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Theme and Variation Among Silencing Proteins in *Saccharomyces cerevisiae* and *Kluyveromyces lactis*

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ABSTRACT

The cryptic mating type loci in *Saccharomyces cerevisiae* act as reservoirs of mating type information used in mating type switching in homothallic yeast strains. The transcriptional silencing of these loci depends on the formation of a repressive chromatin structure that is reminiscent of heterochromatin. Silent information regulator (Sir) proteins 2–4 are absolutely required for silencing. To learn more about silencing, we investigated mating type and Sir proteins in the yeast *Kluyveromyces lactis*, which contains cryptic copies of the mating type genes. A functional homolog of *SIR4* from *K. lactis* complements the silencing defect of *sir4* null mutations in *S. cerevisiae. K. lactis sir2* and *sir4* mutant strains showed partial derepression of the silent α*1* gene, establishing that the silencing role of these proteins is conserved. *K. lactis sir2* mutants are more sensitive than the wild type to ethidium bromide, and *K. lactis sir4* mutants are more resistant phenotypes that are not observed for the corresponding mutants of *S. cerevisiae.* Finally, the deletion of *sir4* in the two yeasts leads to opposite effects on telomere length. Thus, Sir proteins from *K. lactis* have roles in both silencing and telomere length maintenance, reflecting conserved functional themes. The various phenotypes of *sir* mutants in *K. lactis* and *S. cerevisiae*, however, revealed unanticipated variation between their precise roles.

HAPLOID strains of *Saccharomyces cerevisiae* contain three loci that encode mating type information. The *MAT* locus is expressed and thus determines the mating type, whereas two additional loci, *HML* (usually encoding the α information) and *HMR* (usually encoding **a** information) are not expressed despite the presence of functional promoters and structural genes. Transcriptional repression of the cryptic mating type loci, known as silencing, occurs by the formation of a repressive chromatin structure (Laurenson and Rine 1992). Evidence for an unusual chromatin structure associated with these loci includes the involvement of histones H3 and H4 in silencing (Kayne *et al.* 1988; Thompson *et al.* 1994), as well as the observation that the *HMR* locus is resistant to DNA modifying enzymes both *in vivo* (Singh and Klar 1992) and in isolated nuclei (Loo and Rine 1994). Marker genes close to telomeres are also silenced by telomere position effect (Gottschling *et al.* 1990), and a similar phenomenon was recently observed for marker genes located in the rDNA locus (Smith and Boeke 1997; Bryk *et al.* 1997). Position effects on gene expression are widespread phenomena. Silencing in yeast is reminiscent of heterochromatic gene inactivation, which underlies the phenomena of *X*-chromosome inactivation in mammals and position effect variegation in Drosophila (Grigliatti 1991; Rastan 1994). Thus, telomeres and the *HML* and *HMR* loci are presumably the yeast counterpart to heterochromatin.

Silencing of the cryptic mating type loci requires a combination of regulatory sites called silencers, as well as dedicated proteins. The most thoroughly studied silencer, *HMRE*, contains a binding site for ORC, a protein complex that is involved in replication initiation, as well as binding sites for two widely used transcriptional activators, Rap1p and Abf1p. Mutations in the genes encoding Orc2p (Foss *et al.* 1993; Micklem *et al.* 1993), Orc5p (Loo *et al.* 1995a), Rap1p (Sussel and Shore 1991), and Abf1p (Loo *et al.* 1995b) lead to derepression of transcription of *HML* and *HMR.* The silent information regulator (Sir) proteins 2–4 are required for both telomere position effect and cryptic mating type loci silencing in *S. cerevisiae* (Aparicio *et al.* 1991; Ivy *et al.* 1986). Null alleles of the *SIR2*, *SIR3*, or *SIR4* genes lead to a complete derepression of the silent mating type genes (Rine and Herskowitz 1987), whereas *sir1* strains show only a partial derepression of *HML* and *HMR* (Pillus and Rine 1989). Moreover, the requirement for a silencer element can be bypassed by fusing Sir1p, Sir3p, or Sir4p to a Gal4p DNA binding domain and exchanging the silencer for *GAL4* binding sites (Chien *et al.* 1993; Marcand *et al.* 1996), confirming the central role of Sir proteins in silencing. The precise functions of the Sir proteins are still unknown, but recent evidence suggests that they are structural parts of silent chromatin because silent chromatin can be specifically immunoprecipitated using antibodies against Sir2p and Sir4p (Strahl-Bolsinger *et al.* 1997).

Little is known about whether or not Sir-like proteins

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exist in other eukaryotes and what function such potential homologs might have. *SIR2* appears to encode a protein of fundamental function because genes highly homologous to *SIR2* are found from bacteria to humans (Brachman *et al.* 1995). The function of these homologs in organisms other than *S. cerevisiae* remains unknown, except for a *K. lactis SIR2* gene homolog. This gene partially complements a *S. cerevisiae sir2* null allele and is required for growth of *K. lactis* in the presence of the DNA intercalating drug ethidium bromide (EtBr; Chen and Clark-Walker 1994). Sequence or functional homologs of the other Sir proteins have not yet been identified.

In this study, we report the identification of a *K. lactis* functional homolog of *ScSIR4.* The deletion of *K. lactis SIR4* had effects on the resistance to EtBr and also affected telomere length. Finally, neither KlSir4p nor KlSir2p are absolutely required for mating in *K. lactis*, but their absence leads to a partial derepression of the silent $\alpha 1$-gene in *MAT***a** strains.

MATERIALS AND METHODS

Cloning of *KlSIR4*: *S. cerevisiae* strain JRY4577 (*MAT***a** *can1-100 his3-11 leu2-3,-112 lys2Δ trp1-1 ura3-1 sir4::HIS3*) was transformed with a genomic *K. lactis* library in plasmid pAB24 (2 μm *URA3*). Approximately 42,000 transformants were screened by mating to a *MAT*α *ura3-52* strain (FY2), after which diploid colonies were recovered. Library plasmids were isolated, and the complementing activity was confirmed by transformation into JRY4577, followed by mating assays. As a result, three plasmids were found with overlapping but nonidentical inserts that could complement the mating deficiency of JRY4577.

Plasmids: From one of the complementing library plasmids, a 7.8-kb *Sal*I-*Hin*dIII fragment containing the entire *KlSIR4* ORF was cloned into the corresponding sites of pSEYC68 (*CEN ARS URA3*) and pRS426 (2 μm *URA3*; Emr *et al.* 1986; Christianson *et al.* 1992), forming plasmids p199 and p201, respectively. Plasmid p86 contained a 9-kb *Sal*I fragment from one of the library plasmids cloned into the corresponding site of pUC118. Because the insert cloned into p199 and p201 contained two ORFs (Figure 1), three deletion derivatives of p201 were generated to elucidate which ORF was responsible for the complementing activity. The entire upstream ORF in p201 was deleted by a *Hin*dIII-*Msc*I digestion, the staggered ends were filled in with T4 DNA polymerase, followed by blunt end ligation, resulting in plasmid p162. Two deletion derivatives of the *KlSIR4* gene (leaving the upstream gene intact; Figure 1) were generated by *Sac*II digestion of p201 followed by ligation, thus generating plasmid p163. Plasmid p122 was generated by cloning a 5.5-kb *Xba*I-*Hin*dIII fragment from p86 into the *Spe*I-*Hin*dIII sites of pRS426 (Christianson *et al.* 1992). These procedures removed the sequences encoding amino acids 688–1314 (p163) and amino acids 909–1314 (p122) of the predicted KlSir4 protein. An integrative plasmid, in which the sequences encoding amino acids 87–939 of the *KlSIR4* gene were exchanged for a functional *LEU2* gene, was generated in two steps. First, a 2-kb *Sal*I-*Xba*I fragment from pJR990 containing a functional *LEU2* gene was exchanged for an internal 2-kb *Spe*I-*Xho*I fragment in p86, generating plasmid p183. Second, a 5.7-kb *Bgl*II fragment from p183 was cloned into the *Bam*HI site of pRS306 (Sikorski and Hieter 1989), generating plasmid p232. Plasmids capable of replicating in *K. lactis* containing the *KlSIR4* and *ScSIR4* genes were generated by cloning a 6-kb *Pst*I-*Eco*RI fragment from pRS315-*ScSIR4* (S. Okamura, unpublished data) into the corresponding sites of pCXJ18 (Chen 1996) and by cloning a 6-kb *Msc*I-*Hin*dIII fragment from p86 into the *Sma*I-*Hin*dIII sites of pCXJ18, thus generating plasmids p248 and p250, respectively.

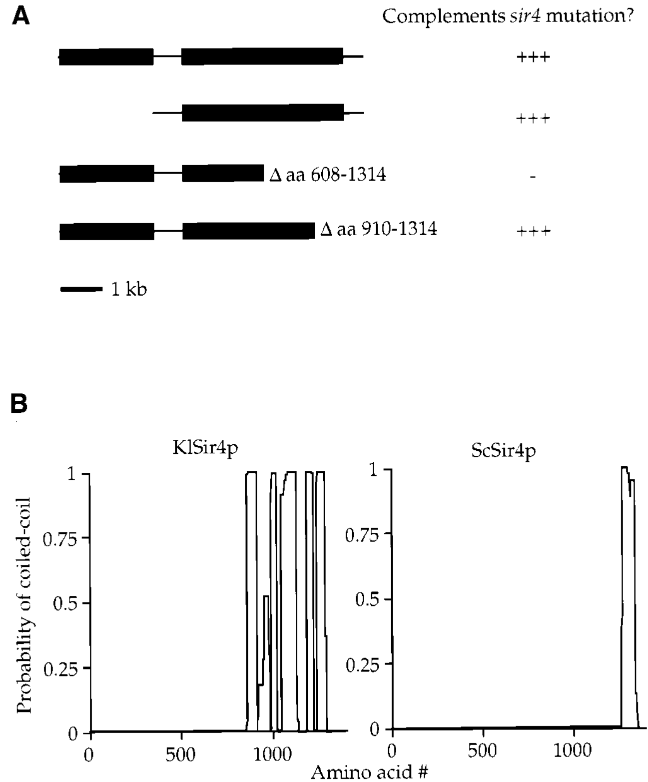

Figure 1.—(A) Identification of *K. lactis SIR4.* Plasmids containing the indicated constructs were introduced into a *MAT***a** *sir4::HIS3* strain (JRY4577) and tested for complementation of the mating defect. +++, efficient complementation; -, no complementation. The left-hand ORF shares 20% identity with ScSir4p; the right-hand ORF shares 21% identity. (B) Predicted coiled-coil domains in ScSir4p and KlSir4p. ScSir4p, 1358 amino acids; KlSir4p, 1314 amino acids. The *KlSIR4* nucleotide sequence appears in the GenBank database under the accession number AF035007.

Sequencing and sequence analysis: The insert of p201 was sequenced on both strands using a Prism sequencing kit (Applied Biosystems, Inc., Foster City, CA) and DNA sequencer (model 373; Molecular Dynamics). Prediction of coiled-coil domains was performed as described (Lupas *et al.* 1991), and homology searches were performed using the BLAST algorithm (Altschul *et al.* 1990).

Strain constructions: The strains used in this study are listed in Table 1. *K. lactis* strain CK213-4C (Chen and Clark-Walker 1994) was transformed with a *Msc*I-linearized pRS306-*sir4::LEU2* (p232) on 5-FOA/plates lacking leucine. This procedure resulted in the replacement of *SIR4* for *sir4::LEU2* (Scherer and Davis 1979), generating strain SAY90. The disruption was confirmed by DNA blot hybridization. Strains SAY90 and CK57-7A (Chen and Clark-Walker 1994) were mated, sporulated, and the resulting tetrads were analyzed. As a result, *sir2 sir4* double (SAY97), *sir2* single (SAY99, SAY102), and *sir4* single mutant (SAY101) strains were obtained for subsequent analysis.

TABLE 1

Yeast strains used in this study

Strain	Genotype	Source
A. *S. cerevisiae*		
JRY4577	*MAT***a** *can1-100 his3-11 leu2-3−112 lys2Δ trp1-1 ura3-1 sir4::HIS3*	This laboratory
JRY4578	As JRY4577 but *MATα*	This laboratory
RBY15	*mataΔp HMLα HMRα leu2-3−112 his3-11,−15 trp1-1 ura3-1 ste14::TRP1*	This laboratory
FY2	*MATα ura3-52*	F. Winston, Harvard Medical School
JRY2726	*MAT***a** *his4*	This laboratory
JRY2728	*MATα his4*	This laboratory
B. *K. Lactis*		
CK213-4C	*MAT***a** *lysA1 trp1 leu2 metA1 uraA1*	X. J. Chen and G. D. Clarke-Walker 1994
WM52V4	*MATα ade1 adeX his7 uraA1*	X. J. Chen and G. D. Clarke-Walker 1994
CK57-7A	*MATα ade1 uraA1 sir2::URA3*	X. J. Chen and G. D. Clarke-Walker 1994
SAY90	*MAT***a** *lysA1 trp1 leu2 metA1 uraA1 sir4::LEU2*	This study
SAY97	*MATα uraA1 leu2* or *LEU2 ade1 metA1 sir2::URA3 sir4::LEU2*	This study
SAY99	*MAT***a** *uraA1 metA1 sir2::URA3*	This study
SAT101	*MAT***a** *uraA1 lysA1 trp1 leu2* or *LEU2 sir4::LEU2*	This study
SAY102	*MAT***a** *uraA1 leu2 lysA1 metA1 trp1 sir2::URA3*	This study

Media: Rich media, sporulation media, and conditions for quantitative matings were as described (Chen and Clark-Walker 1994; Rose *et al.* 1990).

RNA and DNA blot hybridizations: Standard techniques were used (Sambrook *et al.* 1989). DNA and RNA preparations were as described (Rose *et al.* 1990). The *α1* probe (Yuan *et al.* 1993) was obtained by PCR from genomic *K. lactis* DNA using oligonucleotides 5′-ATGAAATCGAATGCTCCAACC-3′ and 5′-CTCAGACTGAGTTCATCAAGG-3′. The telomere probe was an oligonucleotide (5′-GATTAGGTATGTGG-3′) specific for the telomeric repeats. Hybridization and washes were performed at 40° for blots probed by the oligonucleotide and at 65° for the *α1* probe. Quantification of signals were performed using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA) and Imagequant software.

RESULTS

Cloning of *KlSIR4*: Comparisons of similar processes in distantly related organisms offer a powerful approach to identifying themes and variations in evolution from the macroscale (Darwin 1859) to the microscale (Susskind and Botstein 1978). Thus, we chose to explore the similarities and differences between Sir proteins in two budding yeasts, *S. cerevisiae* and *K. lactis.* Based on 18S rRNA sequences, these two yeasts are more closely related to each other than either is related to budding yeasts such as *Candida albicans* or *Yarrowia lipolytica* (Barns *et al.* 1991). Previous work by others (Chen and Clark-Walker 1994) established the existence of a *K. lactis SIR2* homolog (*KLSIR2*) that could complement the *sir2* mutations of Saccharomyces. To learn if *SIR4* was evolutionarily and functionally conserved in *K. lactis,* we transformed a genomic *K. lactis* library in a 2 μm plasmid vector into an *S. cerevisiae* strain carrying a null allele of *sir4.* Saccharomyces *sir4* strains, unable to silence *HML* and *HMR*, are sterile because of the simultaneous expression of **a** and α genes (Laurenson and Rine 1992). Among mating competent transformants that were isolated, there were three plasmids with overlapping but nonidentical inserts. Complementation was specific to the *sir4* mutation because the introduction of these plasmids into *sir2* or *sir3* null strains did not restore mating. The sequence of the insert from one of the complementing plasmids revealed two long ORFs that share a limited homology to *ScSIR4* (Figure 1). The complementing gene was identified by deletion of the left-hand gene from the complementing insert (as drawn), showing that the intact right-hand gene still complemented the phenotype. A deletion removing the sequences encoding the carboxyl-terminal half of this gene (corresponding to amino acids 688–1314) completely abolished the complementing activity, confirming that this gene was necessary and sufficient to complement the *sir4* mutation (Figure 1). A smaller deletion removing sequences corresponding to amino acids 909–1314 of the *KlSIR4* gene still complemented the phenotype, however, defining a region between amino acids 688 and 909 as important, but excluding amino acids 910–1314 as essential for the complementing activity. This *KLSIR4* gene encoded a putative peptide of 1314 amino acids that was subjected to a homology search in the GenBank database. No significant homologies were found, and KlSir4p and ScSir4p shared only 21% sequence identity. Because of this limited homology, a meaningful alignment between the two molecules could not be assembled. Despite their limited homology, KlSir4p had a predicted carboxyl-terminal coiled-coil

domain similar to that suggested for ScSir4p (Diffley and Stillman 1989; Figure 1). The extreme carboxyl terminus of ScSir4p is essential for silencing (Kennedy *et al.* 1995), suggesting that this coiled-coil domain plays an important role in Sir4p function. Interestingly, the predicted coiled-coil domain of KlSir4p is more extended than that of ScSir4p, and the truncation that removed most but not all of the carboxyl-terminal coiled-coil domain still complemented the *sir4* null mutation in *S. cerevisiae* (Figure 1). The truncation that removed the entire coiled-coil domain, however, did not complement the phenotype, which is consistent with a coiled-coil domain being essential for Sir4p function. Thus, these homologs showed low sequence similarity, but they had structural similarity and functional conservation.

Phenotypic characterization of *KlSIR4* in *S. cerevisiae*: The ability of the *KlSIR4* gene to complement the *Scsir4* mutation depended on the copy number of the plasmid carrying the *KlSIR4* gene in an unusual way. Quantitative mating determinations (a measure of silencing) revealed that *KlSIR4* on a low copy plasmid complemented the mating deficiency very well—in fact, more efficiently than the same gene in high gene dosage (Figure 2). This 10-fold difference in complementation efficiency between the high- and low-copy plasmids was observed only in the *MAT***a** strain. In *MAT*α strains, both plasmids only partially restored silencing, suggesting that KlSir4p silenced *HML*α more efficiently than *HMR***a.** Moreover, KlSir4p was unlikely to be expressed at levels significantly different from ScSir4p because *LacZ* gene fusions to *ScSIR4* and *KlSIR4* produced similar levels of β-galactosidase, 5 units and 7 units, respectively, when assayed in *S. cerevisiae.*

The inefficient silencing in *MAT***a** strains by the high-copy *KlSIR4* plasmid was reminiscent of the ability of ScSir4p to derepress *HML* and *HMR* when overexpressed (Marshall *et al.* 1987). We tested to see if

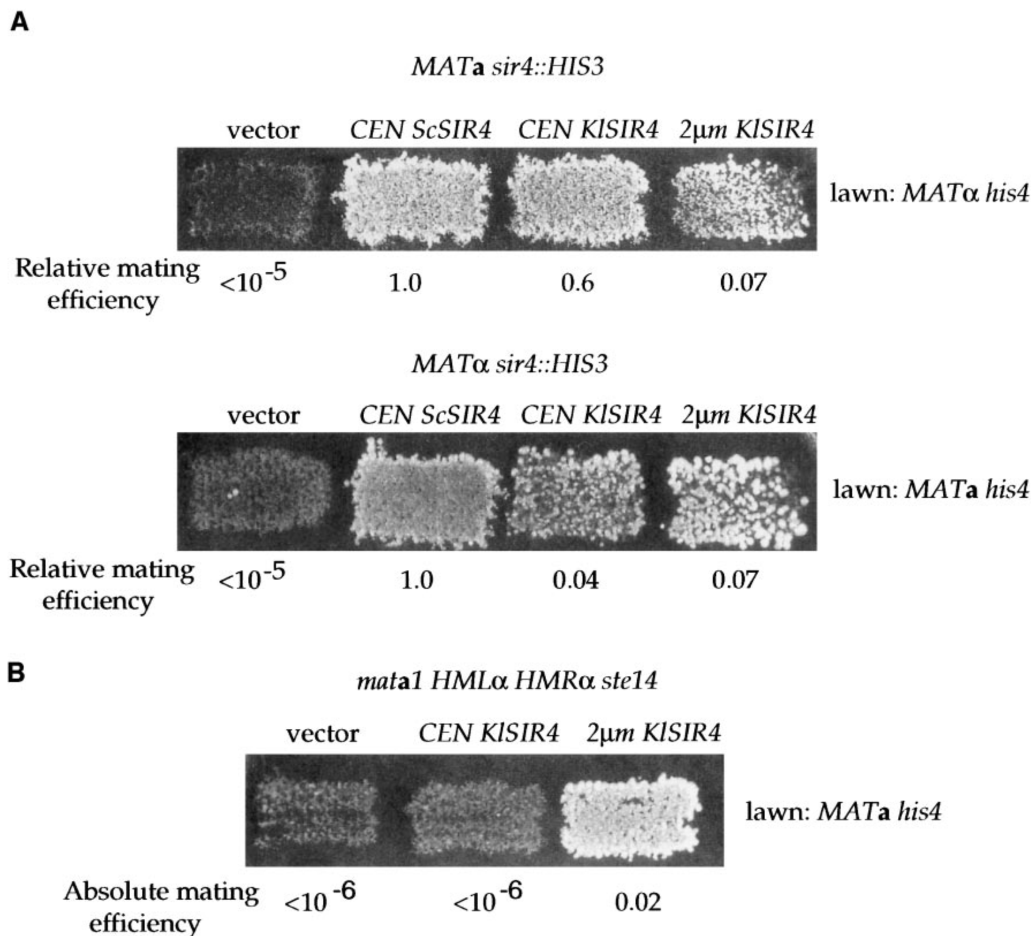

Figure 2.—(A) *KlSIR4* complements the mating deficiency of *S. cerevisiae sir4*-null strains. Patch matings involving a *MAT***a** *sir4::HIS3* strain (JRY4577; upper panel) and a *MAT*α *sir4::HIS3* strain (JRY4578; lower panel) containing the indicated plasmids. Mating type tester strains were as indicated on the right. *CEN* denotes low-copy number, and 2 μm denotes high-copy number. Relative mating efficiencies, as determined by quantitative matings, are shown below the patches. The absolute mating efficiencies of JRY4577/*CEN ScSIR4* and JRY4578/*CEN ScSIR4* were 0.1 and 0.7, respectively. (B) *KlSIR4* disturbed silencing at the *HM-loci* when overexpressed. Patch matings involving a *mat***a***1 HML*α *HMR*α *ste14::TRP1* strain (RBY15), containing the indicated plasmids are shown. Abbreviations are as described above. Absolute mating efficiencies are indicated below the patches.

overproduction of KlSir4p could disrupt silencing at *HML* and *HMR* by using a strain in which loss of silencing leads to the ability to mate as an α strain. *KlSIR4* in high-copy number disrupted silencing in this strain efficiently, and no interference was seen when the gene was present on a low-copy plasmid (Figure 2). Because this strain had wild-type copies of all the Saccharomyces *SIR* genes, it was likely that high levels of KlSir4p interfered with the function of a Saccharomyces protein required for silencing.

K. lactis* strains had stable mating types and silent mating type genes:** To characterize the mating-specific functions of KlSir2p and KlSir4p, we had to determine if mating in general in *K. lactis* and *S. cerevisiae* is similar. The ability of *KlSIR4* to silence *HML* and *HMR* in Saccharomyces implied that *K. lactis* might also have cryptic copies of mating type genes. To determine if *K. lactis* contained both silent and expressed mating type genes, we used DNA blot hybridizations. The *K. lactis* α*1* gene (Yuan *et al.* 1993) was used as a probe in genomic DNA hybridization experiments. If the *S. cerevisiae* model were to be recapitulated in *K. lactis*, we would expect to see one invariantly sized restriction fragment hybridizing in strains of either mating type, as well as a second α*1*-specific band present only in *MAT*α strains. Indeed, this was the observed result (Figure 3). Because wild-type *MATa** strains did not express the α*1*-transcript (see below), we deduced that the invariant band seen on the DNA blot must represent a cryptic locus, and that the unique locus found only in *MAT*α strains was expressed. Therefore, *K. lactis* did have a cryptic copy of α*1*, and its expression most likely resulted from a genomic rearrangement. Moreover, the presence of a single hybridizing band in the genome of multiple *MAT***a** strains indicated that the rate of mating type interconversion in these strains must be low.

Mutations in *KlSIR2* and *KlSIR4* did not abolish mating in *K. lactis*: Others have shown that *K. lactis sir2* strains have a moderate mating defect (Chen and Clark-Walker 1994), but the phenotype is not as dramatic as that seen in *S. cerevisiae sir2* strains. In our hands, the mating defect of *sir2*-null strains was barely detectable in quantitative mating determinations in which dilutions of the strains tested were spread on a high density lawn of the opposite mating type. We found, however, that the *sir2* strains required a high density of the mating partner to mate efficiently. Patch matings, in which approximately equal numbers of the two mating partners were mixed, resulted in less mating of the *sir2* strains (Figure 3).

To study the role of the *SIR4* gene in *K. lactis*, we deleted the gene in a *MAT***a** strain. The resulting mutant was viable and showed no growth defect compared to the wild-type parent. Moreover, mating in both mating types appeared unaffected by the deletion (Figure 3, data not shown).

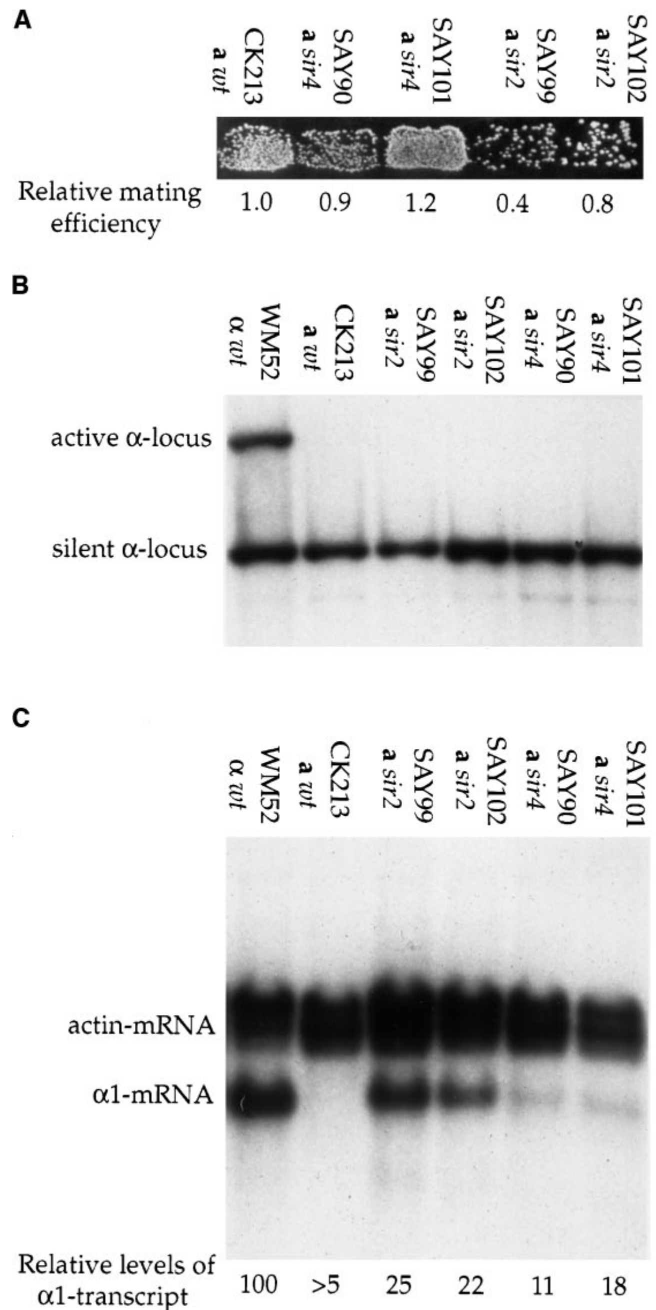

Figure 3.—(A) The absence of Sir proteins did not abolish mating in *K. lactis.* Patch matings of the indicated strains are shown. The *MAT*α mating type tester strain was WM52. Relative mating efficiencies, as determined by quantitative matings, are shown below the patches. The absolute mating efficiency of the *K. lactis MAT***a** strain (CK213) was 0.7. (B) The absence of Sir proteins did not lead to increased mating type interconversions. DNA blot hybridization of *K. lactis* chromosomal DNA, digested with *Xba*I, from the indicated strains. The positions of the active and silent α loci are indicated on the left. (C) Sir proteins were required for repressing the silent α*1* gene transcription. RNA blot hybridization of total *K. lactis* RNA from the indicated strains. The position of the actin mRNA, serving as a control for the amount of mRNA in each lane, as well as the α*1* mRNA, are indicated on the left. The relative amounts of α*1* mRNA in the various strains are indicated below the lanes.

Sir proteins of *K. lactis* have a role in silencing: Although mutations causing defects in silencing in *S. cerevisiae* lead to decreased mating efficiency, only a small subset of mutations affecting mating efficiency do so through defects in silencing. To investigate if mutations in *sir2* and *sir4* lead to derepression of the silent mating type loci, we performed an RNA blot hybridization on RNA from various *MAT***a** strains probing for the α*1*-transcript (Figure 3). The control *MAT***a** strain, as expected, did not express the α*1* transcript. The *sir2* and *sir4* mutant strains, however, showed derepression of the silent α*1*-locus, but not to the same extent as the control *MAT*α strain. In Saccharomyces, the α*1* gene is partially repressed by the **a***1*/α*2* repressor in *sir* strains, so we would not necessarily expect the α*1* levels in *sir2* and *sir4* mutants to be equal to that of the *MAT*α strains. The difference between the impact of a *sir2* mutation and a *sir4* mutation on α*1* levels in *K. lactis*, however, was unexpected. The silent α*2* transcript was also partially derepressed in *sir* strains (data not shown). Therefore, null alleles of *sir2* and *sir4* caused a partial derepression of silent mating type genes in *K. lactis* that did not abolish mating ability, revealing a surprising difference between *K. lactis* and *S. cerevisiae.*

Mutations in *sir* genes affected the sensitivity of *K. lactis* to EtBr: *sir2* mutant strains in *K. lactis*, but not *S. cerevisiae*, are hypersensitive to DNA intercalating agents such as the drug EtBr (Chen and Clark-Walker 1994). We investigated the relative EtBr sensitivities of a *sir2* strain, a *sir4* strain, and a *sir2 sir4* double-mutant strain. As expected, the *sir2* mutant was at least four orders of magnitude more sensitive to EtBr than were the wild-type strains (Figure 4). Surprisingly, the *sir4* mutant was at least 10-fold more resistant to EtBr than the parental strain. The *sir2 sir4* double-mutant strain was EtBr sensitive, showing that the *sir2* mutation was epistatic to *sir4* by this phenotype. Thus, both Sir proteins were involved in the response of *K. lactis* to EtBr, but in opposite directions.

The role of Sir proteins in telomere metabolism in *K. lactis*: The deletion of *SIR4* in *S. cerevisiae* leads to shorter telomeres (Kennedy *et al.* 1995; Palladino *et al.* 1993), so we investigated the size of the telomeres in *K. lactis sir2*, *sir4*, and *sir2 sir4* double-mutant strains. The deletion of *SIR4* in *K. lactis* led to telomeres that were ~200 bp longer (Figure 4). This phenotype could be partially complemented by a plasmid carrying *KlSIR4*, but not by a plasmid carrying *ScSIR4.* Increased gene

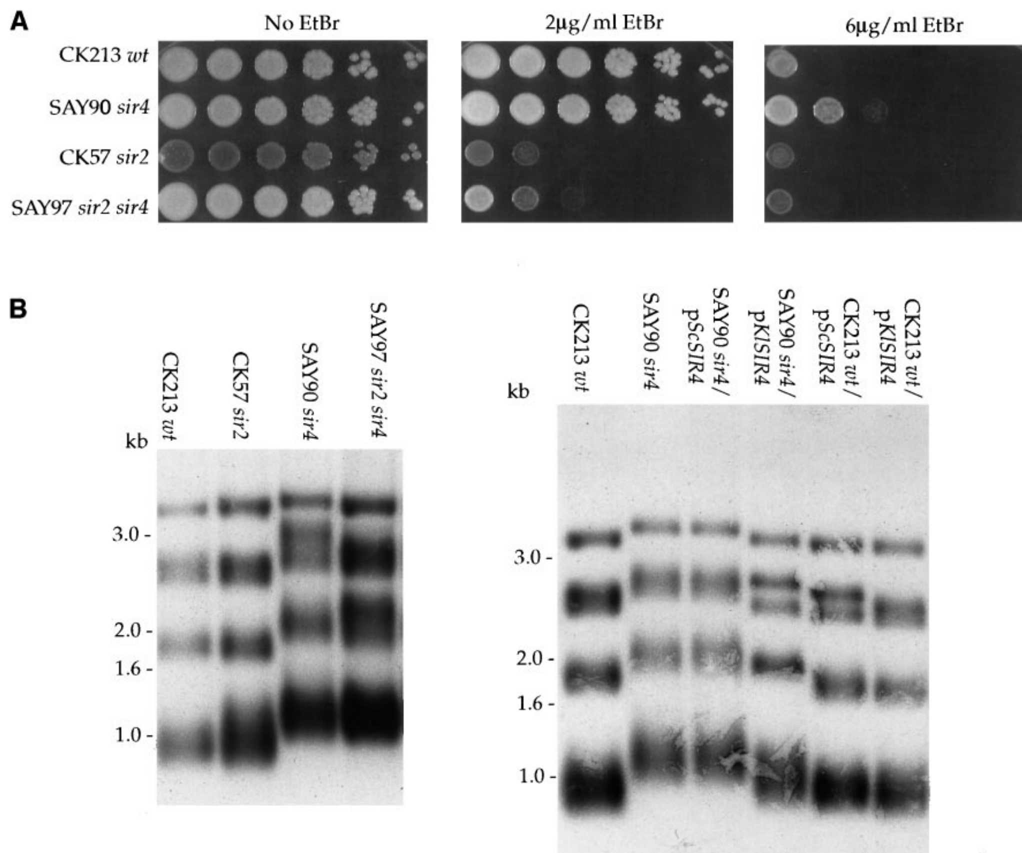

Figure 4.—(A) Sir proteins regulated the sensitivity of *K. lactis* to EtBr. Serial dilutions (10-fold) of overnight cultures of the strains indicated were spotted onto either YEPD or YEPD plates containing 2 or 6 μg/ml EtBr. (B) Elongated telomeres in *K. lactis sir4*-null strains. DNA blot hybridization of *K. lactis* genomic DNA digested with *Eco*RI, from the indicated strains. p*ScSIR4*, a plasmid carrying *ScSIR4*; p*KlSIR4*, a plasmid carrying *KlSIR4.* Size markers in kilobasepairs are on the left.

dosage of either *Sc*- or *KlSIR4* did not affect telomere length in wild-type cells. Thus, the deletion of *SIR4* in *K. lactis* and *S. cerevisiae* had opposite effects on telomere length. The *K. lactis sir2* mutant had telomeres of apparent wild-type length, but *sir2 sir4* double-mutant strains had long telomeres (Figure 4). Thus, by this phenotype, the *sir4* mutation was epistatic to the *sir2* mutation, whereas by the EtBr resistance phenotype, *sir2* was epistatic to *sir4.*

DISCUSSION

In this report, we have studied mating-type and the genes encoding Sir2p and Sir4p in *K. lactis.* We found that the *K. lactis* laboratory strains had stable mating types and contained cryptic mating type genes. Moreover, based on the DNA blot hybridization data, mating type interconversion in *K. lactis* presumably involves genomic rearrangements.

K. lactis Sir proteins were involved in transcriptional silencing of silent mating type genes, as their Saccharomyces counterparts. The modest effect that *sir2* and *sir4* mutations had on the mating efficiency, however, was surprising. We cannot exclude that *K. lactis* might encode redundant functions for Sir2p and Sir4p, which could explain why strains mutant for the corresponding genes still mate with reasonable efficiency. However, DNA blot hybridizations using probes against these genes at high stringency indicate that closely related sequences do not exist in the *K. lactis* genome (Chen and Clark-Walker 1994; data not shown). Moreover, the phenotypes of *sir2* and *sir4* mutants with respect to EtBr sensitivity, telomere length, and silencing indicate that *K. lactis* lacks any genes whose function is completely redundant with those of *KlSIR2* and *KlSIR4.*

Given the low sequence similarity between *KlSIR4* and *ScSIR4*, it was possible that a gene sharing more sequence identity to *ScSIR4* was present in the *K. lactis* genome. The gene that we identified in this study, however, encodes a protein that is structurally similar to ScSir4p, and this gene complements the mating deficiency of *S. cerevisiae sir4* strains to almost wild-type levels when present on a single copy vector. Moreover, in complementation experiments of *S. cerevisiae sir4* strains with Saccharomyces plasmid libraries, only plasmid-borne *SIR4* has been found to complement the mutation. These arguments suggest that the gene we identified indeed encodes the *K. lactis* equivalent to *ScSIR4*, but we cannot exclude that this gene is in fact a low-copy number suppressor.

Because both KlSir2p and KlSir4p are required for complete silencing of the silent α locus, we expected *MAT***a** *sir2* and *sir4* mutants to have a dramatic mating defect if haploid specific genes are repressed by an **a**1/α2 repressor, as observed for *S. cerevisiae.* We tested this assumption by determining whether a plasmid-borne copy of a *K. lactis* α locus would block mating of a *MAT***a** strain. A plasmid encoding the *K. lactis* α*1* and α*2* genes, when introduced into *MAT***a** strains, almost abolished mating under conditions with a limited number of mating partners. Surprisingly, these strains still mated efficiently when a large surplus of mating partners were present (data not shown). Thus, *K. lactis* was able to mate despite the simultaneous expression of both **a** and α information, at least under these conditions. This behavior is fundamentally different from comparable *S. cerevisiae* mutants. The **a**1/α2 repressor of *K. lactis* is perhaps unable to completely repress the transcription of haploid-specific genes.

With respect to telomere length, *sir4* mutations lengthened telomeres in *K. lactis* and shortened telomeres in *S. cerevisiae.* In *S. cerevisiae*, the DNA-binding protein Rap1p binds to telomeric sequences (Longtine *et al.* 1989; Lustig *et al.* 1990) and interacts with Sir4p via its carboxyl terminus (Moretti *et al.* 1994). Recently, Shore and co-workers suggested a model for telomere length regulation in *S. cerevisiae* that involved a protein-counting mechanism (Marcand *et al.* 1997). In this model, the precise number of Rap1p molecules bound to the telomere negatively regulates telomere length. Furthermore, interaction between Sir4p and Rap1p limits the amount of Rap1p available for the counting mechanism. Thus, in the absence of Sir4p, more Rap1p molecules bound to the telomeres are counted, explaining the shorter telomeres observed in *sir4* null strains. This model cannot explain the data from this related yeast, however, because *K. lactis sir4*-null strains have long telomeres.

In *K. lactis*, Rap1p also binds telomeres, and mutations that change the sequence of the repeats, and thus decrease Rap1p binding, lead to telomere lengthening (Krauskopf and Blackburn 1996). Moreover, *K. lactis* strains that contain a carboxyl terminal deletion of Rap1p have long telomeres (Krauskopf and Blackburn 1996), similar to the phenotype obtained here in *Klsir4* strains. Perhaps a counting mechanism in *K. lactis* maintains the number of Sir4p/Rap1p heteromeres that are bound to telomeres to regulate telomere length. Further investigations are required, however, to elucidate if KlSir4p has a direct interaction to KlRap1p and telomeres.

In contrast to *S. cerevisiae* Sir proteins, KlSir2p and KlSir4p controlled the sensitivity of cells to EtBr. Surprisingly, *sir2* and *sir4* mutations had opposite effects on EtBr sensitivity, indicating that Sir2p and Sir4p have opposing functions by this phenotype. One explanation for the observed phenotypes would be that Sir2p was required for resistance to EtBr, and that Sir4p limited the effective level of Sir2p. Thus, *sir2* mutants would be sensitive to EtBr, and in the absence of Sir4p, there would be more Sir2p available for conferring resistance, thereby making *sir4* mutants resistant to EtBr. We have observed that wild-type cells grown in the presence of EtBr exclude the drug from the cells, whereas *sir2* cells

accumulate EtBr, suggesting that this accumulation causes the EtBr sensitivity (our unpublished observation).

In Saccharomyces, Sir2p, Sir3p, and Sir4p have been found together in a multicomponent complex (Moazed and Johnson 1996). In contrast, the data from *K. lactis* implies that Sir2p has a function that was different from Sir4p. Recently, ScSir2p was shown to be required for efficient rDNA silencing, whereas Sir4p appears to interfere with rDNA silencing (Smith and Boeke 1997). Thus, in both Saccharomyces and *K. lactis*, the Sir proteins appear to have functions that are independent of each other. These data imply either that some Sir2p or Sir4p function independently of the Sir2p-Sir3p-Sir4p complex, or that the complex has multiple different roles.

Each species shares fundamental processes with other species and differs from other species by certain specializations. At least one silencing protein (Sir2p) is conserved among many phyla and kingdoms, indicating that its function is important. Studies like the one reported here have helped us discriminate between fundamental themes and species-specific variations, and they should prove equally valuable across a broad range of phenomena.

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