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The *Kluyveromyces lactis KEX1* gene encodes a subtilisin-type serine proteinase

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KEX1 is a chromosomal gene required for the production of the killer toxin encoded by the linear DNA plasmid pGKL-1 of *Kluyveromyces lactis*. The nucleotide sequence of the cloned *KEX1* gene has been determined. The deduced structure of the KEX1 protein, 700 amino acids long, indicated that it contained an internal domain with a striking homology to the sequences of the subtilisin-type proteinases, and a probable transmembrane domain near the carboxyl terminus. The results confirm the hypothesis that the product of the gene *KEX1* of *K. lactis* is a proteinase involved in the processing of the toxin precursor.

Killer toxin; Protein processing; Nucleotide sequence

1. INTRODUCTION

The killer toxin of *Kluyveromyces lactis* is encoded by the linear DNA plasmid pGKL-1 and secreted to the culture media. The *kex1* mutation of the host chromosome [1] leads to a non-killer phenotype. We have previously cloned the *KEX1* gene, and demonstrated, by in vivo complementation, that this gene was functionally related to the *KEX2* gene [2] of *Saccharomyces cerevisiae* [3]. The latter is known to code for a proteinase which converts the precursor proteins of M1 toxin and α-factor into mature molecules which are secreted [4–6]. Comparison of the amino acid sequence of the *K. lactis* toxin subunits [7] and the killer plasmid gene sequence [8,9] also suggested that a KEX2-type enzyme should be involved in the processing of the toxin precursor in *K. lactis*. The sequence of the *KEX1* gene confirmed this expectation.

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The nucleotide sequence presented here has been submitted to the EMBL/GenBank database under the accession number X07038

2. MATERIALS AND METHODS

2.1. *Strains*

The *K. lactis* (*K. marxianus* var. *lactis* [10]) strains used have been previously described [3,11].

2.2. *DNA sequencing*

DNA fragments of the *KEX1* gene region were subcloned into the multi-functional vectors pTZ18R and pTZ19R (Pharmacia). Serial deletions of cloned DNA were generated according to Lin et al. [12]. Single-stranded DNA was sequenced by the dideoxy chain termination method [13].

2.3. *RNA analysis*

Total RNA was extracted from the strain 2359/152. Poly(A)$^+$ RNA was isolated by two passages over an oligo(dT)-cellulose (BRL) column, essentially as described in Maniatis et al. [14]. RNA was electrophoresed through an agarose gel containing formaldehyde, transferred to a nitrocellulose membrane, then hybridized with probe according to Maniatis et al. [14].

3. RESULTS AND DISCUSSION

3.1. *Nucleotide sequence of the* KEX1 *gene of* K. lactis

The *KEX1* gene has previously been identified in a *K. lactis* DNA bank by complementation of the *kex1* mutation [3]. The complementing region of the cloned fragment contained a single large open reading frame of 2100 bp. A radioactive probe

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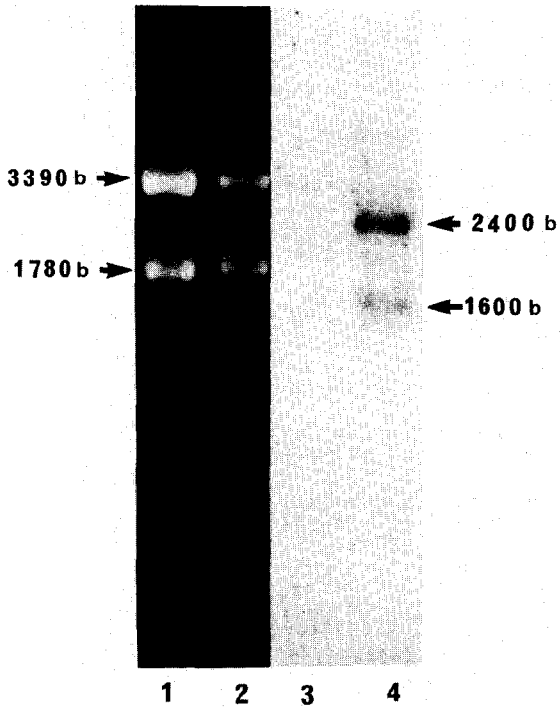

Fig.1. Northern blot analysis of the *KEX1* gene mRNA. Molecular mass markers are cytoplasmic ribosomal RNAs of *K. lactis*. Lane contents: lanes 1 and 3, 15 μg total RNA; lanes 2 and 4, 10 μg *K. lactis* poly(A^+) enriched RNA. Lanes 1 and 2 show ethidium-stained RNA; lanes 3 and 4, hybridised RNA.

containing 80% of the cloned *KEX1* gene and a flanking 1 kb fragment was used to detect the *KEX1* transcript (fig.1). Hybridization with poly$(A)^+$ enriched RNA detected a major RNA of 2400 bases, consistent with the length of the open reading frame. A weak signal of a 1600 base-long RNA was also detected, which corresponded to a second gene starting 800 bp downstream of the *KEX1* gene (not studied).

The DNA sequence of the *KEX1* gene and its flanking regions is shown in fig.2. The deduced protein sequence contained three methionine codons in the N-terminal region. If we assume that the first one is the translational start codon, the 5′-leader region of the gene shows a few typical features of a yeast gene: a TATA box (−188 to −185), an almost canonical CAAT box (−115 to −106) and a purine at −3. The 3′-flanking region is AT rich and has several termination codons in frame.

3.2. *Amino acid sequence of the KEX1 product*

The open reading frame can code for a protein of 700 amino acids. The main features of this putative protein are summarized in fig.3. The hydrophobicity profile [15] reveals three major hydrophobic regions: the 18 amino acid residues at the amino-terminus, the 14 amino acids long central segment and the largest hydrophobic region near the carboxyl-terminus. This last region presents several characteristics of the membrane-spanning domain found in transmembrane proteins [15,16]. This sequence is immediately followed by a short stretch of positively charged amino acids. This structure is supposed to play a role in the anchoring of the protein in the membrane [17]. The supposed transmembrane domain is also preceded by clusters of serine and threonine residues (603–643). This domain is thought to serve as attachment sites for *O*-linked carbohydrate chains [16]. There are also five potential *N*-glycosylation sites (Asn-X-Thr/Ser) in the KEX1 protein.

3.3. *The* KEX1 *encoded protein has a domain highly homologous to the subtilisin-type proteinases*

The deduced amino acid sequence of the KEX1 protein showed a region of striking homology with a family of serine proteases: subtilisin BPN′ from *Bacillus amyloliquefaciens* [18], thermitase from *Thermoactinomyces vulgaris* [19] and the alkaline extracellular protease AEP from *Yarrowia lipolytica* [20]. Fig.4 is an optimized sequence comparison of the four proteins. Maximum of homology was observed around the essential amino acids of the active site (corresponding to Asp-164, His-202, Ser-373 of the KEX1 sequence). The residues of the active site triad are situated at about the same relative positions in all four proteins. In addition, the KEX1 sequence contained a cysteine (Cys-206) localized 5 residues after the active site His, as in the case of thermitase and proteinase K from *Tritirachium album* [19].

The KEX1 sequence is much longer than the other three enzymes compared, but the homology is localized exclusively within the 155–388 region, without significant expansion or deletions.

Although the nucleotide sequence of the *KEX2* gene of *S. cerevisiae* has not been published, the structure of the KEX1 protein deduced from the

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                                                                -221       -211
                                                              TTCTATCG AATCGGGTGC

      -201       -191       -181       -171       -161       -151       -141
TCTTCATGTG TTACACGTCT TTTATACAGC ATAAAAATAA AGGCCATTCC AAAAAGTTGT ACAATACTAA

      -131       -121       -111       -101        -91        -81        -71
GGGTCTAGTA CAGCTAGACA AATTAGGTGC AATCTCTAAA TCAGGATATC AGCTCTACGC CGGGCAAGTC

       -61        -51        -41        -31        -21        -11         -1
ATTGAATAAG ATTTTCCACT TACTTATTAC CTTTTTCCCT TAATATTCCT TAATTTTCAG AACGATAGTT

                  15                  30                  45                  60
MET Ile Leu Ser Ser Gln Leu MET Leu Ala Leu Ile Ala Val Ser Gly Tyr Gly Lys Ala
ATG ATC CTA TCG TCG CAG CTC ATG CTA GCT TTA ATA GCA GTG TCA GGA TAC GGT AAA GCA

                  75                  90                 105                 120
MET Gln Val Pro Lys Lys Asp His Glu Asn Arg Gln Tyr Phe Ala Ile Glu Ser Tyr Asp
ATG CAA GTT CCT AAA AAA GAC CAC GAA AAT AGG CAG TAT TTT GCA ATT GAA TCT TAT GAT

                 135                 150                 165                 180
Asp Val Gly Asn Leu Leu Ala Glu His Ser Asp Trp Ser Phe Glu His Asp Val Arg Gly
GAT GTA GGT AAT CTA CTA GCG GAA CAC AGT GAC TGG AGT TTC GAG CAC GAT GTT CGA GGC

                 195                 210                 225                 240
Leu Ala Asn His Tyr Val Phe Ser Lys Pro Leu Gln Ser Leu Gly Lys Arg Asp Ala Ile
CTT GCC AAT CAC TAT GTG TTC TCG AAA CCG TTG CAG AGT TTG GGT AAA CGA GAT GCG ATT

                 255                 270                 285                 300
Asp Thr Gly Tyr Ser Glu Asn Ile Ile Asp Phe His Asp Leu Pro Pro Val Gln Leu His
GAC ACA GGA TAT TCA GAA AAC ATC ATT GAT TTC CAC GAT CTA CCC CCC GTT CAG TTA CAC

                 315                 330                 345                 360
Lys Arg Leu Pro Ile Gly Asp Ser Ser MET Glu Gln Ile Gln Asn Ala Arg Ile Leu Phe
AAA AGA TTG CCT ATT GGG GAT TCT AGT ATG GAA CAA ATC CAG AAC GCT AGA ATT CTT TTC

                 375                 390                 405                 420
Asn Ile Ser Asp Pro Leu Phe Asp Gln Gln Trp His Leu Ile Asn Pro Asn Tyr Pro Gly
AAT ATT TCT GAT CCA TTG TTT GAT CAG CAG TGG CAC TTG ATC AAT CCA AAC TAC CCT GGA

                 435                 450                 465                 480
Asn Asp Val Asn Val Thr Gly Leu Trp Lys Glu Asn Ile Thr Gly Tyr Gly Val Val Ala
AAT GAC GTT AAC GTA ACT GGT TTA TGG AAA GAA AAC ATC ACT GGC TAT GGT GTA GTG GCA

                 495                 510                 525                 540
Ala Leu Val Asp Asp Gly Leu Asp Tyr Glu Asn Glu Asp Leu Lys Asp Asn Phe Cys Val
GCA TTG GTG GAT GAT GGA TTG GAT TAT GAG AAC GAA GAT TTA AAA GAC AAT TTC TGT GTT

                 555                 570                 585                 600
Glu Gly Ser Trp Asp Phe Asn Asp Asn Asn Pro Leu Pro Lys Pro Arg Leu Lys Asp Asp
GAA GGT TCT TGG GAT TTT AAT GAC AAC AAC CCA TTG CCG AAG CCA AGG CTA AAA GAT GAT

                 615                 630                 645                 660
Tyr His Gly Thr Arg Cys Ala Gly Glu Ile Ala Ala Phe Arg Asn Asp Ile Cys Gly Val
TAC CAT GGT ACC CGC TGC GCA GGT GAA ATA GCG GCT TTC CGT AAT GAT ATT TGT GGG GTT

                 675                 690                 705                 720
Gly Val Ala Tyr Asn Ser Lys Val Ser Gly Ile Arg Ile Leu Ser Gly Gln Ile Thr Ala
GGT GTC GCC TAT AAC TCT AAG GTA TCC GGT ATC AGA ATT TTG TCA GGC CAG ATC ACA GCC

                 735                 750                 765                 780
Glu Asp Glu Ala Ala Ser Leu Ile Tyr Gly Leu Asp Val Asn Asp Ile Tyr Ser Cys Ser
GAA GAT GAG GCT GCT TCA TTA ATT TAT GGA CTA GAC GTT AAT GAT ATT TAC TCT TGC TCG

                 795                 810                 825                 840
Trp Gly Pro Ser Asp Asp Gly Lys Thr MET Gln Ala Pro Asp Thr Leu Val Lys Lys Ala
TGG GGT CCA TCT GAT GAC GGT AAA ACT ATG CAA GCG CCG GAT ACA TTA GTA AAA AAG GCA

                 855                 870                 885                 900
Ile Ile Lys Gly Val Thr Glu Gly Arg Asp Ala Lys Gly Ala Leu Tyr Val Phe Ala Ser
ATC ATA AAA GGT GTA ACA GAA GGA CGA GAT GCA AAA GGT GCA CTA TAT GTA TTT GCG AGT

                 915                 930                 945                 960
Gly Asn Gly Gly MET Phe Gly Asp Ser Cys Asn Phe Asp Gly Tyr Thr Asn Ser Ile Phe
GGG AAT GGT GGT ATG TTT GGC GAC AGC TGC AAC TTT GAC GGC TAC ACA AAC TCT ATA TTT

                 975                 990                1005                1020
Ser Ile Thr Val Gly Ala Ile Asp Trp Lys Gly Leu His Pro Pro Tyr Ser Glu Ser Cys
TCT ATC ACT GTA GGT GCC ATT GAT TGG AAG GGC CTA CAT CCT CCA TAT TCT GAA TCA TGT

                1035                1050                1065                1080
Ser Ala Val MET Val Val Thr Tyr Ser Ser Gly Ser Gly Asn Tyr Ile Lys Thr Thr Asp
TCT GCT GTA ATG GTT GTT ACT TAT TCT TCG GGA TCA GGA AAT TAC ATA AAA ACA ACA GAT

                1095                1110                1125                1140
Leu Asp Glu Lys Cys Ser Asn Thr His Gly Gly Thr Ser Ala Ala Ala Pro Leu Ala Ala
TTA GAC GAA AAA TGT TCC AAT ACG CAT GGA GGC ACT TCA GCT GCA GCT CCT CTT GCA GCT

                1155                1170                1185                1200
Gly Ile Tyr Thr Leu Val Leu Glu Ala Asn Pro Asn Leu Thr Trp Arg Asp Val Gln Tyr
GGT ATA TAT ACT TTA GTG CTG GAA GCT AAC CCG AAC TTA ACA TGG CGA GAT GTA CAA TAC
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                1215                1230                1245                1260
Leu Ser Ile Leu Ser Ser Glu Glu Ile Asn Pro His Asp Gly Lys Trp Gln Asp Thr Ala
CTC TCA ATA TTG AGC TCT GAG GAA ATA AAT CCG CAC GAT GGA AAG TGG CAG GAT ACA GCT

                1275                1290                1305                1320
MET Gly Lys Arg Tyr Ser His Thr Tyr Gly Phe Gly Lys Leu Asp Ala Tyr Asn Ile Val
ATG GGA AAG CGT TAT TCT CAC ACA TAT GGA TTT GGA AAA CTT GAT GCA TAT AAC ATT GTC

                1335                1350                1365                1380
His MET Ala Lys Ser Trp Ile Asn Val Asn Pro Gln Gly Trp Leu Tyr Leu Pro Thr Ile
CAT ATG GCA AAA AGT TGG ATC AAT GTA AAC CCA CAA GGT TGG CTT TAC CTT CCT ACA ATC

                1395                1410                1425                1440
Val Glu Lys Gln Ser Ile Ser Asn Ser Asp Glu Val Ile Glu Ser Thr Val Ser Val Ser
GTT GAA AAA CAG TCT ATC AGT AAT TCA GAT GAA GTT ATA GAA TCC ACA GTC TCA GTT TCT

                1455                1470                1485                1500
Ala Glu Glu Phe Lys Gln Asn Asn Leu Lys Arg Leu Glu His Val Thr Val Thr Val Asp
GCT GAA GAG TTT AAA CAA AAT AAC CTA AAA AGG TTG GAA CAT GTC ACT GTA ACT GTC GAT

                1515                1530                1545                1560
Ile Asp Ala Pro Tyr Arg Gly His Val Leu Val Asp Leu Ile Ser Pro Asp Gly Val Thr
ATA GAC GCA CCT TAC CGT GGA CAT GTC TTA GTA GAT CTA ATA TCG CCT GAT GGA GTT ACA

                1575                1590                1605                1620
Ser Thr Leu Ala Thr Ala Arg Arg Leu Asp Lys Asn Arg Tyr Gly Phe Gln Asn Trp Thr
TCT ACC TTA GCG ACA GCT AGA CGT TTA GAT AAA AAC CGC TAT GGT TTT CAA AAT TGG ACT

                1635                1650                1665                1680
Phe MET Ser Val Ala His Trp Gly Ser Ser Gly Val Gly Ser Trp Lys Leu Lys Val Lys
TTC ATG TCT GTC GCG CAC TGG GGC TCT AGT GGA GTT GGA AGC TGG AAA TTA AAA GTA AAG

                1695                1710                1725                1740
Ser Thr His Asp Asn Glu Ile Val Thr Leu Lys Ser Trp Arg Leu Lys MET Phe Gly Glu
TCT ACG CAT GAT AAT GAA ATT GTA ACA CTC AAA TCT TGG AGA TTA AAG ATG TTT GGA GAA

                1755                1770                1785                1800
Thr Ile Asp Ala Lys Lys Ala Lys Val Ile Ser Tyr Gly Asn Asp Lys Glu Asp Ala Glu
ACT ATC GAT GCA AAG AAG GCC AAA GTG ATA TCA TAT GGA AAT GAC AAA GAG GAT GCT GAA

                1815                1830                1845                1860
Val Lys Ser Thr Glu Ser Lys Thr Thr Thr Pro Thr Ala Gln Thr Ser Ser Phe Thr Thr
GTT AAG AGT ACC GAA TCT AAA ACC ACA ACT CCC ACT GCA CAA ACT TCG TCA TTC ACG ACG

                1875                1890                1905                1920
Thr Ser Gly Glu Glu Thr Ser Gly Ala Asn Lys Leu Pro Arg Pro Glu Gln Ala Ala Gln
ACT TCT GGA GAA GAA ACA TCT GGT GCA AAT AAG TTG CCT CGT CCC GAA CAG GCT GCC CAG

                1935                1950                1965                1980
Leu Tyr Leu Ala Ile Phe Val Ile Gly Ala Ile Val Ile Ile Ile Tyr Tyr Leu Phe Phe
TTA TAC TTG GCA ATT TTT GTC ATT GGT GCG ATA GTC ATC ATA ATT TAC TAT TTG TTT TTC

                1995                2010                2025                2040
Leu Lys Ser Arg Arg Ile Ile Arg Arg Ser Arg Ala Glu Ala Tyr Glu Phe Asp Ile Ile
TTA AAA TCA AGA AGA ATA ATC AGA AGG TCT AGA GCA GAA GCT TAT GAA TTT GAT ATC ATT

                2055                2070                2085                2100
Asp Thr Asp Ser Glu Tyr Asp Ala Ser Ile Asn Lys Leu Gln Ser Leu Tyr Leu Val Lys
GAT ACC GAC TCA GAA TAC GAT GCT TCG ATT AAC AAA CTG CAG AGT CTA TAT CTG GTG AAG

                2115                2130                2145                2160

TAA ATG ATG ATA ACC TTG AAG ACT TTA ACT TCG ACA TAA ATG AAG AAG AGC TCT CAC CCC

                2175                2190                2205                2220

GTG AAA GTT CAA GCA ATA ATC CTT CGG GAA TGA ATC TCT GGA ATC TTT CGA CAT CTC CTG

                2235                2250                2265                2280

ATC ATA CAA GCA ATT TAC TAG GGC AAA ACT CGA TTC CCA ACA AAT AGA AAG ACA TAG CAT

                2295                2310                2325                2340

AAC AGT TCA TAA GAG ATA AAT CTC AAA ATA CTG ACG TTT TCA TAT AGA AGA TAG GTT TTT
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Fig.2. Nucleotide sequence of the *KEX1* gene of *K. lactis*. The amino acid sequence of the coding region, predicted from the nucleotide sequence, is given above the nucleotide sequence. Probable TATA and CAAT sequences are underlined.

gene sequence appears to have an overall organization analogous to that reported for the KEX2 protein [5,21].

3.4. *The* kex1 *mutation in* K. lactis *is concerned with the secretion of the killer toxin*

Fig.5 illustrates the absence of the bands corresponding to the toxin subunits in the culture fluid of a *kex1* mutant, and the restoration of the

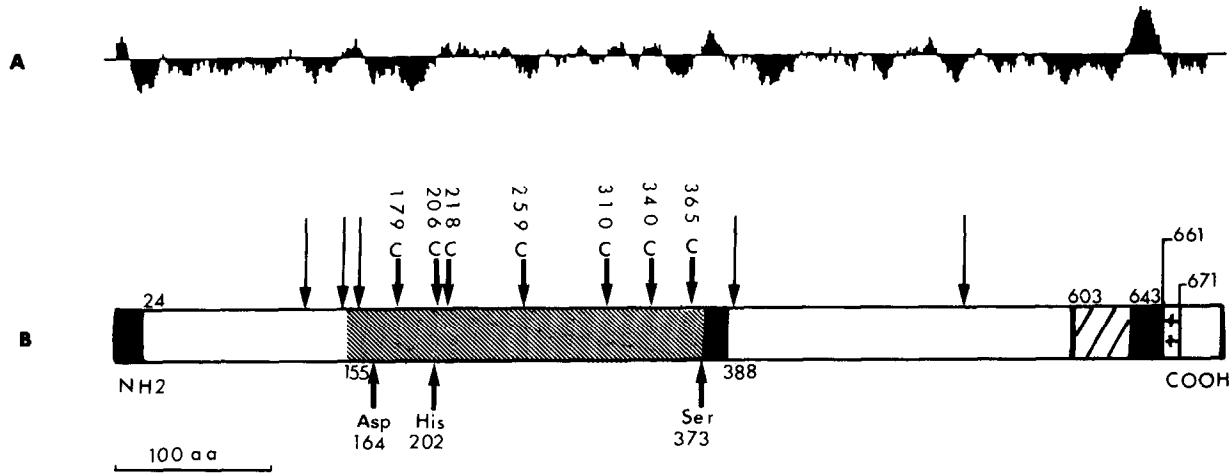

Fig.3. Main structural features of the deduced KEX1 gene product. (A) Hydropathy profile of the KEX1 product; (B) structure of the KEX1 protein. (■) Hydrophobic domains; (▩) region sharing sequence homology with subtilisin-type proteins. The three essential amino acid residues of the potential catalytic site are indicated. Putative *N*-glycosylatable sites are shown by arrows. Cysteine positions are also indicated (c). (▨) Thr-Ser rich region; (⊞) positively charged residues. Numbers indicate the amino acid positions in the deduced protein sequence.

bands in the transformant clones. The β- and γ-subunits can be seen on this gel; the α-subunit was visualized on another gel (not shown). The wild-type killer strain MW98-8C and the isonuclear plasmid-less mutant strain VD1, were used to identify the toxin subunits.

Biological roles of the *K. lactis KEX1* gene remain to be studied further. It is known that homozygous *kex1* diploids are deficient for sporulation [3].

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Fig.4. Amino acid homology between the KEX1 gene product of *K. lactis* (K) and a variety of subtilisin-type proteinases. All sequences are aligned with respect to the KEX1 gene product. Numbers to the left and to the right indicate respectively the amino acid in the protein sequence at which the homology starts and ends. The numbers above the sequences correspond to the KEX1 protein. Amino acids homologous to the *KEX1* gene are boxed. Asterisks indicate the essential amino acids of the active site, known for subtilisin. Subt-BPN' (S), subtilisin BPN' from *Bacillus amyloliquefaciens*. Thermitase (T), thermitase from *Thermoactinomyces vulgaris*. AEP lipolytica (A), alkaline extracellular protease of *Yarrowia lipolytica*.

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(For fig.5, see overleaf.)

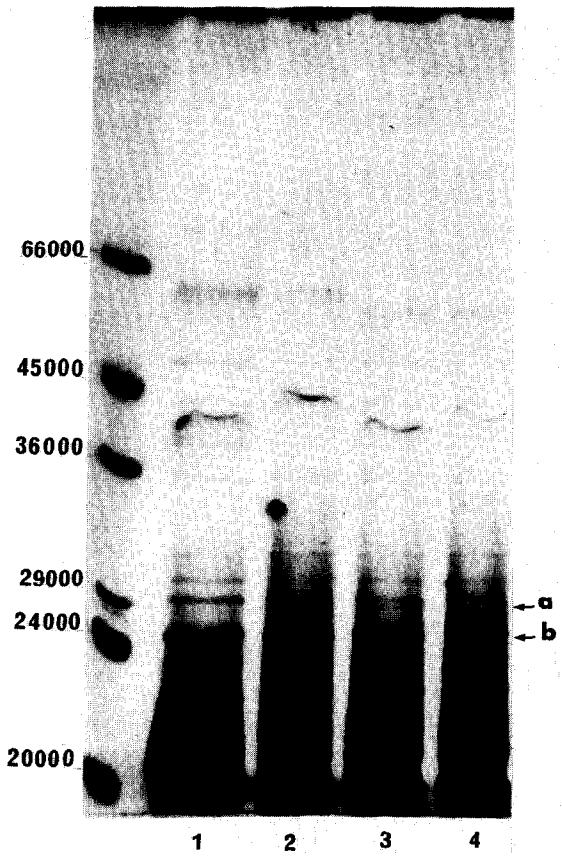

Fig.5. Secreted polypeptides in the wild-type and the *kex1* mutant strains. Polypeptides secreted into the culture medium were tested by SDS-polyacrylamide gels electrophoresis containing 11% acrylamide. Using Minicon B15 membranes (Amicon) culture supernatants were concentrated approx. 50-fold before loading 10 μl in each gel-slot. The gel was silver stained according to Merril et al. [22]. The positions of the molecular mass markers are indicated. Lanes: 1, MW105-2D, *kex1* mutant strain; 2, MW105-2D strain, transformed with KEp6-pc7M plasmid carrying *KEX1* gene [3]; 3, MW98-8C wild-type killer strain; 4, MW98-8C without killer plasmids (VD1). a = β-subunit; b = γ-subunit.