Contribution of the response regulators Ssk1p and Skn7p in the pseudohyphal development, stress adaptation and drug sensitivity in the opportunistic yeast *Candida lusitaniae*.

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Running title : Role of Ssk1p and Skn7p response regulators in *Candida lusitaniae*.

SUPPLEMENTAL MATERIAL

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Strains and standard growth conditions. *C. lusitaniae* strains **(Table S2)** were routinely cultivated in liquid YPD medium (1% yeast extract, 2% peptone, 2% glucose), at 35°C, under agitation (250 rpm). Solid media were prepared with 2% agar (Sigma).

Sequence analysis. The complete ORFs of *S. cerevisiae* ScYpd1p (Genbank accession number SCU62016), ScSsk1p (Z73178) ScSkn7 (YSCSKN7) and *C. glabrata* CgYpd1p (XM_448442), CgSsk1p (XM_445541) CgSkn7p (XM_446363) were retrieved from the NCBI database. Similarity searches in the yeast databases were performed with the BLAST algorithm (2) using CaYpd1p (AF213247), CaSsk1p (AF084608) and CaSkn7p (AF510732) of *C. albicans*. The complete open reading frames of the *C. lusitaniae YPD1, SSK1* and *SKN7* genes and corresponding homologues from *C. guillermondii* (CguiYpd1p: PGUG_05889.1; CguiSsk1p: PGUG_01054.1; CguiSkn7p: PGUG_02177.1) and *C. tropicalis* (CtYpd1p: CTRG_05488.3; CtSsk1p: CTRG_03669.3; CtSkn7p: CTRG_05246.3) were retrieved from their respective genome databases available on the Broad Institute Fungal Genome web site (http://www.broad.mit.edu).

Plasmid constructions. The backbone plasmids pG-*YPD1*, pG-*SSK1* and pG-*SKN7* were built by cloning in pGEM-T easy vector (Promega) PCR amplification fragments overlapping the *YPD1* (2555-bp), *SSK1* (5097-bp) and *SKN7* (4126-bp) genes, respectively. All the primers used for PCR are listed in **Table S3** and were synthesized by Invitrogen.

To obtain plasmid pG-Δ*YPD1*/GUN, plasmid pG-*YPD1* was digested with AgeI (producing compatible ends with XmaI) and XbaI to release a 602-bp central fragment from the *YPD1* gene. The resulting digested plasmid was ligated to the GUN fragment (consisting of the *C. lusitaniae URA3* gene flanked on both sides by a non-coding 327-bp repeat named REP) previously amplified from pGUN (6) with primers XMAUN1 and XBAUN2 **(Table S3)** and digested with XmaI and XbaI. In the same way, plasmids pG-Δ*SSK1*/GUN and pG-Δ*SKN7*/GUN were constructed from plasmids pG-*SSK1* and pG-*SKN7*, respectively, replacing a BglII-digested central fragment from the *SSK1* and *SKN7* genes by a GUN sequence amplified with primers BGLUN1 and BGLUN2 **(Table S3)**. Thus, the three resulting plasmids pG-Δ*YPD1*/GUN, pG-Δ*SSK1*/GUN and pG-Δ*SKN7*/GUN harbour 5'end-*YPD1-GUN-YPD1*-3'end, 5'end-*SSK1-GUN-SSK1*-3'end and 5'end-*SKN7-GUN-SKN7*-3'end disruption cassettes, respectively.

The complementation vectors were obtained by digestion of plasmids pG-*SSK1* and pG-*SKN7* with NotI to release fragments overlapping *SSK1* and *SKN7* genes, respectively. Finally, these fragments were subcloned into the unique NotI restriction site from plasmid pVAX-*URA3* (6), resulting in complementation plasmids pVAX-*URA3-SSK1* and pVAX-*URA3-SKN7*, respectively.

Disruption of the *YPD1, SSK1* and *SKN7* genes in *C. lusitaniae*. The 5'end-*YPD1-GUN-YPD1*-3'end, 5'end-*SSK1-GUN-SSK1*-3'end and 5'end-*SKN7-GUN-SKN7*-3'end disruption cassettes (released by digestion with NotI from plasmids pG-Δ*YPD1/GUN*, pG-Δ*SSK1/GUN* and pG-Δ*SKN7/GUN*, respectively) were separately used to transform the

strain 6936 *ura3*$_{[\Delta 360]}$ to prototrophy by the electroporation procedure, as previously described (10). Correct insertion of the disrupting cassette was checked at each locus by Southern analysis of the genomic DNA of a subset of 24 Ura$^+$ randomly selected transformants. Genomic DNAs were digested with PstI or AgeI and membranes were hybridized with YPD1, SSK1 or SKN7 digoxigenin-labelled DNA probes (PCR DIG probe synthesis kit, Roche Molecular Biochemicals), according to the experiment. Hybridization pattern revealed that homologous integration of the 5'end-*SSK1-GUN-SSK1*-3'end and 5'end-*SKN7-GUN-SKN7*-3'end cassettes at the corresponding target loci occurred in half of transformants analyzed and was derived from gene replacement, resulting in the genotypes *ura3*$_{[\Delta 360]}$, *ssk1Δ::REP-URA3-REP* (abbreviated *ssk1::GUN*), and in *ura3*$_{[\Delta 360]}$, *skn7Δ::REP-URA3-REP* (abbreviated *skn7::GUN*), respectively **(Fig. S2)**. For the remaining Ura$^+$ transformants, the hybridization pattern revealed that they were derived from gene replacement at the *ura3* locus (results not shown).

We failed to obtain homologous integration of the 5'end-*YPD1-GUN-YPD1*-3'end cassette at the corresponding target locus. Southern blot analysis of 24 Ura$^+$ transformants revealed that they only derived from ectopic integrations of the disruption cassette (50%) or from gene replacement at the *ura3* locus (50%) **(Fig. S3)**.

In order to obtain the double mutant genotype *ssk1*Δ *skn7*Δ, a representative *skn7::GUN* Ura$^+$ transformant was plated onto YNB supplemented with 5FOA and uracil and the genotype *ura3*$_{[\Delta 360]}$, *skn7Δ::REP* (abbreviated *skn7::REP*) was assigned to the 5FOA-resistant clones **(Fig. S2)**. The linear 5'end-*SSK1-GUN-SSK1*-3'end was used to transform the strain *skn7::REP* and the double mutant genotype *ura3*$_{[\Delta 360]}$, *skn7Δ::REP, ssk1Δ::REP-URA3-REP* (abbreviated *skn7::REP ssk1::GUN*) was assigned to transformants harbouring the expected hybridization profile as screened in the **Fig. S2.** The linear DNA 5'end-*SSK1-GUN-SSK1*-3'end and 5'end-*SKN7-GUN-SKN7*-3'end disruption cassettes were also used to transform the strain PC1(α) (genotype *ura3*$_{[\Delta 360]}$ *MAT*α) (6) to prototrophy in order to generate genotypes *MAT*α, *ura3*$_{[\Delta 360]}$, *ssk1*Δ ::*REP-URA3-REP* (abbreviated *ssk1::GUN*α), and *MAT*α, *ura3*$_{[\Delta 360]}$, *skn7Δ::REP-URA3-REP* (abbreviated *skn7::GUN*α), respectively **(Table S2)**

Complementation of the *ssk1* and *skn7* null mutant alleles. To obtain reintegrant strains, linearized plasmids pVAX-*URA3-SSK1* and pVAX-*URA3-SKN7* were used to transform to prototrophy the *ssk1::REP* (counter-selected by cultivating *ssk1::GUN* strain onto 5-FOA-containing YNB medium) and *skn7::REP* mutants, respectively. We verified by Southern blot **(Fig. S2)** that homologous integration of the whole plasmids pVAX-*URA3-SSK1* and pVAX-*URA3-SKN7* occurred at the *ssk1* and *skn7* loci, respectively, resulting to the relevant genotypes *ura3*$_{[\Delta 360]}$, *ssk1Δ::[REP, pVAX-URA3-SSK1]* (abbreviated *ssk1 + SSK1*) and *ura3*$_{[\Delta 360]}$, *skn7Δ::[REP, pVAX-URA3-SKN7]* (abbreviated *skn7 + SKN7*) **(Table S2)**.

Determination of generation time. The generation time of each strain was determined in YPD and YNB liquid media following the protocol described in (5). Values reported in **Table S2** correspond to the average values of three independent experiments.

Sensitivity test for stress responses, methylglyoxal (MG) and antifungal compounds. Each strain was incubated overnight at 35°C in liquid YPD medium, washed, serially diluted (10^2 to 10^5 dilutions) in distilled water, and spotted (4 µl) onto solid YPD medium. This medium was supplemented with 1.5 M of NaCl, KCl or sorbitol (Sigma-Aldrich), with 1 to 8 mM H_2O_2, or with 25 mM MG (Sigma-Aldrich). Spotted cells were also exposed to UV for 12 s (2880 J/m^2) using a UV table (Fischer Bioblock Scientific). For temperature sensitivity, YPD plates were incubated for 1 day at 43°C.

Stock solutions of fluconazole (FLC, ICN Biomedicals Inc.) and flucytosine (5FC, Sigma-Aldrich) were prepared by dissolving these antifungal agents in water at concentrations of 3.2 mg ml^{-1} and 12.8 mg ml^{-1}, respectively. Stock solution of amphotericin B (AmB, Bristol-Myers Squibb) (1.6 mg ml^{-1}) was prepared in dimethylsulfoxide, as iprodione (100 mg ml^{-1}) and fenpiclonil (100 mg ml^{-1}), kindly provided by P. Leroux (INRA, Versailles, France). For FLC (0.25 to 16 µg ml^{-1}) and 5FC (0.25 to 16 µg ml^{-1}) antifungal susceptibility testing, we used RPMI 1640 AutoMod (modified for autoclaving) agar plates. For AmB susceptibility testing (0.25 to 16 µg ml^{-1}), we used the antibiotic medium 3 (Difco Laboratories) supplemented with 2% agar whereas YPD agar plates were employed to test iprodione (1 to 50 µg ml^{-1}) and fenpiclonil (1 to 50 µg ml^{-1}) susceptibility.

Mating test. Genetic crosses were performed under the same conditions as described previously (11, 23). The reference mating-type tester strains were 6936 *MAT***a** and Cl38 *MAT*α strain. The number of conjugation tubes and produced tetrades (echinulate ascospores) was evaluated on three representative replicates for each unilateral (*ssk1::GUN MAT***a** X Cl38 *MAT*α and *skn7::GUN MAT***a** X Cl38 *MAT*α) and bilateral (*ssk1::GUN MAT***a** X *ssk1::GUN MAT*α and *skn7::GUN MAT***a** X *skn7::GUN MAT*α) genetic cross (11, 23).

Pseudohyphal growth study and morphological observations. The pseudohyphal growth was triggered spotting a 5-µl drop (10^6 cells) on YCB solid medium [1,17% Yeast Carbon Base (Difco laboratories)] supplemented with sorbitol (0.5 to 1.5 M), NaCl (0.3 to 1 M), KCl (0.3 to 1 M), or H_2O_2 (0.5 to 5 mM).

Pseudohyphae length was measured from the edge of the spotted colony after 48 h of growth with an inverted Leitz microscope fitted with a micrometer eye-piece. All pictures were taken with Olympus BX41 microscope using DP-controller software. Pictures of spotted cells producing pseudohyphae were taken directly on agar plates at 100X magnification.

TABLE S1. Characterization of genes potentially encoding HPt and RRs and deduced proteins in *C. lusitaniae*

Gene	Acc. number [a]	Supercontig: position	Protein length (aa)	Identity (%)[b]				
				Sc	Cg	Ca	Ct	Cgui
YPD1	EU401919	7 : 400231-400674	148	35	39	52	65	68
SSK1	EU401920	8 : 172732-174573	613	21	17	41	37	44
SKN7	EU401921	4 : 466961-468397	478	41	40	49	52	54

[a] These sequence have been submitted to the GenBank database
[b] Abbreviations : Sc, *S. cerevisiae*; Cg, *C. glabrata*; Ca, *C. albicans*; Ct, *C. tropicalis*; Cgui, *C. guilliermondii*

TABLE S2. *Candida lusitaniae* strains

Strain	Genotype	Mating type	Generation time (h) [d]	
			YPD	YNB
6936 [a]	wild type	*MAT***a**	1.28 ±0.02	1.50 ±0.05
6936 *ura3* $_{[\Delta 360]}$	*ura3*$_{[\Delta 360]}$	*MAT***a**	ND	ND
PC1 (α) [b]	*ura3*$_{[\Delta 360]}$	*MAT*α	ND	ND
ssk1::GUN [c]	*ura3*$_{[\Delta 360]}$*, ssk1Δ ::REP-URA3-REP*	*MAT***a**	1.75 ±0.03	1.67 ±0.05
skn7::GUN	*ura3*$_{[\Delta 360]}$*, skn71Δ ::REP-URA3-REP*	*MAT***a**	1.29 ±0.05	1.53 ±0.07
*ssk1::GUN*α	*ura3*$_{[\Delta 360]}$*, ssk1Δ ::REP-URA3-REP*	*MAT*α	ND	ND
*skn7::GUN*α	*ura3*$_{[\Delta 360]}$*, skn7Δ ::REP-URA3-REP*	*MAT*α	ND	ND
ssk1::REP	*ura3*$_{[\Delta 360]}$*, ssk1Δ ::REP*	*MAT***a**	ND	ND
skn7::REP	*ura3*$_{[\Delta 360]}$*, skn7Δ ::REP*	*MAT***a**	ND	ND
skn7::REP ssk1::GUN	*ura3*$_{[\Delta 360]}$*, skn7Δ ::REP, ssk1Δ ::REP-URA3-REP*	*MAT***a**	1.76 ±0.15	1.75 ±0.12
ssk1+ SSK1	*ura3*$_{[\Delta 360]}$*, ssk1Δ ::REP, pVAX-URA3-SSK1*	*MAT***a**	1.24 ±0.06	1.40 ±0.02
skn7+ SKN7	*ura3*$_{[\Delta 360]}$*, skn7Δ ::REP, pVAX-URA3-SKN7*	*MAT***a**	1.25 ±0.07	1.44 ±0.11

[a] Reference strain from Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands).
[b] Described in (6)
[c] Italicized name of strains corresponds to abbreviated genotypes.
[d] The values are means in h ± standard deviations based on three individual replicates. ND : not determined.

TABLE S3. Oligonucleotides

Primer	**Sequence (5' to 3')**	**Use for PCR amplification of**
YPDS	AGTTGCCACTGCGAGCGGCAGAGAACC	*YPD1* gene (upstream primer)
YPDR	GCTGGTTATCTTGGTGCCGCGTTGAACC	*YPD1* gene (downstream primer)
SSKS	TCAGAAGAGAGTCAGACTACCAAGAGGGTC	*SSK1* gene (upstream primer)
SSKR	TGATGAGCCTTCAGCCGAAGCGCTTGTACC	*SSK1* gene (downstream primer)
SKNS	GGGCTAGGCATGGGATTATGCTCGTGCGCC	*SKN7* gene (upstream primer)
SKNR	CGCCACAGCACAGGTGCTGGTGTATGACCC	*SKN7* gene (downstream primer)
BGLUN1	CTGACAAGATCTCCCGACGTCGCATGCTCC	GUN sequence (upstream primer, BglII site)
BGLUN2	CTCAGAAGATCTCCAAGCTATTTAGGTGACAC	GUN sequence (downstream primer, BglII site)
XBAUN1	CTGACATCTAGACCCGACGTCGCATGCTCC	GUN sequence (upstream primer, XbaI site)
XMAUN2	CTCAGACCCGGGCCAAGCTATTTAGGTGACAC	GUN sequence (downstream primer, XmaI site)

TABLE S4. Pseudohyphal differentiation of *C. lusitaniae* strains on various supplemented YCB media.

Strain	**YCB**	**+ sorbitol (1 M)**	**+ NaCl (0.5 M)**	**+ KCl (0.5 M)**	**+ H_20_2 (1 mM)**
WT (6936)	986 ±33	407 ±36	273 ±22	367 ±22	400 ±37
ssk1::GUN	657 ±54	113 ±8	273 ±16	233 ±50	500 ±14
skn7::GUN	933 ±29	147 ±57	267 ±16	347 ±16	0
skn7::REP ssk1::GUN	673 ±22	113 ±8	287 ±16	180 ±51	0
ssk1+ SSK1	940 ±37	407 ±22	273 ±33	353 ±8	466 ±22
skn7+ SKN7	933 ±33	433 ±8	273 ±29	327 ±16	420 ±14

The length of pseudohyphae was measured from the edge of the spotted colony. The values are means in µm ± standard deviations based on three individual replicates.

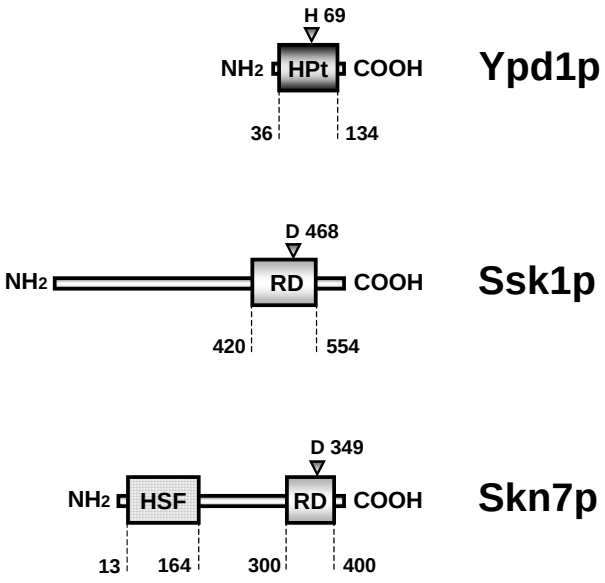

FIG. S1. Structure of Ypd1p, Ssk1p and Skn7p proteins from *C. lusitaniae.* HPt : histidine containing phosphotransfer domain ; RD : receiver domain ; HSF : Heat shock factor DNA-binding domain. The positions of phosphorylatable residues histidine (H) or aspartate (D) are indicated.

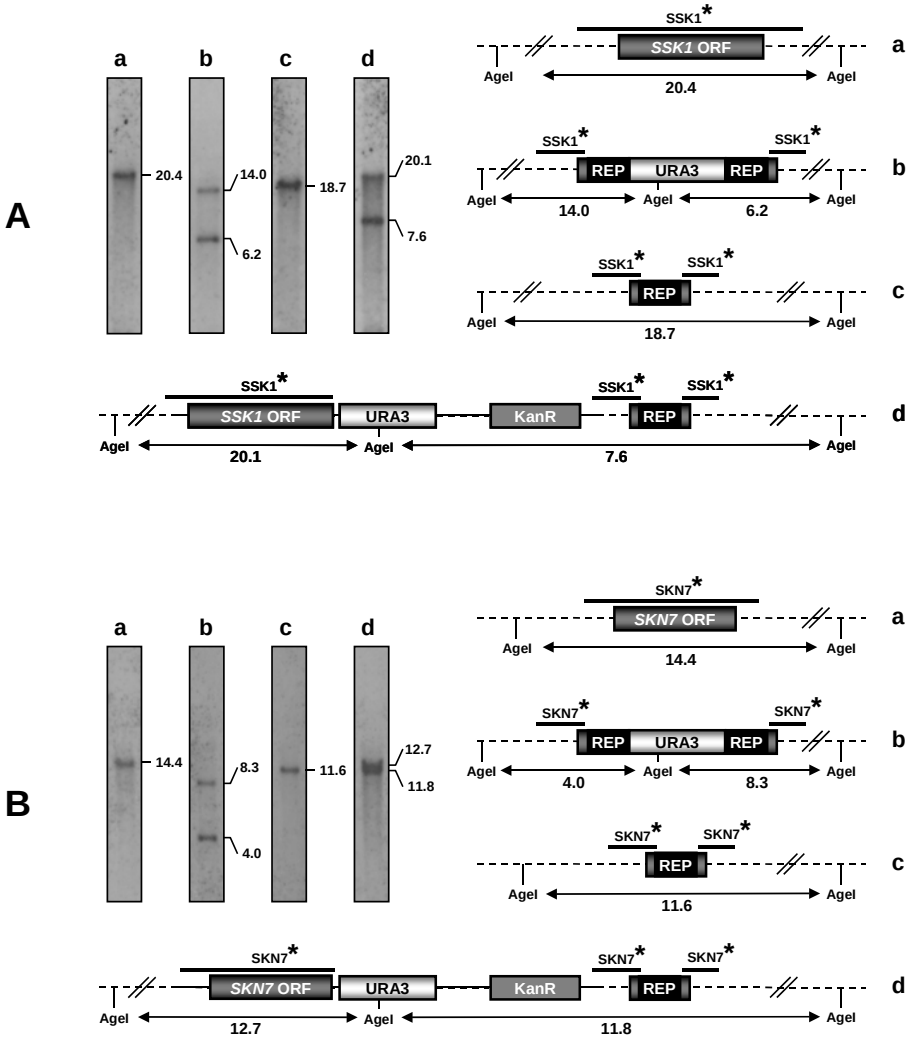

FIG. S2. Southern blot hybridization and schematic representation of resident loci *SSK1* and *SKN7* and of molecular events that occurred in transformants. Signals revealed by the labeled probes (each marked with an asterisk) correspond to those expected from the genomic restriction map. (A) Hybridization pattern with SSK1 probe of AgeI-digested genomic DNA from 6936 *ura3*$_{[\Delta 360]}$ (a), a representative transformant *ssk1::GUN* (b), a 5FOA-resistant derivative *ssk1::REP* (c) and a reintegrant strain *ssk1*+SSK1 (d). (B) Hybridization pattern with SKN7 probe of AgeI-digested genomic DNA from 6936 *ura3*$_{[\Delta 360]}$ (a), a representative transformant *skn7::GUN* (b), a 5FOA-resistant derivative *skn7::REP* (c) and a reintegrant strain *skn7*+SKN7 (d). DNA fragment sizes are indicated in kilobases.

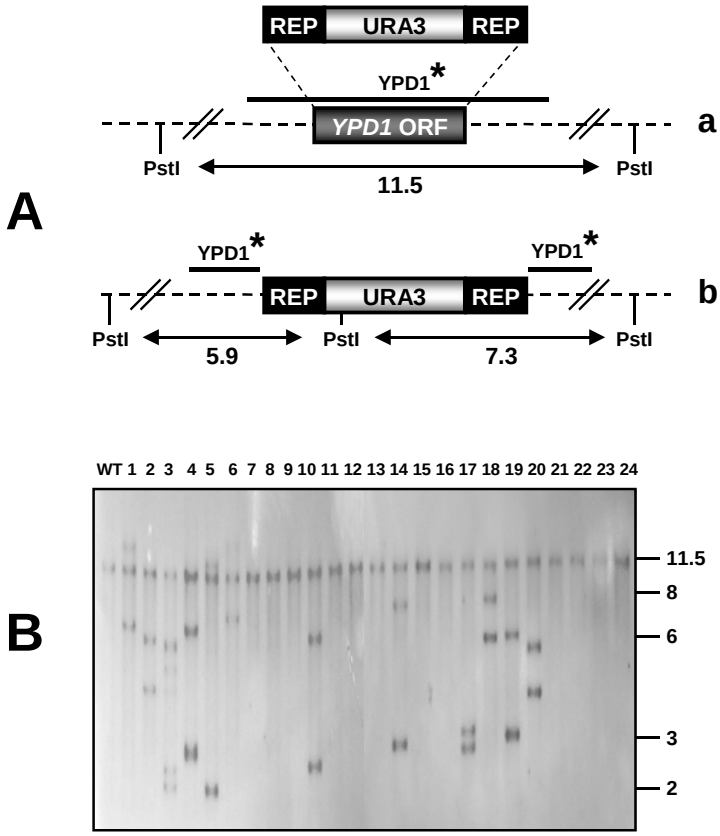

FIG. S3. Southern blot hybridization and schematic representation of resident locus *YPD1*. (A) Expected molecular events from the genomic restriction map. (B) The hybridization pattern is shown with YPD1 probe of PstI-digested genomic DNA from 6936 *ura3*$_{[\Delta 360]}$ (WT) and 24 Ura^{+} randomly selected transformants. Transformants derived from ectopic integrations of the disruption cassette (strains 7, 8, 9, 11, 12, 13, 15, 16, 21, 22, 23, 24) or from gene replacement at the *ura3* locus (strains 1, 2, 3, 4, 5, 6, 10, 14, 17, 18, 19, 20). For each transformant, the *YPD1* locus is not affected (11.5 kb DNA fragment). DNA fragment sizes are indicated in kilobases.