceutical antibodies (Stoger *et al.*, 2000; Yuan *et al.*, 2000) and as a means to block physiological factors or plant pathogens (Le Gall *et al.*, 1998; Conrad & Fiedler, 1998). Most recombinant scFv molecules have been expressed in *Escherichia coli* cells, but results were variable whether or not the scFv was fused with a reporter protein (Fernandez *et al.*, 2000; Fernandez & De Lorenzo, 2001). ScFv accumulation as insoluble inclusion bodies, needing solubilization and refolding, has been reported to be a frequent problem (Smallshaw *et al.*, 1999; Sanchez *et al.*, 1999; Cho *et al.*, 2000), triggering the search for alternative hosts. High yields of secreted, active scFv were reported using the prokaryote *Proteus mirabilis*, ranging from 40 to 200 mg (l culture medium\(^{-1}\)) depending on the type of expressed scFv (Rippmann *et al.*, 1998). Expression of scFv antibody in insect cells has been described (Kretzschmar *et al.*, 1996), but production of insoluble material was also observed or low secretion levels were obtained when scFv antibody was co-expressed with a signal peptidase (Ailor *et al.*, 1999). A few attempts using yeast cells have been described, including successful expression in *Pichia pastoris* (Ridder *et al.*, 1995; Eldin *et al.*, 1997; Luo *et al.*, 1998; Fischer *et al.*, 1999; Freyre *et al.*, 2000; Andrade *et al.*, 2000) and in *Saccharomyces cerevisiae* where levels up to 20 mg l\(^{-1}\) were reached after tuning gene expression and overexpressing chaperones or foldases (Shusta *et al.*, 1998).

Problems encountered with heterologous expression are often linked to the heterologous protein expressed and are particularly observed with scFv proteins. It is thus important to have a range of possible hosts to investigate the best one for efficient scFv production. *Yarrowia*...
lipolytica and Kluyveromyces lactis, two non-conventional yeasts, can efficiently secrete heterologous proteins (Gellissen & Hollenberg, 1997; van der Berg et al., 1990; Fleer, 1992; Muller et al., 1998; Dominguez et al., 1998). Both are generally recognized as safe (GRAS) organisms. Y. lipolytica is able to secrete approximately 1 g alkaline extracellular protease (AEP) l−1 into the medium under optimal conditions (Barth & Gaillardin, 1997), suggesting good potential for secretion of heterologous proteins. The promoter of the XPR2 gene encoding AEP has been used to direct heterologous protein expression in Y. lipolytica (for examples see Park et al., 1997; Muller et al., 1998). Regulation of this promoter is very complex thus limiting its industrial use. A hybrid promoter has been constructed, based on tandem copies of upstream activator sequences from the XPR2 promoter (Mazdak et al., 1999). This hybrid promoter (hp4d) is weakly affected by environmental conditions and drives a quasi-constitutive protein expression (Mazdak et al., 2000). Similarly, K. lactis was reported to secrete high-molecular-mass proteins (Wesolowski-Louvel et al., 1996).

To analyse the scFv secretory capacity of these two yeasts, we have constructed expression vectors that allow secretion of the anti-p21ras scFv (Y28), a single-chain antibody derived from the neutralizing mAb Y13-259 (Werge et al., 1990). Expressed genes were placed under the control of the quasi-constitutive promoter hp4d for Y. lipolytica (Mazdak et al., 2000) and of the lactose-inducible LAC4 promoter for K. lactis. Y28 was fused to different pre- or prepro- regions, or to Arxula lactose-inducible ∆270 xpr2-322 axp2- conditions and drives a quasi-constitutive protein expression (Mazdak et al., 2000). Similarly, K. lactis was reported to secrete high-molecular-mass proteins (Wesolowski-Louvel et al., 1996).

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**METHODS**

**Strains and growth conditions.** Escherichia coli strains DH5α [F′ endA1 hsdR17 (rK mK) supE44 thi-1 recA1 gyrA (Nal) relA1 Δ(lacZYA-argF)U169 deoR (racB dlacA[lacZM15] and TG1 [l(lac pro A) B] supE thi hsdS5 F′ traD36 pro A B lacI lacZ M15] were used as host strains for bacterial transformations and plasmid propagation. BL21(DE3) (Novagen) [F′ompT hsdS5 (rK mK) gal dcm (DE3)] was used as a protein expression host.

The Yarrowia lipolytica strain P01 (MATa ura3-302 len2-270 xpr2-322 asxG49 xpr2::SUC2) (Mazdak et al., 2000) was used as recipient. The Kluyveromyces lactis strain MW98-8C (Matα ura3-Arg4 lys2 A K’ pKD1) was kindly provided by M. Wesolowski-Louvel, Université Claude Bernard, Villeurbanne, France. Strain FB05 (CBS29 591; patent no. FR9109854; Rhône-Poulenc Rorer SA) was obtained after disruption of the 3-phosphoglycerate kinase gene (Fournier et al., 1990) in the CBS1065 background (CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands).

E. coli cells were grown in LB medium (1% bactotryptone, 1% yeast extract, 0.5% NaCl) with 100 μg ampicillin ml−1 at 37 °C. Y. lipolytica cells were cultivated either on rich YPD medium (1% yeast extract, 1% bactopectone, 1% glucose) at 28 °C or on minimal medium (0.67% yeast nitrogen base without amino acids; Difco) with 0.8% glycerol or 2% stachar (Prolabo) as carbon source in 50 mM phosphate buffer, pH 6.8, at 28 °C. K. lactis strains were grown on glucose rich medium (YPD) or on minimal medium (YNB) with 1% starch, 2% glucose or 2% lactose as carbon source and the appropriate amino acids and bases, as described by Rose et al. (1990). Starch plates were used for halo assays of glucoamylase activity (Tokunaga et al., 1993). Transformed strains were grown in the presence of G418 (200 mg l−1).

**Pigments**

Construction of Y. lipolytica integrative expression vectors. p0 is a pBluescript derivative containing the Y. lipolytica URA3 gene on an EcoRI–HindIII fragment from pLNA156 (R. W. Wing & D. M. Ogrydziak, unpublished), the hp4d hybrid promoter of pLNA993 (Mazdak et al., 2000) between Clal and SfiI as a PCR fragment amplified with oligonucleotides 5′-GGATCATA- GGCTCTCAAGGGCATCGTC-3′ and 5′-GGATCTGTCG- CACTGGCCCTGAGATGTGTTGATGTTGATG-3′, a polylinker (SfiI, BamHI, EcoRI, Ndel, XbaI) obtained by annealing 5′-TGCGGAGATCTAGTACATTATAGAATATGATTGAGTCC-3′ and 5′-GGGCGGCTAGAATAAGGATTTTGCC-3′, and a NotI–KpnI fragment from an Ascl site obtained by PCR from pBUPAEP (D. Swennen, unpublished) with 5′-AAAGAAAAGCCGGCCGGTCATCTCCGGGACCTCCCTGCG-3′, 5′-ATGCAAACCCTGTGTTAGGCA-3′, 5′-GGGGCCGCGCTAGAATAACGATAGTGTGTCGCC-3′ and 5′-GGGCTACCTGAGAAATGAAATAGCACTG-3′. Thus, targetted integration into the XPR2 promoter can be obtained after Ascl digestion of the construct.

PDA is a p0 derivative containing a BamHI–BamHI fragment from pBSg/GAA/C (provided by D. M. Ogrydziak, unpublished) with 5′-AAAGAAAAGCCGGCCGGTCATCTCCGGGACCTCCCTGCG-3′, 5′-ATGCAAACCCTGTGTTAGGCA-3′, 5′-GGGGCCGCGCTAGAATAACGATAGTGTGTCGCC-3′ and 5′-GGGCTACCTGAGAAATGAAATAGCACTG-3′. pPY28 is another p0 derivative containing a Y28 fragment (patent no. WO94/29446; Rhône-Poulenc Rorer SA) amplified with oligonucleotides 5′-GGGAATTCCTCGGCAAGGGTG- CAGCTGAGGAG-3′ and 5′-GGATCTTATATATATGAT- CAGTACTTCTCTCG-3′, digested with Fspl and EcoRI, and a synthetic fragment encoding the XPR2 signal sequence using oligonucleotides 5′-TGCCATGACCGCTTACGCC-3′ and 5′-GGCGAGGAGGTCGAGGAG-3′. pPY28 results from the insertion into p0 of the same Fsp1–EcoRI Y28 fragment and of a SfiI–NotI PCR fragment encoding the XPR2 pre-pro-sequence amplified from pBUPAEP using oligonucleotides 5′-AAAGAACGCAACCCACCT-3′ and 5′-AAAGGCCTTCTGGGATTGAGAAAGCCAGG-3′. pPY28 results from the insertion into p0 of a SfiI–HindIII fragment amplified from the pBSg/GAA/C (D. M. Ogrydziak et al., 1996) containing the Y28 fragment and of a SfiI–NotI fragment containing an Ascl site obtained by PCR from pBUPAEP (D. Swennen, unpublished) with 5′-AAAGAAAAGCCGGCCGGTCATCTCCGGGACCTCCCTGCG-3′, 5′-ATGCAAACCCTGTGTTAGGCA-3′, 5′-GGGGCCGCGCTAGAATAACGATAGTGTGTCGCC-3′ and 5′-GGGCTACCTGAGAAATGAAATAGCACTG-3′. Thus, targetted integration into the XPR2 promoter can be obtained after Ascl digestion of the construct.
fragment from pGAA and a HindIII–NotI fragment from the *K. lactis* pKNH5 plasmid.

**Construction of *K. lactis* replicative expression vectors.** The deoxyribozyme oligonucleotides 5′-AGCTTGGTTAATTTAAGGGGCC-CCCGCCCTATAGGGGCCGCCAGCCGCTAGGCGGCCGCCCCCTTAAATACCCGA-3′ were paired and ligated into the HindIII site of the replicative pKD-1-based expression vector pYG1043 (Fleeer *et al.*, 1991), derived from pYGI023 (patent no. FR9109854; Rhône-Poulenc Rorer SA), to introduce three unique restriction sites (PalI, Fsel and NotI) and a stop codon between the Fsel and NotI sites, generating pKNH18. The gene encoding glucoamylase was amplified from pBscG/GAA/c by PCR with the primers 5′-GGTTAATTTAAAACATGCTAGCTACGATCCTTGGC-3′ and 5′-CCCCGGGCGCTAGCTACGATCCTTGCGC-3′ to clone the chimeric sequence as a PalI–Fsel fragment, generating pKNH6.

The sequence encoding the fusion of the Kex2p-like site and Y28 was amplified by a two-round PCR using the scFv sequence as template with two forward primers, 5′-TCTTCAAAGGATGGCCGATGCGTCAGTTGCAAG-3′ and 5′-GCGGGCCTAGGTCAGCAAACGATCTCTCAACGATGCTGCAGC-3′ and the reverse primer 5′-CCCCGGCCTACCGTTGGC-3′ to clone the Y28 sequence as a Fsel–NotI fragment into pKNH18, generating pKNH23. The Fsel–NotI fragment from pKNH23 was cloned into the glucoamylase-encoding plasmid pKNH6, generating pKNH5, encoding the fusion between the glucoamylase gene and the Y28 gene (patent no. WO94/29446; Rhône-Poulenc Rorer SA), separated by the Kex2p-like encoding sequence.

The sequence encoding Y28 devoid of the Kex2p-like site was made by PCR amplification using the forward primer 5′-GGGGGGCCGGATAGGCATGCGTTGACAGCTGACAG-3′ and the reverse primer 5′-CCCCGGGCGCTACGATCCTACGCTGTCACAC-3′ to clone the Y28 sequence as a Fsel–NotI fragment into pKNH18, generating pKNH33, and into pKNH6, generating pKNH1.

The glucoamylase signal sequence (Bui *et al.*, 1996b) was amplified by PCR using pKNH6 as a template with the primers 5′-GGTTAATTTAAAACATGCTAGCTACGATCCTTGGC-3′ and 5′-CCCCGGGCGCTACGATCCTTCGACGTGTCAGC-3′ and was cloned as a PalI–Fsel fragment into pKNH23 and pKNH33, generating pKNH11ss and pKNH11ss, respectively.

**Glutathione S-transferase (GST) fusions.** cDNA encoding the C-terminal part of β-amyloid precursor (β-APP, aa 650–695) (negative control) and the full length Harvey-Ras (Ha-Ras<sup>1018</sup>) (Ha-Ras<sup>1018</sup>) were cloned into pGEX4T1 (Pharmacia).

**DNA techniques.** Standard techniques have been used according to Sambrook *et al.* (1989). Enzymes were supplied by Gibco-BRL. Life Technologies and New England Biolabs. All vectors were checked by sequencing on a fluorescent DNA sequencer (ABI Biosystems Perkin-Elmer) according to the supplier.

**Transformation procedures.** *E. coli* strains were transformed by the method of Chang & Miller (1988). *Y. lipolytica* strain transformations were carried out according to Xuan *et al.* (1990). *K. lactis* strains were transformed by the method of Dohnen *et al.* (1991).

**Protein determination.** Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad) or by the method of Lowry using bovine serum albumin as standard.

### Solubilization of Y28 inclusion bodies.

*E. coli* BL21(DE3) cells transformed with the pet29a (Novagen)–Y28 plasmid were grown at 28°C. Fusion protein synthesis was induced by adding 0.5 mM IPTG to the culture medium and incubating for 3 h. Cells were collected and lysed by sonication in 10 mM Tris/HCl, pH 8, 1 mM EDTA, 1 mM PMSF buffer and the pellet was washed three times in the same solution. Inclusion bodies were solubilized in 4 M urea, 10 mM DTT, 10 mM Tris/HCl, pH 8, 1 mM EDTA, 1 mM PMSF, for 2 h at 28°C with gentle agitation or in 0.6% SDS. Solubilized proteins from the supernatant after 15 min centrifugation at 10000 g were analysed by Western blotting and the protein concentration was measured.

**Protein analysis by Western blotting.** Aliquots from yeast cultures were centrifuged (450 g), PMSF (1 mM) was added to supernatant and cells were washed and resuspended in 0.5 ml 10 mM Tris/HCl, pH 8, 1 mM EDTA, 1 mM PMSF, and lysed by TCA precipitation. Samples were subjected to SDS–PAGE analysis (homogeneous 10% acrylamide or 4–20% Tris-glycine Novex gels) using the buffer system of Laemmli (1970). Proteins were detected by Coomassie blue staining or transferred onto a nitrocellulose membrane (Protran BA 85; Schleicher & Schuell) and subjected to immunodetection by a standard procedure (Sambrook *et al.*, 1989). Rabbit serum directed against scFv was kindly provided by Dr J. L. Teillaud (INSERM U 255, France). Rat monoclonal anti-Ras antibody Ab-1 (clone Y13-259) was from Calbiochem. Horseradish-peroxidase-labelled secondary antibodies were obtained from Interchim and phosphatase-conjugated antibodies from Promega.

**Protein–protein interaction assays.** GST fusion proteins were produced in *E. coli* BL21(DE3), resuspended in TE buffer (10 mM Tris/HCl, pH 7.5, 1 mM EDTA) containing 0.2% Triton X-100, then bound to glutathione agarose (Sigma) following standard procedures (Smith & Johnson, 1988). Supernatant from a 120-h-old rich medium culture of *Y. lipolytica* carrying either pPY28, pPPY28 or p0 [mixed with v-H-ras (Ab-1) mAbs (Calbiochem) as positive control] was mixed with GST–Ras or GST–CAPP glutathione agarose in equal volumes. Proteins present in medium from transformed *K. lactis* cultures were ethanol-precipitated or filtered onto a Sephadex G25 column (Pharmacia) equilibrated in TE buffer before mixing with GST fusions. Samples were incubated with 100 µl GST fusions bound to agarose beads for 1 h at room temperature in PBS buffer (50 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM KCl, 1 mM MgSO<sub>4</sub>, pH 7) containing 0.2% Triton X-100. Beads were washed with 30 column volumes of PBS buffer and 0.2% Triton X-100. Bound proteins were eluted from the resin by heating the samples in SDS-PAGE loading buffer at 95°C for 10 min. Proteins from equivalent volumes of total, unbound and bound fractions were separated by SDS-PAGE, then subjected to immunodetection.

**Glucoamylase assay.** Aliquots of supernatant cultures were incubated in 40 mM citrate buffer (pH 4) containing 0.8% soluble starch (Prolabo) in a final volume of 0.5 ml. After incubation at 50°C for 20 min, the reaction was stopped as described by the manufacturer by addition of 1 ml glucose (trinder) reagent (Sigma) to quantify the released amount of glucose (modified from Böttner *et al.*, 1987). The halo assay was done on yeast cells grown on starch-containing medium for 2–7 d. Haloes surrounding the transformants were detected by the absence of iodine-staining (I<sub>2</sub>/KI) of the hydrolysed starch (Tokunaga *et al.*, 1993).

**N-terminal sequence determination.** Automated N-terminal Edman sequencing was performed using a Perkin Elmer
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2 Glucoamylase
5 scFv

Fusions cloned into p0
1 Glucoamylase
2 scFv
3 scFv

ProY28 → Y28
PGAA-Y28

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Fig. 1. (a) Schematic representation of proteins encoded by Y. lipolytica plasmids. 1, GAA signal peptide; 2, AEP signal peptide; 3, AEP proregion; 4, Kex2p-like site; 5, myc-tag. Open arrows, signal peptidase cleavage sites; filled arrows, Xpr6p (Kex2p-like) cleavage sites. (b) Western blot analysis of Y. lipolytica strains carrying p0, pPY28, pPPY28 or pGAA-Y28: SDS-PAGE analysis of TCA-precipitated intracellular proteins from a culture at OD600 of 1.5 on rich medium (YPD) at 48 h (P), or extracellular proteins from 8µl supernatant of the same culture (S). Following electro-transfer, the membrane was probed with rabbit serum directed against scFv.

RESULTS

Secretion of A. adeninivorans glucoamylase (glucan 1,4-α-glucosidase, EC 3.2.1.3)

Y. lipolytica. Plasmid p0, used to express the different constructions, is a pBluescript vector containing the Y. lipolytica URA3 gene as a selection marker, the hp4d hybrid promoter and a recombinant XPR2 terminator. The glucoamylase coding sequence was cloned between SfiI and BamHI sites to generate pGAA so as to direct glucoamylase to the secretory pathway thanks to its own signal sequence (Fig. 1). p0 and pGAA were integrated as single copy at the chromosomal XPR2 terminator locus of the Y. lipolytica strain P01f. P01f is derived from strain W29 (ATCC 20460) (Gaillardin et al., 1973) which is devoid of extracellular proteases (Madzak et al., 2000). Y. lipolytica strains carrying pGAA could grow on starch as sole carbon source, confirming secretion of active glucoamylase, whereas untransformed strains did not. A delay was observed on minimal starch medium for the strain carrying pGAA due to low expression of the hp4d promoter at the onset of exponential phase (Madzak et al., 2000). Growth on rich or minimal glycerol media was similar for both strains. Similar levels of glucoamylase activity were measured at the end of the exponential phase on both rich and minimal media.

K. lactis. The A. adeninivorans glucoamylase gene was fused to the lactose-inducible LAC4 promoter and cloned into a multicopy pKD-1-based expression vector (Fleer et al., 1991). Expression plasmids pKN18 (no insert) and pKNH6 (glucoamylase-encoding) were used to transform two different K. lactis strains, MW98-8C and FB05. Strains transformed with the glucoamylase-encoding plasmid were able to grow on soluble starch as sole carbon source, indicating that the heterologous enzyme was secreted and functional in K. lactis, as described by Bui et al. (1996b). Glucoamylase activity could be visualized on solid medium by the formation of a halo surrounding yeast colonies caused by starch degradation (Fig. 2b). Glucoamylase inhibitors α-glucosonic acid lactone and β-cyclodextrin were found to inhibit halo formation when added into solid culture medium (not shown). Transformed strains were grown on glucose-containing medium until stationary phase and culture medium was subjected to SDS-PAGE analysis (Fig. 3a, b). The glucoamylase secreted by MW98-8C migrated as a diffuse band between 90 and 120 kDa, as described by Bui et al. (1996b). The enzyme secreted by strain FB05 was more difficult to detect than that produced by strain MW98-8C, reflecting either a more complex glycosylation leading to a more diffuse band or higher glucoamylase degradation by FB05 cells.

Applied Biosystems Procise 494A sequencer with reagents and methods of the manufacturer. Supernatants were ethanol-precipitated (70% final ethanol). Samples were separated by SDS-PAGE electrophoresis and transferred onto SequiBlot PVDF membrane (Bio-Rad). Proteins were analysed at the picomole level.
Secretion of anti-Ras scFv

**Y. lipolytica.** Three expression plasmids based on the p0 vector were constructed to secrete scFv protein (Y28). Two of them targeted Y28 to the secretory pathway with the *Y. lipolytica* XPR2 signal sequence without (pPY28) or with (pPPY28) the XPR2 pro-sequence. In both constructs, a myc-tag was fused to the Y28 C-terminal end (Fig. 1a). The third construction (pGAA-Y28) (Fig. 1a) used glucoamylase as a carrier and reporter protein separated from the Y28 sequence by a Kex2p-like processing site (GRPNVISKR), the fusion being exactly the same as the one used in *K. lactis* plasmid pKNH5. *Y. lipolytica* strain P01f was transformed by linearized p0, pPY28, pPPY28 and pGAA-Y28, and resulting strains were checked for single-copy homologous integration. Intracellular and extracellular Y28 proteins from 2-d stationary-phase cultures of these strains in rich medium were probed with the anti-scFv antibody (Fig. 1b). Since intracellular protein samples were approximately 10 times less concentrated than extracellular samples, this indicates accumulation of mature Y28 proteins (31 kDa band) in the cells. Extracellularly unprocessed or partially processed proteins were detected in extracts from cells transformed with the proregion-driven construct (pPPY28): the 45 kDa band corresponds to unprocessed pro-Y28 precursor, the 31 kDa band to the Y28 protein and the other bands result from non-specific degradation. The pGAA-Y28-carrying strain secreted a low amount of Y28 which appeared correctly cleaved and migrated slightly faster than pPPY28-derived material due to the absence of the myc-tag. Very low glucoamylase activity was detected in supernatants of 2-d-old cultures of this strain (see Fig. 6). Two faint bands cross-reacted with the Y28 antisera in p0 (negative control) intracellular samples. Western blot analysis with anti-Y28 serum of rich medium culture supernatants indicated that the maximum level of secreted Y28 was obtained after 2 d and remained stable for 8 d (data not shown).

**K. lactis.** The replicative plasmid construct pKNH5 used for the expression of the anti-Ras scFv (Y28) encodes an in-frame fusion of scFv DNA fragment separated from the glucoamylase gene by an artificial hexapeptide linker containing a Kex2p-like processing site (Contreras et al., 1991). A similar construct devoid of the Kex2p-like site (pKNH1) was obtained (Fig. 2a). *K. lactis* strains MW98-8C and FB05 were transformed with pKNH5 and pKNH1. Strains containing the scFv-fusion-encoding plasmids were able to hydrolyse starch on solid medium. In that case, however, the diameter of the halo was smaller than in strains expressing glucoamylase only (Fig. 2b). Transformed strains were grown until late stationary phase on glucose-containing medium. Cells expressing scFv fusion proteins failed to grow on lactose-containing medium (not shown). One explanation of this phenotype is that Y28 overexpression is toxic to *K. lactis*. In contrast, in cells grown on glucose-containing medium a basal level of Y28 expression allowed Y28 accumulation in late-stationary-phase cultures (Fig. 3a). FB05 was found to secrete more scFv than MW98-8C (Fig. 3b). SDS-PAGE and Western blot analysis of proteins from 10-fold concentrated culture
medium revealed that Y28 was secreted by strains transformed with pKNH5, migrating as a 30 kDa band which cross-reacted with an anti-scFv serum (Fig. 3c).

Characterization of secreted Y28

**Y. lipolytica.** To get information about Y28 protein structure, aliquots of culture supernatants of strains carrying pPY28 and pPPY28 were analysed by SDS-PAGE in the presence or absence of β-mercaptoethanol. Western blotting with anti-Y28 serum revealed that oxidized Y28 migrated slightly faster than reduced Y28, indicating the probable formation of disulfide bonds (not shown). N-terminal sequencing of Y28 proteins produced by *Y. lipolytica* strains transformed with pPY28 and pPPY28 indicated in both cases that cleavage occurred at the expected position (not shown), either after the usual signal peptide cleavage site or at the Kex2p-like site. In vitro assays showed that Y28 produced by *Y. lipolytica* was able to bind the antigen bound to glutathione agarose as did the mAb from which Y28 originates (Fig. 4). As expected, neither the Y28 proteins nor the mAb were retained on the GST–cAPP fusion agarose beads.

**K. lactis.** N-terminal sequencing of scFv produced in *K. lactis* transformed with pKNH5 revealed that the Kex2p-like site was cleaved at the predicted position, as was the *A. adeninivorans* signal sequence from the fusion protein encoded by pKNH11ss. Cleavage of the fusion encoded by pKNH1 was found to occur between glycoamylase (624 aa) Tyr617 and Leu618. The scFv produced by *K. lactis* was found to bind to the GST–Ras fusion agarose beads (Fig. 5), as described for Y28 secreted by *Y. lipolytica*.

Comparison of *Y. lipolytica* and *K. lactis* glucoamylase and Y28 secretion capacity

Glucoamylase activity was measured from at least three independent rich medium culture supernatants of *K. lactis* strain FB05 carrying the multicopy replicative plasmids pKNH6, pKNH5 or pKNH18, and of *Y. lipolytica* single-copy integrants of pGAA, pGAA-Y28 or p0, as described in Methods (Fig. 6). These representative results indicated that under these conditions *K. lactis* secreted more glucoamylase, alone or as a reporter protein than *Y. lipolytica*, or that the glucoamylase was in a better conformation or better glycosylated.

Y28 proteins secreted by *Y. lipolytica* (transformed with integrative plasmids pPY28 or pPPY28) and *K. lactis* (FB05 transformed with the replicative plasmid pKNH5) were compared by SDS-PAGE analysis and Western
Fig. 4. In vitro binding of Y28 secreted from *Y. lipolytica* to GST–HaRasVal12 fusion protein. Culture supernatant of *Y. lipolytica* carrying expression plasmids pPY28 (PY28), pPPY28 (PPY28) or the empty plasmid p0 were tested on glutathione agarose beads bound to (a) GST–HaRasVal12 (antigen) or (b) GST–cAPP (negative control). Anti-Ras rat antibody (Ac) Y13-29 from which Y28 originates was used as positive control and mixed with control p0 supernatant (Ac + p0). Equivalent volumes of total (lanes 1), unbound (lanes 2) and bound (lanes 3) fractions were immunoprobed with anti-myc antibodies (PY28 and PPY28 supernatants), revealing the Y28 band at 31 kDa, or with anti-rat IgG (Ac + p0), revealing heavy and light chains of the antibody at 50 and 30 kDa, respectively.

Fig. 5. In vitro binding of Y28 from *K. lactis* to a GST–HaRasVal12 fusion protein. Medium from culture of FB05 transformed with pKNH18 (F18), pKNH6 (F6) or pKNH5 (F5) were filtered onto Sephadex G25 (G) (a and b) or ethanol-precipitated (P) (c and d), then tested on glutathione agarose beads bound to GST–cAPP (negative control) (a and c) or GST–HaRasVal12 (antigen) (b and d). Anti-Ras rat antibody (Ac) Y13-259 was added to the medium where indicated. Equivalent volumes of total (lanes 1), unbound (lanes 2) and bound (lanes 3) fractions were immunoprobed with anti-rat IgG, revealing heavy and light chains of Y13-259 at 50 and 30 kDa, respectively, or with anti-scFv antibodies to detect Y28 at 30 kDa.
blotting with anti-Y28 serum (Fig. 7). The Y28 protein secreted by K. lactis had a lower molecular mass than the scFv produced by Y. lipolytica because of the absence of the myc-tag. Comparison of the signals to those of known amounts of scFv produced in E. coli as inclusion bodies (not shown) allowed us to estimate that Y28 concentration was 20 mg l\(^{-1}\) for Y. lipolytica and 10 mg l\(^{-1}\) for K. lactis, in shake flask culture, corresponding to 2 and 1% of total cell proteins, respectively.

**DISCUSSION**

Expression/secreton plasmids were constructed to allow secretion of a recombinant scFv antibody, anti-Ras (Y28), by two non-conventional yeasts, Y. lipolytica and K. lactis. This scFv protein was secreted by both yeasts using different pre- or prepro-regions as in-frame fusions with a reporter protein (A. adeninivorans glucoamylase), separated or not by a peptide containing a Kex2p-like processing site. Expressed genes were placed under the control of a Y. lipolytica strong quasi-constitutive promoter or a K. lactis strong inducible promoter. The constructions were integrated in the Y. lipolytica chromosome as single copies and expressed as extrachromosomally multicopies in K. lactis strains. Near-wild-type yeast strains were used here to allow comparison of both yeasts. The results indicated that more glucoamylase, expressed alone and with its own signal sequence, was produced by K. lactis than by Y. lipolytica as measured by *in vitro* glucoamylase assay. This may of course reflect copy number differences of the expression cassettes, or, less likely, differences in folding or secretion efficiency. Glucoamylase fusion to the scFv sequence favoured Y28 expression in K. lactis when compared to fusions devoid of the enzyme sequence. This suggests that, as described for glucoamylase fusions in *Aspergillus* (for review see Gouka *et al*., 1997), the level of heterologous protein production in K. lactis may be improved by fusing it to a well secreted protein. On the contrary, Y28 was more efficiently produced by Y. lipolytica when expressed as a fusion to a homologous pre- or prepro-sequence than when fused to the A. adeninivorans glucoamylase. Production yields reached 10 and 20 mg l\(^{-1}\) in K. lactis and Y. lipolytica cultures, respectively. We noticed that, even with a single-copy integration of the Y. lipolytica construct, intracellular accumulation of mature Y28 protein was detectable, indicating saturation of the secretion pathway. The quantity of secreted mature scFv proteins in Y. lipolytica transformed by pPPY28, in which identical amounts of properly processed and unprocessed Y28 proteins were obtained, may be increased by overexpressing the XPR6 gene encoding a dibrasic processing endoprotease homologous to *S. cerevisiae* Kex2p (Enderlin & Ogrydziak, 1994).

K. lactis strains MW98-8C and FB05 transformed with constructs encoding Y28 failed to grow on expression-inducing medium, suggesting that Y28 overexpression was toxic to cells. Similarly, transformants selected for enhanced copy number of the expression plasmid in Y. lipolytica, as described by Le Dall *et al*., (1994), led to severe loss of viability (unpublished). Even though the reasons for scFv toxicity are unknown, attempts at improving the production yields are under way. The use of a promoter inducible in late stationary phase could be more appropriate for anti-Ras scFv protein production. Overexpression of chaperone/foldase has been shown to improve secretion levels in *S. cerevisiae* (Shusta *et al*., 1998) and overexpression in Y. lipolytica of Kat2p (Lee & Ogrydziak, 1997) and Sls1p (Boisrame *et al*., 1996), two endoplasmic resident proteins that are known to interact for efficient co-translational translocation of secreted proteins (Boisrame *et al*., 1998) might have similar effects.

More generally, improving fermentation conditions may significantly increase secretion yields: using similar constructs and alternative growth conditions enhances chymosin secretion in Y. lipolytica from 20 to 160 mg l\(^{-1}\) (Madjak *et al*., 2000). Although some differences in

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**Fig. 6.** Time course of glucoamylase production in supernatant of K. lactis or Y. lipolytica grown on rich medium (YPD). △, K. lactis pKNH6; □, Y. lipolytica pGAA; ■, K. lactis pKNH5; ▲, Y. lipolytica pGAA-Y28; ○, Y. lipolytica p0; ●, K. lactis pKNH18.

**Fig. 7.** SDS-PAGE analysis of 4 µl culture supernatant of Y28 secreted by Y. lipolytica (Yl) strains carrying pPY28 or pPPY28 (120 h YPD culture) and K. lactis (Kl) carrying pKNH5 (144 h YPD culture). Following electrottransfer, the membrane was probed with rabbit serum directed against scFv.
secretion efficiency were observed between both yeasts, their secretion capacity for the proteins analysed is approximately equivalent and indicates that these two non-conventional yeasts may be considered as valuable alternative hosts to secrete recombinant scFv.

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