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Modulation of Chaperone Gene Expression in Mutagenized *Saccharomyces cerevisiae* Strains Developed for Recombinant Human Albumin Production Results in Increased Production of Multiple Heterologous Proteins[▽]

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The yeast *Saccharomyces cerevisiae* has been successfully established as a commercially viable system for the production of recombinant proteins. Manipulation of chaperone gene expression has been utilized extensively to increase recombinant protein production from *S. cerevisiae*, focusing predominantly on the products of the protein disulfide isomerase gene *PDI1* and the hsp70 gene *KAR2*. Here we show that the expression of the genes *SIL1*, *LHS1*, *JEM1*, and *SCJ1*, all of which are involved in regulating the ATPase cycle of Kar2p, is increased in a proprietary yeast strain, developed by several rounds of random mutagenesis and screening for increased production of recombinant human albumin (rHA). To establish whether this expression contributes to the enhanced-production phenotype, these genes were overexpressed both individually and in combination. The resultant strains showed significantly increased shake-flask production levels of rHA, granulocyte-macrophage colony-stimulating factor, and recombinant human transferrin.

The intrinsic commercial value of heterologous proteins has driven a wide range of studies of the optimization of yeast strains for use as production "cell factories." A number of reviews exist covering the virtues of different systems for this purpose (12, 27, 31). While the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris* do not compete with filamentous fungi such as *Aspergillus* or *Trichoderma* spp., or bacterial systems such as *Escherichia coli* or *Bacillus* spp., in terms of productivity, they provide a proven, safe alternative without the complexity or high costs of mammalian cell culture. Currently, out of 21 FDA-approved therapeutic proteins produced using yeasts, 19 are derived from *S. cerevisiae* (28).

Factors affecting heterologous protein production include the properties of the target protein, the host strain, vector system, promoter choice (mRNA availability), leader sequences, translation signals, processing and folding in the endoplasmic reticulum (ER), and secretion (24). If any one of these is suboptimal, it can create a bottleneck leading to poor production yields (21). Thus, although production from *S. cerevisiae* has been reported in the g/liter range, titers are often 100- to 1,000-fold lower (10, 32).

The use of different expression systems makes comparative assessment of strain improvement approaches difficult, although manipulation of certain groups of genes, and in particular the ER chaperones, has been shown to have widely beneficial effects on the secreted production of at least some heterologous proteins (21, 31). Data indicate that ER function is frequently a major bottleneck in production of secreted heterologous proteins, thus providing a target for optimization (37, 49). This will provide the focus of data discussed here.

Manipulation of the ER luminal environment has mainly focused on the products of the protein disulfide isomerase gene *PDI1* and the hsp70 gene *KAR2* (33). While overexpression of *PDI1* has relatively consistent effects on protein production across a range of protein substrates, the effects of *KAR2* overexpression are much more varied (32). This was also observed in our mutagenized, enhanced-production yeast strains, with *PDI1* duplication and multicopy overexpression increasing secretion of human transferrin significantly (10, 17, 39), while *KAR2* duplication had little or no effect (unpublished data).

Kar2p is the major hsp70 chaperone present in the ER lumen and participates in protein translocation and folding, ER-associated degradation (ERAD), and regulation of unfolded protein response (UPR) signaling (1, 9, 15, 18, 19, 25, 45, 48, 49). Essential to all of these activities is the ATP-dependent cycle of protein binding and release (8, 34). The ATPase cycle is promoted by two sets of cochaperones. The first, consisting of Sec63p, Jem1p, and Scj1p (hsp40-type chaperones present in the ER), promotes ATP hydrolysis, while Sil1p and Lhs1p promote nucleotide exchange. In a recent study, it was demonstrated that Kar2p and Lhs1p, which is also an ER-resident hsp70, share coordinated ATPase cycles (44).

We hypothesized that stimulating the ATPase cycle of Kar2p via overexpression of the cochaperones listed above, both individually and in combination, might promote ER function for heterologous protein production. Further evidence that this might be the case was provided by an analysis of mRNA levels for these genes in our mutagenized, enhanced-production strains. With the exception of *SEC63* (which was consequently excluded from subsequent analysis), transcript levels were significantly increased, with *SIL1* showing a greater-than-sixfold increase relative to the progenitor strain.

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TABLE 1. Yeast strains used in this study

Strain name	Genotype
DB1	*MAT***a** *leu2-3 leu2-112*
DB1 *ura3*	*MAT***a** *leu2-3 leu2-112 ura3*
DS569	*MAT***a** *leu2-3 leu2-112*
DYB7	*MAT***a** *leu2-3 leu2-112 ubc4 ura3 yap3*::*URA3 lys2 hsp150*::*LYS2*
DYB7 *ura3*	*MAT***a** *leu2-3 leu2-112 ubc4 ura3 yap3*::*ura3 lys2 hsp150*::*LYS2*

MATERIALS AND METHODS

Yeast strains and growth conditions. Yeast strains are summarized in Table 1. The yeast strains DB1 and DS569 have been described previously (39). DYB7 was derived from the strain D88 (17), which was in turn derived from DS569. DS569 and DYB7 contain unknown mutations resulting from chemical mutagenesis. Strains were routinely grown in buffered minimal medium with 2% (wt/vol) dextrose (BMMD) (41) or complete medium with 2% (wt/vol) dextrose (yeast extract-peptone-dextrose [YEPD]) at 200 rpm and 30°C. Log-phase cultures were grown to an optical density at 600 nm (OD_{600}) of 2; stationary-phase cultures were grown for 4 to 5 days postinoculation, as specified. Analysis of heterologous protein production in stationary-phase cultures therefore represents an analysis of accumulated products, taking into account different growth phases up to the point of sampling.

Protein expression plasmids. Plasmids are summarized in Table 2. All expression plasmids were of the disintegration format, being based on the native *S. cerevisiae* 2μm plasmid, whereby the *E. coli* origin of replication and ampicillin resistance marker are removed by site-specific recombination upon transformation into yeast (6). The recombinant human albumin (rHA) expression plasmid pAYE329 has been described previously and consists of the human serum albumin (HSA) cDNA under the control of the *GPD1* promoter (40). The expression cassette for pDB2109 consists of *PRB1* promoter, HSA/*MF*α*-1* fusion secretion leader, granulocyte-macrophage colony-stimulating factor (GM-CSF) cDNA, and *ADH1* terminator. The expression cassette for pDB3213 consists of *PRB1* promoter, proprietary preleader sequence, human transferrin cDNA, and *ADH1* terminator. In addition, pDB3213 contains a copy of the *PDI1* gene cloned into the XcmI site in the inverted repeat region after *REP2*.

Chaperone overexpression plasmids. Chaperone overexpression constructs were initially made in the plasmid pTPA02. pTPA02 is based on pBST+ (41) but contains a polylinker containing the following restriction sites (5′ to 3′): MluI, AleI, BbvCI, FseI, NarI, PspOMI, RsrII, SbfI, XhoI, PacI, PmeI, and EcoRI. After constructs were completed in this plasmid, they were transferred to the centromeric plasmid YCplac33 (11) (also containing a polylinker to facilitate cloning). Primer sequences are shown in Table 3.

The *ACT1* promoter and terminator were amplified by PCR from DB1 genomic DNA using the primers A01-A02 and A03-A04, respectively. PCR products were purified and restriction digested with PacI/XbaI (promoter) and XbaI/PmeI (terminator). The two fragments were gel extracted and ligated in a three-way ligation with PacI/PmeI-digested pTPA02 to create pTPA03.

The spliced form of *HAC1* (*HAC1*^{i}) was amplified by PCR from cDNA derived from DB1 using the primers A05 and A06. The PCR product was gel extracted, digested with XbaI, and repurified. This fragment was ligated with XbaI-digested pTPA03, resulting in insertion of *HAC1*^{i} between the *ACT1* promoter and terminator present in pTPA03, creating pTPC01. Restriction digests of putative plasmids were carried out to check for the correct orientation of the *HAC1*^{i} open reading frame (ORF) relative to the *ACT1* promoter/terminator.

TABLE 2. Plasmids used in this study[a]

Plasmid	Description	Reference
pSAC35	2μm expression vector (2μm *LEU2 ampR*)	42
pAYE329	2μm rHA expression vector (2μm *LEU2 ampR*)	45
pDB2109	2μm GM-CSF expression vector (2μm *LEU2 ampR*)	
pDB3213	2μm rTf expression vector (2μm *LEU2 PDI1 ampR*)	
pBST+	General purpose cloning vector (*ampR*)	44
YCplac33	Yeast shuttle vector (*CEN4 URA3*)	11

[a] All plasmids except for YCplac33 were from Novozymes Biopharma UK.

TABLE 3. Oligonucleotide primers used for overexpression plasmid construction

Primer name	Sequence (5′–3′)[a]
A01	CTAGGTAACTTAATTAA(PacI)GGGTAAGCTGCCACAGCA
A02	CTACGTACTCTAGA(XbaI)TGTTAATTCAGTAAATTTTC
A03	CTAGACTCTAGA(XbaI)TCTCTGCTTTTGTGCGCG
A04	CATGCTACGTTTAAAC(PmeI)GATGATCATATGATACAC
A05	CTAGTCTCTAGA(XbaI)ATGGAAATGACTGATTTTGAAC
A06	CTAGTCTAGA(XbaI)TCATGAAGTGATGAAGAAATC
A07	CGATCACCGATGTG(AleI)GTTGTTTCCGGGTGTACAATATGG
A08	CCTATAGCAACAAAAGCTGTTAAAAATAAAAGCCTTAAAACGTTTCGCATTGTATATGAGATAGTTGATTG
A09	CGGTAGTACCTGCAGG(SbfI)AAGCAACAGGCGCGTTGGAC
A10	GGCAACAACAATAAAGATAGTATCAAATGTATATATAATTTTGGAATCATTTTGTAATTAAAACTTAGATTAGATTGC
A11	CACAATATTTCAAGCTATACCAAGCATACAATCAACTATCTCATATACAATGCGAAACGTTTTAAGGCT
A12	GCATGCTGAGG(BbvCI)GTGCCACTATAATATTAATGTGC
A13	CACGCTTACTGCTTTTTTCTTCCCAAGATCGAAAATTTACTGAATTAACAATGATTCCAAAATTATATATAC
A14	GCATCTCGAG(XhoI)GACTTTGAGACCTGTGATC
A15	GCATGGCCGGCC(FseI)ACCATATGGAGGATAAGTTGG
A16	ACCTAGTCTAGA(XbaI)TTTGTTTTGTGTGTAAATTTAG
A17	GCATGGGCCC(PspOMI)AGATTCCTGACTTCAACTCAAG
A18	GATCTAGTCTAGA(XbaI)TGTTTTATATTTGTTGTAA
A19	CTAGATCTCTAGA(XbaI)ATGGTCCGGATTCTTCC
A20	GCATGGCGCC(NarI)CCACGGCAGGGCAGTTGGCAC
A21	CTAGATCTCTAGA(XbaI)ATGATACTGATCTCGGG
A22	CGATCGGTCCG(RsrII)AGGGAAATAAGGCAGATCAAAG

[a] Restriction endonuclease sites are underlined.

The *ADH1* and *TEF1* promoters and *LHS1* and *SCJ1* ORFs (including native terminator regions) were amplified by PCR from DB1 genomic DNA using the primers A07-A08, A09-A10, A11-A12, and A13-A14, respectively. The promoters and ORF PCR products were then joined by overlap extension PCR (36). The promoter-ORF fusion fragments were digested with AleI/BbvCI for *ADH1*prom/*LHS1* and SbfI/XhoI for *TEF1*prom/*SCJ1* and subsequently ligated into pTPA02 digested with the same enzymes to create the plasmids pTPC03 and pTPC05.

The *TDH1* and *PGK1* promoters and *SIL1* and *JEM1* ORFs (including native terminator regions) were amplified by PCR from DB1 genomic DNA using the primers A15-A16, A17-A18, A19-A20, and A21-A22, respectively. All fragments were gel extracted and digested with the following enzymes: FseI/XbaI (*TDH1*prom), PspOMI/XbaI (*PGK1*prom), XbaI/NarI (*SIL1* ORF), and XbaI/RsrII (*JEM1* ORF). The promoter and ORFs were then ligated with pTPA02 and digested with the same enzymes (i.e., those present on promoter forward

TABLE 4. qRT-PCR probe/primer sequences

Gene name	Forward primer (5′–3′)	Reverse primer (5′–3′)	Probe (5′–3′)	Probe binding coordinates (relative to start codon)
ACT1	CCCAGAAGCTTTGTTCCATCCTT	ATGATGGAGTTGTAAGTAGTTTGGTCAA	CAGATTCCAAACCCAAAACA	795–814
LHS1	ACACTACTCAGCCCGTTACAATAGA	GTAAACTTTGCACCACCTAGATGTG	ATTTGAAGGATATGGGTATAATC	789–811
SIL1	GACATGTACGAAAATGACGATACAAATCT	TCGTTTGCCCACTCTTGCA	TTTGACGACCAATTCTC	940–956
SCJ1	GGCGCAGGTGGATTCCA	CGCCAGGACCTCCATGAC	CATATTCGAACGGATGTTTC	342–361
JEM1	CCTCTCCACGCACATCGA	TGCTTGTCGAGGATTGTTTCGTAAT	TCGTTAGCTGCTGCTATCA	592–610
HAC1^{i}	ACAATTCAATTGATCTTGACAATTGG	TCAATTCAAATGAATCAAACCTGAC	CGTAATCCAGAAGCGCA	652–668

primer and ORF reverse primer) in a three-way ligation. The resultant plasmids were named pTPC02 (*TDH1*prom/*SIL1*) and pTPC04 (*PGK1*prom/*JEM1*).

The multiple chaperone overexpression plasmids pTPC06 to pTPC08 were created by adding promoter/ORF constructs from pTPC02, pTPC03, and pTPC05 to pTPC04 in a serial manner by digestion with the same restriction enzymes used to ligate fragments into pTPA02 (i.e., sites found in promoter forward primer and ORF reverse primer).

Finally, overexpression constructs were transferred to YCplac33 plasmids (containing a polylinker with an AleI site cloned between BamHI and EcoRI sites). Overexpression constructs were transferred to YCplac33 using AleI/BclI (BamHI-compatible site for BclI in YCplac33) and AleI/XhoI (SalI-compatible site for XhoI in YCplac33). The YCplac33 plasmids corresponding to each of pTPC01 to pTPC08 are named pTPC11 to pTPC18.

RNA extraction. Yeast strains were grown to an OD_{600} of 2 in BMMD and harvested by centrifugation at 3,000 × *g* for 3 min. Supernatants were poured off, and pellets were resuspended in a small volume of growth medium (approximately 0.8 ml per 50-ml culture) and transferred to a microcentrifuge tube. Two-hundred-microliter aliquots were released drop by drop into caps from 20-ml universal tubes (Sterilin) containing liquid nitrogen and stored at −80°C. RNA extractions were carried out on cell pellets as described previously (14) using a Braun dismembrator and Trizol reagent (Invitrogen). RNA was quantified using A_{260} readings. RNA quality was assessed using an Agilent bioanalyzer, and samples with a 28S:18S rRNA ratio of greater than 1.8 were deemed satisfactory for quantitative reverse transcriptase-PCR (qRT-PCR).

DNase digestion/cDNA preparation. Ten micrograms of total RNA was added to 10 μl of 10× DNase digestion buffer, 5 μl of RQ1 RNase-free DNase (Promega), and nuclease-free H_2O to a 95-μl final volume. Samples were incubated at 37°C for 3 h. An additional 5 μl of RNase-free DNase was then added, and samples were incubated for a further 3 h. Digested RNA samples were frozen at −20°C and purified the following day using an RNeasy cleanup column (Qiagen). Columns were eluted with 40 μl of nuclease-free water. One microgram of DNase-digested total RNA was used in a 20-μl Superscript III (Invitrogen) reverse transcription reaction mixture as described in the manufacturer's instructions.

qRT-PCR. TaqMan probes/primers were designed using the Applied Biosystems "assay-by-design" service with the exception of *HAC1*^{i}, which was designed using Primer Express software (Applied Biosystems). The *HAC1*^{i} probe binds across the exon-exon boundary formed upon splicing. Probe/primer sequences are shown in Table 4. All reagents were supplied by Applied Biosystems or Invitrogen (Ultrapure water). Reactions were set up with 25-μl volumes comprising 12.5 μl 2× Universal PCR master mix, 1.25 μl 20× TaqMan probe/primer mix, 5 μl cDNA template (original cDNA diluted 100×), and water to 25 μl. For *HAC1*^{i}, instead of the 20× probe/primer mix, 2.5 μl of 2.5 μM probe and 2.25 μl of each 10 μM primer were added.

Reactions were carried out on an Applied Biosystems 7500 system as described in the manufacturer's instructions. Data were analyzed using the relative standard curve method using *ACT1* as the reference gene.

ELISAs. Enzyme-linked immunosorbent assays (ELISAs) were performed using a human albumin ELISA quantitation kit (Bethyl Laboratories) as described in the manufacturer's instructions. Ninety-six-well plates were scanned using a SpectraMax plate reader (Molecular Devices), and data were interpreted using SoftMax Pro software (Molecular Devices).

SDS-polyacrylamide gel electrophoresis (PAGE)–densitometry. For rHA gels, 20 μl of culture supernatant was run on a 12% (wt/vol) acrylamide nonreducing sodium dodecyl sulfate (SDS)-polyacrylamide gel (Invitrogen) in MOPS (morpholinepropanesulfonic acid) buffer at 200 V for 50 min next to standards of known concentrations. Quantification was performed using LabWorks software (UVP) by comparison to the rHA standard curve. Results were normalized to OD_{600} readings to account for differences in growth. For GM-CSF gels, 30 μl of culture supernatant was run on a 4 to 12% (wt/vol) acrylamide nonreducing SDS-polyacrylamide gel (Invitrogen) in morpholineethanesulfonic acid buffer at 200 V for 35 min. Relative quantification was performed using LabWorks software (UVP). Gels were stained using Gel-code blue stain (Pierce).

Rocket immunoelectrophoresis. Culture supernatants were loaded at 5 μl per well in 1% (wt/vol) agarose gels containing goat polyclonal antitransferrin (human) antiserum (Calbiochem) at 40 μl per 50-ml gel (7). The rocket immunoelectrophoresis gels were run at 20 V/cm for 120 min using a Tris/Tricine-based system (22) and stained with PhastGel Blue R (Pharmacia Biotech). Standards were human plasma holotransferrin (Calbiochem) at 100, 50, 20, and 10 μg/ml (all added at 5 μl per well).

RESULTS

Mutagenized production strains show increased transcription of the cochaperone-encoding genes *SIL1*, *LHS1*, *JEM1*, and *SCJ1*. Transcript levels for the genes *SIL1*, *LHS1*, *JEM1*, and *SCJ1* were investigated using qRT-PCR (Fig. 1). All four genes showed increased expression in the strain DS569 [pAYE329] compared to DB1[pAYE329]. This was not a re-

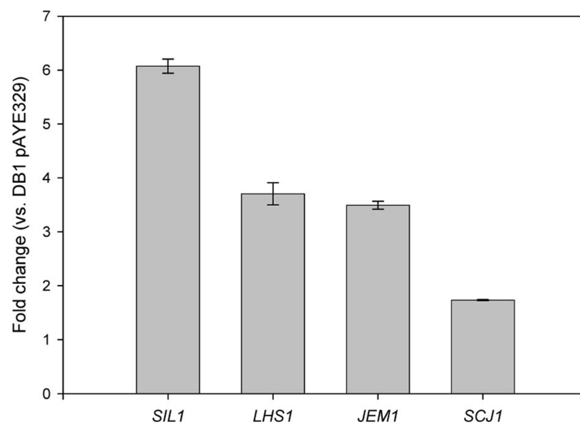

FIG. 1. qRT-PCR analysis of chaperone gene expression in DS569 [pAYE329]. All analysis was carried out on log-phase cells. Data are presented as changes from DB1[pAYE329] to DS569[pAYE329]. qRT-PCR data shown are normalized to *ACT1* transcript levels. All values are averages of duplicate measurements on duplicate RNA samples, each of which contained RNA derived from three independent cultures. Error bars indicate standard deviations ($n = 2$).

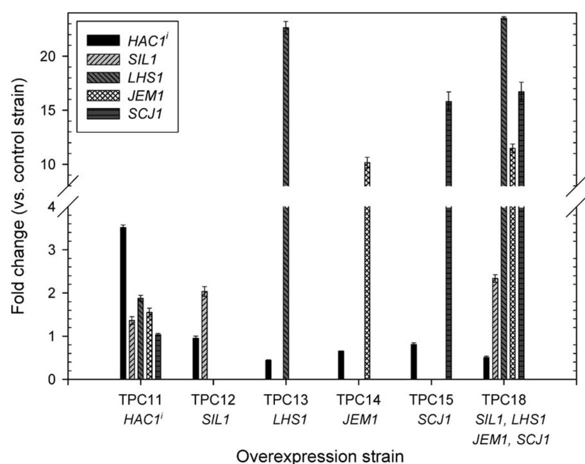

FIG. 2. Confirmation of gene overexpression by qRT-PCR. All analysis was carried out on log-phase cells. Genes overexpressed in each of the TPC strains are given underneath strain names. Data shown are normalized to *ACT1* transcript levels and presented as changes over the levels of the control strain, DB1 *ura3*[pAYE329, pTPA05]. All values are averages of duplicate measurements with exponential-phase cultures. Error bars indicate standard deviations ($n = 2$). The absence of a bar indicates that this transcript was not measured in this strain.

sult of increased protein production by DS569, as a comparable result was obtained by analysis of nonexpressing variants of the same strains (data not shown).

Cochaperone overexpression results in reduced *HAC1^i* transcript levels. Centromeric plasmids based on YCplac33 were constructed for the overexpression of *SIL1*, *LHS1*, *JEM1*, and *SCJ1* (see Materials and Methods). Promoters of known highly expressed genes were used to direct transcription. In addition, the spliced form of the unfolded protein response transcription factor *HAC1* (*HAC1^i*) was overexpressed individually. Overexpression

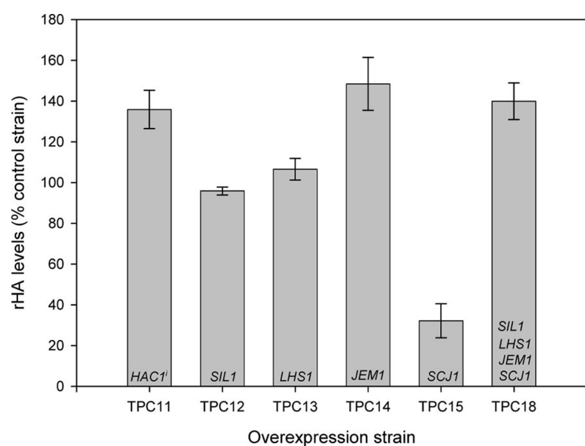

FIG. 3. Log-phase rHA secretion levels in chaperone overexpression strains. All supernatants analyzed were from cultures inoculated to an OD_{600} of 0.05 and grown to an OD_{600} of 2. rHA secretion was assessed by ELISA. All values shown are presented as percentages of rHA secretion compared to the control strain, DB1 *ura3*[pAYE329, pTPA05], and are averages of duplicate measurements. Error bars indicate standard deviations ($n = 2$). Gene names shown in bars indicate genes overexpressed by each strain.

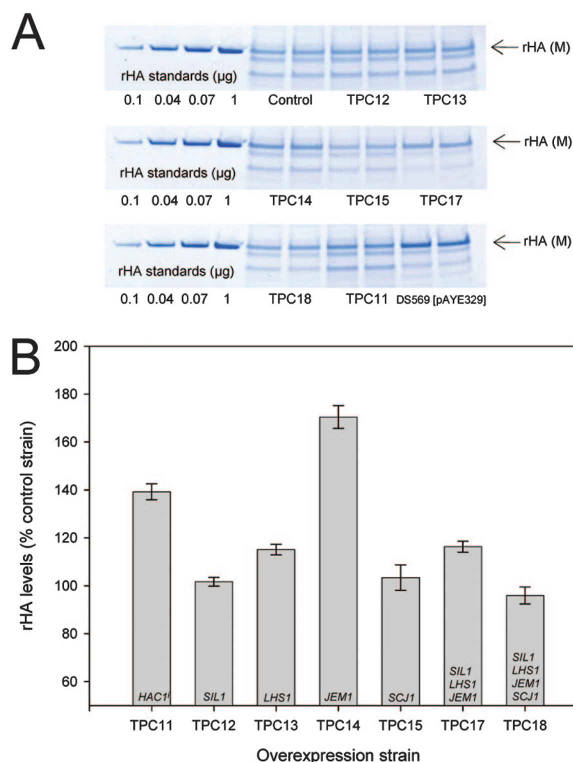

FIG. 4. SDS-PAGE analysis of rHA production from stationary-phase cultures. All supernatants analyzed were from cultures grown for 5 days. The control strain is DB1 *ura3*[pAYE329, pTPA05]. (A) Twenty microliters of culture supernatant was run on 12% nonreducing SDS-polyacrylamide gels next to standards of known concentrations. Mature (M) rHA is indicated by arrows. (B) rHA secretion was assessed by densitometric analysis of SDS-polyacrylamide gels and comparison to an rHA standard curve. Due to differences in final cell densities between strains, values have been normalized to OD_{600} readings. Duplicates shown for each strain represent two individual shake-flask cultures from the same transformant. Error bars indicate standard deviations ($n = 2$). Gene names shown in bars indicate genes overexpressed by each strain.

of *HAC1^i* has been shown previously to increase secreted production of some heterologous proteins in *S. cerevisiae* (47). Centromeric plasmids were then transformed into DB1 *ura3*[pAYE329]. The resultant strains follow the corresponding plasmid names (i.e., TPC13 is DB1 *ura3*[pAYE329 pTPC13]).

qRT-PCR was performed to confirm gene overexpression in strains overexpressing each gene individually and all four cochaperones together (Fig. 2). All genes were successfully overexpressed, though expression levels varied significantly. *SIL1* showed the smallest increase in expression, with transcript levels increasing approximately twofold relative to the control strain, whereas *LHS1* showed the largest increase, at approximately 23-fold. Overexpression of multiple genes from the same plasmid had no detrimental effect on expression levels relative to individual overexpression.

Overexpression of *HAC1^i* resulted in induction of *SIL1*, *LHS1*, and *JEM1* (TPC11, Fig. 2). *LHS1* showed the most dramatic effect, with levels elevated approximately 1.9-fold in TPC11. Unexpectedly, overexpression of *LHS1*, *JEM1*, and all four cochaperones together reduced *HAC1^i* transcript levels

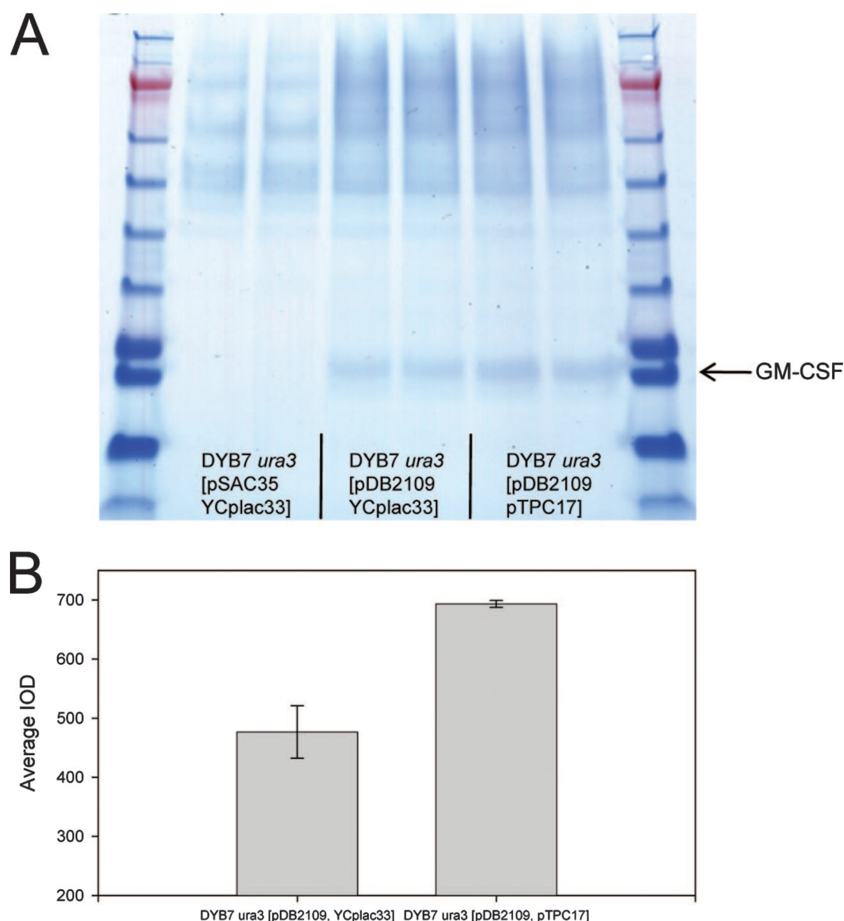

FIG. 5. Increased expression of GM-CSF from DYB7. Two transformants for each strain were inoculated into 10 ml BMMD in 50-ml shake flasks in duplicate and incubated in an orbital shaker at 30°C and 200 rpm for 4 days. Culture supernatants were harvested, and the recombinant GM-CSF titers were compared by SDS-PAGE (A) and densitometric analysis (B). Data are presented as integrated optical density readings (IODs) produced by densitometry. Error bars indicate standard deviations ($n = 2$).

up to approximately twofold. This effect was strongest in the strain overexpressing *LHS1* (TPC13).

Cochaperone overexpression results in increased production of rHA at log phase. To assess the effect of cochaperone overexpression on rHA production, culture supernatants were collected from TPC11 to TPC15 and TPC18 at mid-log phase (OD_{600} of 2). It should be noted that overexpression affected growth rate in a number of the strains used (data not shown). ELISAs were then performed on supernatants (Fig. 3). Overexpression of *HAC1*^{i}, *LHS1*, and *JEM1* individually and *SIL1*, *LHS1*, *JEM1*, and *SCJ1* together had a positive effect on rHA production levels. Overexpression of *SIL1* alone had a negligible effect on production, as might be expected due to low-level overexpression. *SCJ1* overexpression had a detrimental effect on production with rHA levels reduced to less than 40% of those of the control strain.

Cochaperone overexpression results in increased production of rHA in stationary-phase cultures. rHA production was also assessed in stationary-phase cultures. Culture supernatants were analyzed by SDS-PAGE (Fig. 4A), and rHA protein bands were quantified by densitometry and comparison to albumin standard curves (Fig. 4B). As final culture densities varied between strains (within the region of approximately 10%), rHA values obtained were normalized to OD_{600} readings.

Results for TPC11 to TPC14 were strikingly similar to those obtained from log-phase cultures. Due to the negative impact of *SCJ1* overexpression on rHA production at log phase (TPC15, Fig. 3), a further strain overexpressing *SIL1*, *LHS1*, and *JEM1* (TPC17) but not *SCJ1* was included in the stationary-phase analysis.

In contrast to the log-phase data, *SCJ1* had little impact on rHA production in stationary-phase cultures. Of the two multiple overexpression strains, only TPC17 showed an increase in rHA production. TPC18 showed no improvement in production in stationary-phase cultures.

Cochaperone overexpression results in increased production of rTf and GM-CSF from the further-enhanced-production strain DYB7. Due to the promising results obtained with rHA production, cochaperone overexpression was investigated with two additional heterologous proteins, recombinant transferrin (rTf) and GM-CSF. As expression of these proteins was low relative to that of rHA in the strain DB1 (data not shown), expression was performed in the strain DYB7 *ura3*. DYB7 is part of the same series of mutagenized, enhanced-production

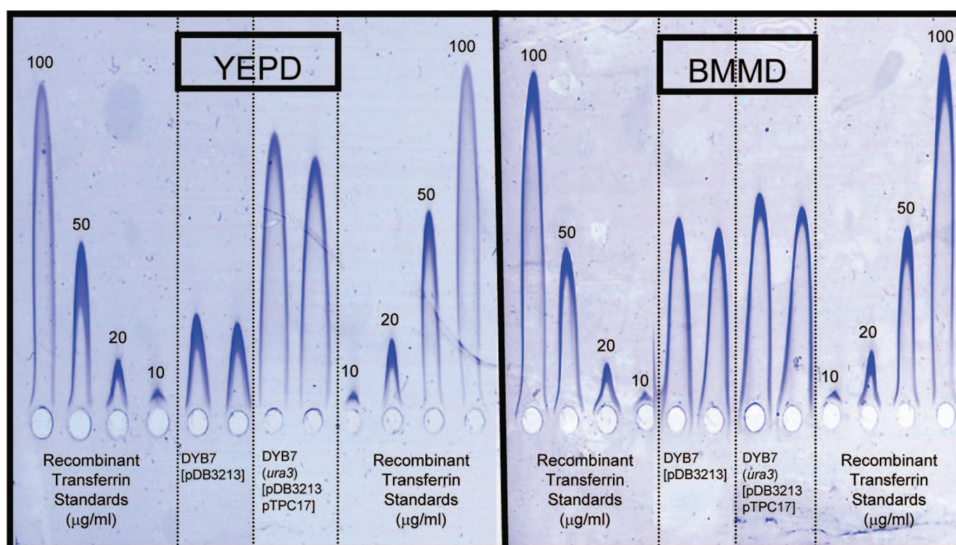

FIG. 6. Increased expression of rTf from DYB7. Transformants of each strain were inoculated into 10 ml BMMD and 10 ml YEPD in 50-ml shake flasks and incubated in an orbital shaker at 30°C and 200 rpm for 4 days. Culture supernatants were harvested, and the rTf titers were compared by rocket immunoelectrophoresis.

strains as DS569. The known genotype (i.e., excluding mutations resulting from chemical mutagenesis) is shown in Materials and Methods.

This part of the study was performed in parallel with the analysis of rHA production in stationary-phase culture. As a result, only pTPC17 (containing *SIL1*, *LHS1*, and *JEM1*) was selected, based on log-phase data. Levels of GM-CSF were elevated approximately 45% upon cochaperone overexpression (Fig. 5). rTf levels were increased even more markedly, although the extent of this effect was shown to be dependent on the growth medium (Fig. 6).

DISCUSSION

The ER provides an environment suitable for the folding and initial maturation of secretory proteins. Two interrelated homeostatic systems, termed the UPR and ERAD, govern the flux of proteins through the ER. Both systems are induced by ER perturbation, either via chemical treatment (e.g., with dithiothreitol to inhibit disulfide bond formation or tunicamycin to prevent N-linked glycosylation) or by overexpression of certain heterologous proteins (4, 16, 46). The yeast ER protein-folding machinery consists of three distinct groups of proteins, hsp70 chaperones (e.g., Kar2p and Lhs1p), along with their cochaperones, the protein disulfide isomerase family (and accessory proteins such as Ero1p and Erv2p) and ERAD/ER quality control proteins (e.g., Der1p and Cne1p) (25).

Altering levels of components of the ER protein-folding machinery has been shown previously to affect UPR signaling. Strains overexpressing *PDI1* showed reduced levels of *HAC1* splicing, corresponding to reduced intracellular levels of scFv (49). This sets a precedent for the unexpected result shown in Fig. 2, indicating that overexpression of *LHS1*, *JEM1*, and all four cochaperones together reduced *HAC1^i^* transcript levels. This effect was most apparent with *LHS1* (TPC13), where *HAC1^i^* transcript levels were reduced more than twofold.

Comparison of data from the analysis of $HAC1^i$ transcript levels and rHA production in overexpression strains (Fig. 2 and 3, respectively) showed that, with the exception of TPC11 (where $HAC1^i$ transcript levels are artificially elevated), strains showing increased rHA production also showed reduced $HAC1^i$ transcript levels. This suggests that ER protein-folding efficiency is improved in these strains.

Therefore, these results support an indirect link between cochaperones of Kar2p and *HAC1* splicing. Maintaining Kar2p in its active, cycling state may both keep luminal load down and keep Ire1p in its inactive form. To complicate this picture, Lhs1p has been shown to have chaperone activity itself, aside from its interaction with Kar2p (29).

The accumulated secreted levels of rHA were analyzed at both log phase and at stationary phase (Fig. 3 and 4). Good correlation was seen between the two sets of results. Overexpression of $HAC1^i$, *LHS1*, and *JEM1* individually produced consistent results, increasing rHA production in both data sets. The strain TPC15, which overexpresses *SCJ1*, and the multiple overexpression strain TPC18 behaved less consistently. While *SCJ1* overexpression appeared to be detrimental to rHA production in log-phase cultures, it had a negligible effect on rHA production in stationary-phase cultures. Conversely, TPC18 showed an approximately 40% increase in rHA production in log-phase cultures but a marginal decrease in stationary-phase cultures.

The reason for the differences in rHA production seen with *SCJ1* and *JEM1* overexpression is unknown (*LHS1* and *SIL1* data presented here are not comparable due to low overexpression of *SIL1*). Cells lacking *JEM1* and *SCJ1* are temperature sensitive and are defective in transport of constitutively misfolded carboxypeptidase Y (CPY*), suggesting overlapping function (26, 38). One aspect that may affect function is localization. Jem1p is an ER membrane protein (though the J domain is found in the ER lumen), whereas Scj1p is a soluble, ER luminal protein (26, 30).

TABLE 5. Characteristics of heterologous proteins

Protein	Leader sequence	Proteolytic processing	Size (kDa)	No. of disulfide bonds	Known modification
rHA	HSA/mating factor α-prepro	Signal peptidase/Kex2p	66.5	17	
GM-CSF	HSA/mating factor α-prepro	Signal peptidase/Kex2p	14.7	2	N glycosylation
rTf	Proprietary (pre only)	Signal peptidase	80	19	

Normalization to optical density readings was used during analysis of rHA production to indicate protein production on a "per-cell" basis. It is true, however, that transcription and translation are affected by growth rate (5). Thus, increased rHA production might be a secondary effect resulting from chaperone overexpression influencing growth rate. In the case of *JEM1* overexpression (TPC14) the general pattern of growth was remarkably similar to that of the control strain, indicating that this is unlikely to be a major factor influencing increased protein production. Furthermore, transcription of *GPD1* and *PRB1* (promoters used in rHA and GM-CSF/rTf expression cassettes, respectively) was not shown to be significantly affected by growth rate (5).

Experimental data also demonstrated that GM-CSF and rTf production was significantly increased with cochaperone overexpression. Data were restricted to combined overexpression of *SIL1*, *LHS1*, and *JEM1* in the strain DYB7. Many studies in this field have shown that the beneficial effects of chaperone overexpression are highly substrate dependent (13, 43). In this case all three proteins, with distinct characteristics (Table 5), showed increased production levels from shake-flask culture.

Analysis of data for rHA production in stationary-phase cultures (Fig. 4) allows dissection of the effects of the individual cochaperones. The data suggest that *JEM1* is likely to be the main driver in increasing heterologous protein production. Due to the specific role of Sec63p as the hsp40 interacting with Kar2p during translocation, this indicates that the function of Kar2p, supporting protein production (as facilitated by *JEM1*), is unrelated to translocation (2, 3, 35).

As previously performed with *PDI1* (10), it would be informative to incorporate *JEM1* along with its native promoter into the 2μm expression plasmid to investigate whether this would increase protein production further. The plasmid used for rTf production already contains a *PDI1* expression cassette and so demonstrates a combined effect of *PDI1*, *SIL1*, *LHS1*, and *JEM1* overexpression. It is key to note that, like all studies of this nature, we are providing only a "snapshot" of the effects of chaperone overexpression on heterologous protein production. Ideally, the levels of gene expression for each overexpressed gene would be tunable to provide a more comprehensive assessment. Such an approach has been used successfully in filamentous fungi to identify optimal levels of BiP (fungal Kar2p) and Pdi1p for the production of the plant sweet protein thaumatin (20, 23).

rTf production from shake-flask culture varied significantly between different growth media (Fig. 6). Increased biomass formation with complete medium (YEPD) normally results in increased protein production relative to minimal medium (BMMD). In this case, the opposite was observed. This may be a result of reduced plasmid stability in nonselective YEPD. The effect of cochaperone overexpression could thus be twofold—increasing productive folding in the ER and reducing the deleterious effects of rTf production on cell physiology and/or growth. This may go some way to explaining the differential effects observed in the two media.

In conclusion, the large number of factors surrounding heterologous protein production, compounded by use of different substrate proteins, ensures that the optimization of the secretion pathway for heterologous protein production is necessarily complex. The term bottleneck is used frequently and is indicative of the problems associated with manipulating single components in a "balanced" system. In the future, large-scale experiments utilizing distinct classes of substrate proteins may be the way forward to gain a fuller understanding. In this way it might be possible to assess protein characteristics, secretory pathway modifications, and consequent production yields in an informative and constructive way.

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