APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1995, p. 1469–1474 Vol. 61, No. 4
0099-2240/95/$04.00+0

Nucleotide Sequence and Expression of *kerA*, the Gene Encoding a Keratinolytic Protease of *Bacillus licheniformis* PWD-1

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Received 9 November 1994/Accepted 17 January 1995

***Bacillus licheniformis* PWD-1 (ATCC 53757) secretes keratinase, a proteolytic enzyme which is active on whole feathers. By amino acid sequence similarity and phenylmethylsulfonyl fluoride inhibition, the keratinase was demonstrated to be a serine protease. The entire nucleotide sequence of the coding and flanking regions of the keratinase structure gene, *kerA*, was determined. A fixed oligonucleotide primer derived from the N-terminal sequence of the purified enzyme and a second random oligonucleotide primer were used in a procedure called PCR walking, which was developed to amplify and sequence the upstream and downstream regions of *kerA*. Another method, PCR screening, was conducted with a λ phage vector with inserted PWD-1 genomic DNA fragments as templates and with the known sequences of the vector arms and the N-terminal sequence of the enzyme as primers. PCR amplification and sequence analysis of the λ library completed the entire *kerA* sequence and established a set of gene deletions. The *kerA* gene shares a 97% sequence identity with the gene encoding subtilisin Carlsberg from *B. licheniformis* NCIMB 6816. The putative promoters, ribosome binding sites, and transcriptional terminators are also similar in these two bacteria. The deduced amino acid sequences indicate only three amino acid differences between the two mature proteases. Northern (RNA) analysis demonstrates that transcriptional regulation controls *kerA* expression on different growth media.**

The isolation and characterization of a feather-degrading bacterium, *Bacillus licheniformis* PWD-1, from a poultry waste digester have previously been reported from this laboratory (13, 21). This bacterium is capable of growing on feathers because of its secretion of a keratinolytic protease, keratinase. Keratinase has been purified, and its enzymatic properties have been characterized (8). Purified keratinase demonstrated considerable thermostability; it hydrolyzes whole feather and a broad range of other protein substrates. These features of the enzyme provide potential applications in feed technology (12), waste management, and fundamental studies of keratinolysis.

Many *Bacillus* serine proteases have been sequenced, cloned, and characterized (7, 14, 16–19). Sequence data demonstrate that the members of the subtilisin family of serine proteases share significant sequence and structural similarities. In this report, we describe evidence that keratinase is also a serine protease and detail the isolation and characterization of the keratinase structural gene, *kerA*, from *B. licheniformis* PWD-1. Two specific methods, PCR walking and PCR screening of a genomic library, were developed for the isolation and sequence analysis of the gene. Northern (RNA) blot analysis was used to study *kerA* expression.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. licheniformis* PWD-1 (ATCC 53757), which was used in this study, is a patented strain described previously (13, 21). It was grown in nutrient broth (Difco Laboratories, Detroit, Mich.) or on feather medium (NaCl, 0.5 g/liter; $MgCl_2 \cdot 6H_2O$, 0.1 g/liter; $CaCl_2$, 0.06 g/liter; KH_2PO_4, 0.7 g/liter; K_2HPO_4, 1.4 g/liter; feather, 10 g/liter) at 50°C. *Escherichia coli* INVαF′ (Invitrogen Corporation, San Diego, Calif.) was grown at 37°C on Luria-Bertani medium supplemented with ampicillin (50 μg/ml).

Keratinase assays and PMSF inhibition. Keratinase was purified as described previously by Lin et al. (8). The activity of keratinase was determined by measuring free amino groups released during enzymatic proteolysis. In 1.0 ml of buffer (0.05 M phosphate buffer, pH 7.5), 16 μg of purified keratinase, which was approximately 0.5 μM on the basis of its molecular weight, was reacted with 10 mg of feather keratin or casein as the substrate. The serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (0 to 5 μM) was added to the reaction mixtures, and specific activity and inhibition were determined.

Amino acid sequence analysis. Purified intact and CNBr-cleaved keratinases (5) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (6) and then were electrotransferred onto polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, Calif.) (2). Protein bands were stained with Coomassie blue and excised for N-terminal amino acid sequence analysis by Edman degradation (4).

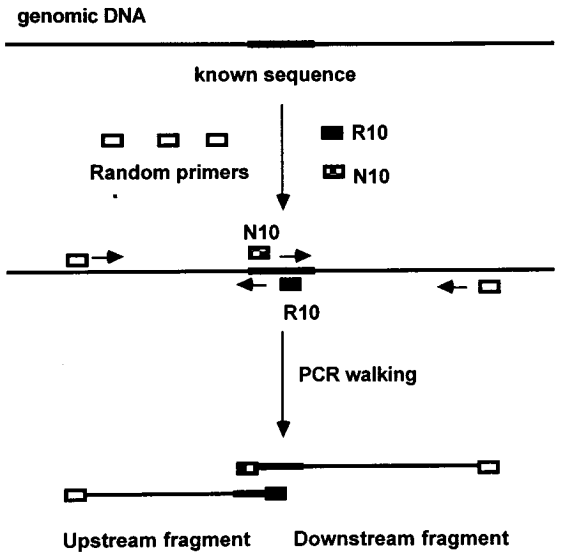

FIG. 1. PCR walking (see Table 1 for the N10 and R10 probe sequences).

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TABLE 1. PCR primers and probe

Type	Sequence[a]
Amino acid	NH_2-A Q T V P Y G I P L I K A D K V Q A Q G F K . . .
	1 20 40 60
DNA: 5′ end	5′GCGCAAACCGTTCCTTACGGCATTCCTCTCATTAAAGCGGACAAAGTGCAGGCTCAAGGCTTTAAG3′
N10	5′GCGCAAACCG→
N25	5′ CAAACCGTTCCTTACGGCATTCCTC→
R10	←CGCCTGTTTC5′ (antisense sequence)

[a] Arrows indicate the direction of the newly synthesized DNA strand that would result if that oligonucleotide was used as the PCR primer. The position of each oligonucleotide in the table reflects the location of it in the gene sequence.

Preparation of DNA and RNA. Strain PWD-1 was grown overnight in 100 ml of nutrient broth medium at 50°C, and the genomic DNA was extracted by standard methods (1). The DNA was further purified by CsCl gradient centrifugation and was stored in Tris-EDTA buffer.

Total RNA of strain PWD-1 was extracted by the method of Ausubel et al. (1). Cells for RNA isolation were collected from feather medium at 24 h and from nutrient broth at 12 h, the times at which they were approximately 2 to 4 h before stationary phase in each medium. The feather medium was filtered through cheesecloth to remove feather residues, and the cells were collected by centrifugation. Total nucleic acid was ethanol precipitated and dissolved in distilled water. Samples were treated with DNase I (100 U/ml) at 37°C for 1 h. Total RNA was ethanol precipitated and suspended in distilled water. All buffers and distilled water used in RNA isolation were treated with diethyl pyrocarbonate to inhibit RNase activity.

PCR walking. The method is presented schematically in Fig. 1. On the basis of the N-terminal keratinase sequence, which is identical with the published sequence of mature subtilisin Carlsberg (7), the same DNA sequence of 66 nucleotides was determined by this method, and oligonucleotide N10 was synthesized (see Table 1 for details of primer and probe design). A series of 20 random decanucleotides (10-mers) were selected to serve as distal PCR primers. Primer N10 was paired with individual random primers in the following PCR reactions (26 μl): 0.3 μM (each) primer, 200 μM (each) deoxynucleoside triphosphate, 5 μM Tris-HCl (pH 8.0), 10 μM NaCl, 1.5 μM $MgCl_2$, 10 μM EDTA, 0.5 μM dithiothreitol, 5% glycerol, 0.1% Triton X-100, 40 ng of PWD-1 genomic DNA as template, and 1.7 U of *Taq* DNA polymerase. The mixtures were overlaid with mineral oil, and the reactions were carried out with the Minicycler (M. J. Research, Inc., Watertown, Mass.). Initial template denaturation was programmed for 2 min at 95°C. Thereafter, the cycle profile was programmed as follows: 1 min at 35°C (annealing), 1.5 min at 72°C (extension), and 45 s at 92°C (denaturation). This profile was repeated for 35 cycles, and the final 72°C extension step was increased to 5 min. The reaction mixture was subjected to 1.2% agarose gel electrophoresis. To identify the bands amplified by the paired fixed primers, single primer reactions as controls were carried out by the same procedure.

PCR walking was also used to isolate the region upstream of *kerA*. An antisense primer, R10, was synthesized and paired with individual random primers in the PCR. Oligonucleotide N25 (Table 1), located between N10 and R10, was used to probe for both 5′ and 3′ sequences among PCR products. Oligonucleotides were synthesized by Keystone Laboratories, Inc. (Menlo Park, Calif.), and random 10-mers were purchased from Operon Technologies, Inc. (Alameda, Calif.). All enzymes were from Promega Corporation, Inc. (Madison, Wis.).

PCR screening of λ clones. The method is shown in Fig. 2. PWD-1 genomic DNA was partially digested with restriction enzyme *Sau*3A1 (5′-GATC-3′) and then ligated to *Xho*I-digested λ GEM-11. For PCR analysis of the library DNA (7.5 to 300 pg of DNA used), oligos N25 (Table 1) and T7 (5′-TAATACGACT CACTATAGGG-3′) or SP6 (5′-GATTTAGGTGACACTATAG-3′) were used as primers. The other components used in the reaction mixture (26 μl) were the same as those used in PCR walking. After initial denaturation at 94°C for 1 min, the cycle profile was 2 min at 50°C, 2 min at 72°C, and 1 min at 92°C (35 cycles and a final 5-min extension at 72°C).

Southern and Northern blotting. The oligonucleotide probe N25 was 5′ end labelled with [γ-^{32}P]ATP (10). PCR products were subjected to 1.2% agarose gel electrophoresis and then were blotted onto nylon membrane. Hybridization was carried out at 60°C for at least 16 h (15), and washing was done by standard protocol (10) at 60°C.

Total RNA isolated from cells grown on feather medium or nutrient broth was subjected to electrophoresis on 1.0% agarose gel with 6% formaldehyde and

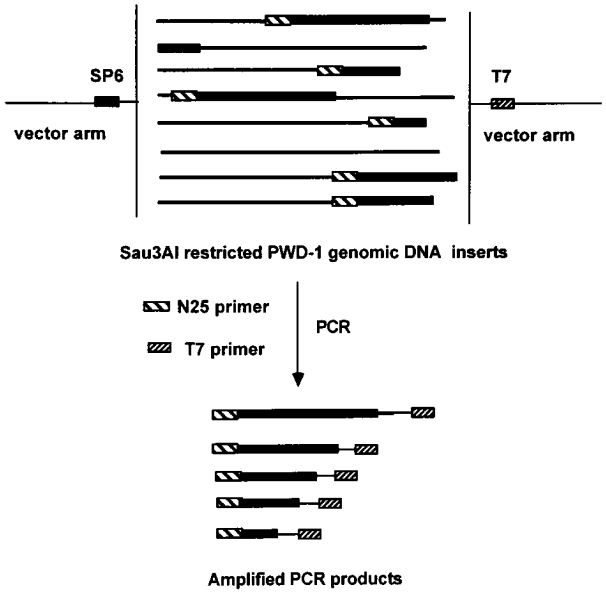

FIG. 2. PCR screening of the λ library (see Materials and Methods for the SP6 and T7 promoter sequences and Table 1 for the N25 sequence).

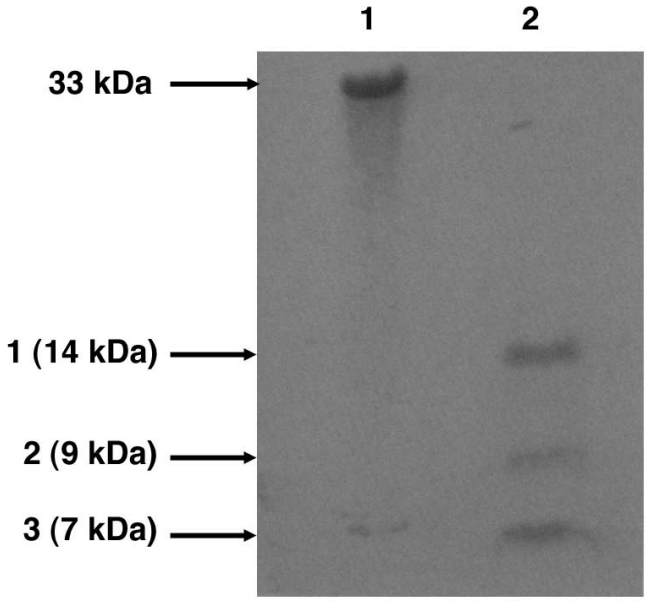

FIG. 3. SDS gel electrophoretic analysis of keratinase and its CNBr-cleaved fragments. Lane 1, intact keratinase; lane 2, CNBr-cleaved keratinase fragments. The N-terminal amino acid sequences are as follows: 1, H_2N-AQTVPYGIP LIKADKVQAQGFK . . .; 2, H_2N-KQAVDNAYARGVVVVAAA . . .; 3, H_2N-ASP. . . .

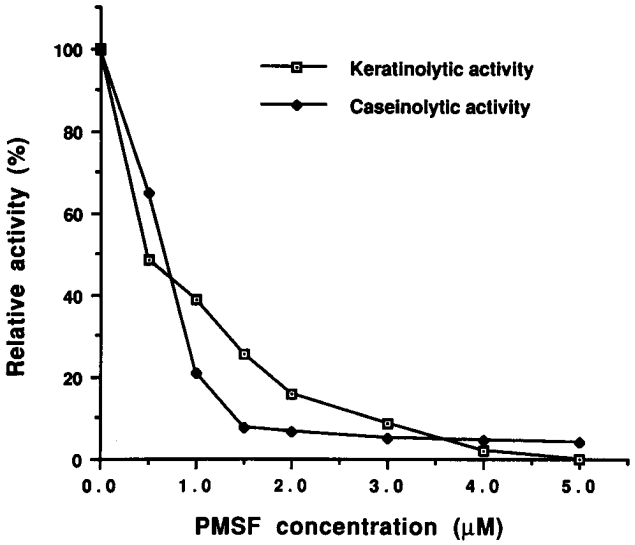

FIG. 4. Inhibition of keratinase by PMSF.

then was blotted onto nylon membrane. A 683-bp *kerA* fragment probe generated by PCR walking was labelled with [α-^{32}P]dATP by nick translation. Hybridization was carried out at 60°C for 16 h, and the membrane was washed by standard protocol (10) at 60°C.

Cloning and sequencing. Products from either PCR walking or PCR screening of λ clones were directly ligated to plasmid pCRII (Invitrogen Corporation) and transformed into *E. coli* INVαF′ competent cells. The standard procedures of the manufacturer were used throughout.

Putative *kerA* clones were screened by PCR with primers complementary to vector DNA flanking the cloning site, and then the products were detected with the 683-bp *kerA* probe. DNA sequences were determined by the dideoxy cycle sequencing method (11) on a model ABI 373 automated DNA sequencer (DNA Sequencing Laboratory, University of Florida, Gainesville).

RESULTS

CNBr cleavage and sequence analysis of purified keratinase. CNBr-cleaved keratinase showed three major polypeptides by SDS-PAGE (Fig. 3). These three bands, along with the intact keratinase, were excised from the gel and subjected to amino acid sequence analysis. The results demonstrated that fragment 1 and intact keratinase share the same N-terminal sequence; fragments 2 and 3 displayed internal sequences (see the legend to Fig. 3). Each of these amino acid sequences of keratinase were found to be identical to portions of the published amino acid sequence for subtilisin Carlsberg (7).

Inhibition of keratinase by PMSF. The identity of the amino acid sequences of the keratinase fragments with that of subtilisin Carlsberg suggested that keratinase is a member of the subtilisin family of serine proteases, which are readily inhibited by PMSF. When PMSF was added to keratinase enzyme reactions with either feather keratin or casein as the substrate, proteolytic activity was inhibited (Fig. 4). At an inhibitor-to-enzyme (PMSF-to-keratinase) molar ratio of 2:1 ([PMSF] = 1.0 μM), the keratinolytic and caseinolytic activities of the enzyme remained at 38.8 and 21.2%, respectively; at a 6:1 ratio ([PMSF] = 3.0 μM), the remaining activities were 8.5 and 5.2%. No keratinolytic activity was observed with PMSF at or above 5 μM.

Isolation of *kerA* by PCR walking. PCR with a fixed primer (N10) in conjunction with specific random primers yielded prominent amplification products that were not observed with reactions with random primers alone. In downstream PCR walking, products from the primer N10-P14 (5′-CCCGCTACAT-3′) reaction hybridized with the N25 probe. After PCR conditions were optimized, a major N25-hybridizing DNA fragment of 683 bp was obtained (Fig. 5). In upstream PCR walking, a 575-bp N25-hybridizing fragment was identified from the R10-P15 (5′-CTCCCTGCCAA-3′) primed reaction (Fig. 6). Both fragments were isolated and cloned into plasmid pCRII for DNA sequence analysis (see below).

PCR screening of the λ library. With the λ library as the template and N25-SP6 or N25-T7 as the primer, PCR-amplified DNA fragments in the size range of approximately 300 to 2,000 bp were obtained (Fig. 7), and several bands hybridized with the distal 683-bp *kerA* probe. The PCR products from N25-T7 were cloned into plasmid pCRII to generate five groups of recombinant plasmids with different insert sizes (Fig. 8). They were further screened and purified. The largest positive insert (928 bp) was sequenced to confirm the partial *kerA* gene as obtained from PCR walking and to complete the 3′ downstream region. A translation stop codon (TAA) and a potential transcriptional terminator sequence were observed in the 928-bp insert.

***kerA* nucleotide sequence.** All upstream and downstream keratinase gene fragments obtained by PCR walking and PCR

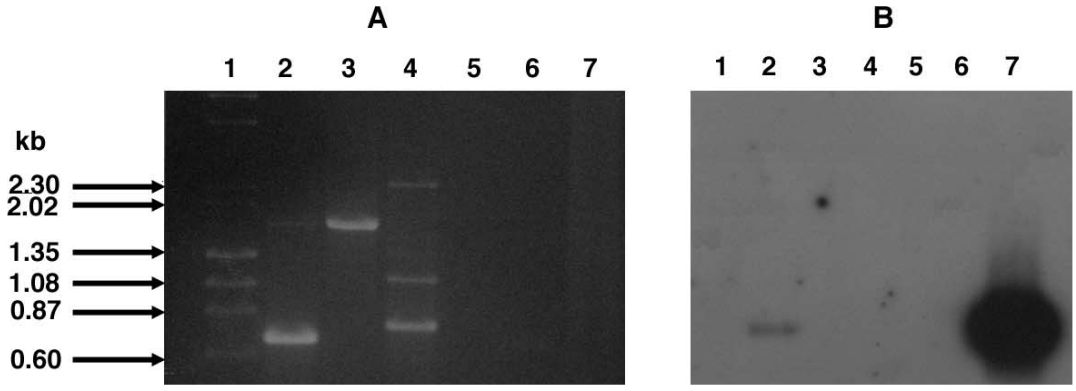

FIG. 5. Downstream PCR walking. Agarose gel (1.2%) electrophoresis of PCR products amplified by N10-P14 (A) and autoradiograph of the same PCR products after they were hybridized with a ^{32}P-labelled N25 probe on a nylon membrane (B). Lane 1, DNA molecular weight markers; lanes 2, 3, and 4, PCR products amplified with N10-P14, N10 alone, and P14 alone, respectively; lane 5, nontemplate DNA control; line 6, non-*Taq* DNA polymerase control; lane 7, PWD-1 genomic DNA completely digested with *Pst*I as positive control. Only lane 2 (N10-P14) is positive for hybridization.

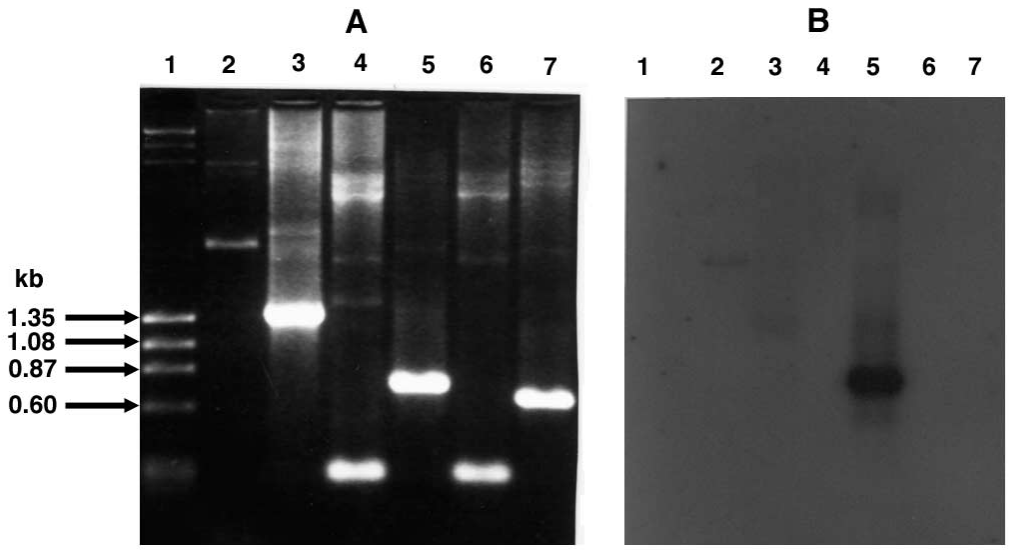

FIG. 6. Upstream PCR walking. Agarose gel (1.2%) electrophoresis of inserted DNA fragments (A) and autoradiograph of the same DNA after it was hybridized with a ^{32}P-labelled N25 probe on a nylon membrane (B). Lane 1, DNA molecular weight markers; lane 2, plasmid pCRII with *kerA* fragment (683 bp) insert as positive control; lanes 3 to 7, amplified inserted DNA fragments with universal primers (SP6-T7 promoter sequences) on plasmid pCRII. The original inserts were amplified with R10 and P8, P10, P15, P20, and P17, respectively. Only lane 5 (R10-P15) is positive for hybridization. The exact size of each DNA fragment on the gel is equal to the insert plus vector portions on both cloning sides which were amplified by universal primers.

screening of the λ library were sequenced. The results are presented in Fig. 9. The determined sequence was confirmed by more than three independent sequencing reactions conducted by both PCR walking and PCR screening of the λ library.

Northern blotting. The 683-bp *kerA* gene fragment from downstream PCR walking was labelled with [α-^{32}P]dATP by nick translation and used as a probe for Northern hybridization. When 30 μg of total RNA isolated from cells grown on feather medium or nutrient broth was employed for Northern blotting only RNA from cells grown on feather medium hybridized to the probe (Fig. 10). Differential synthesis of this 1.3-kb *kerA* RNA demonstrated transcriptional regulation of keratinase synthesis.

DISCUSSION

The PWD-1 keratinase is believed to be a serine protease on the basis of its inhibition by PMSF (Fig. 4) and the demonstrated high sequence similarity between it and subtilisin Carlsberg (Fig. 9). The two enzymes are, however, produced by different strains of *B. licheniformis*, namely, thermophilic PWD-1 (21) and mesophilic NCIMB (formerly NCIB) 6816 (3).

Unlike conventional PCR, which uses two primers of known sequences (9), and random amplified polymorphic DNA (RAPD)-PCR, which uses entirely random primers (20, 22), a new method called PCR walking was employed for gene isolation (Fig. 1). In PCR walking, a known sequence was used as

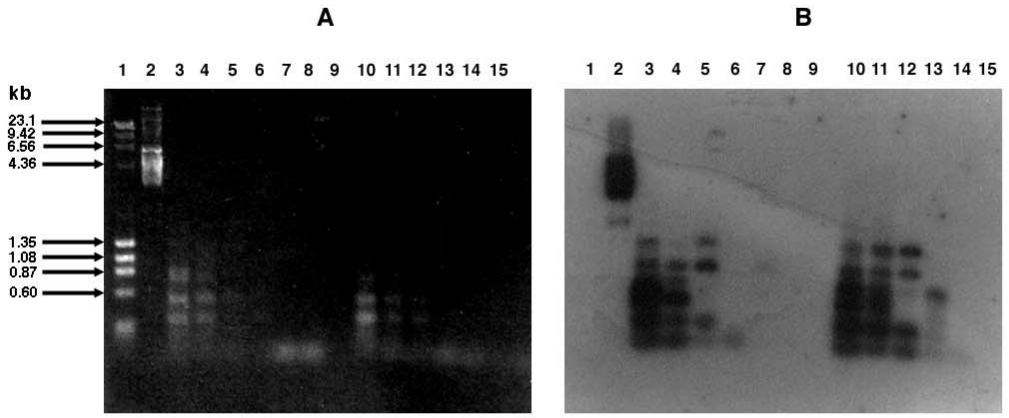

FIG. 7. PCR amplification of potential *kerA* fragments in the λ library with N25-SP6 and N25-T7 primers (A) and autoradiography of the amplified DNA after it was hybridized with the ^{32}P-labelled *kerA* fragment (683 bp) (B). Lane 1, DNA markers; lane 2, plasmid pCRII with the *kerA* fragment (683 bp) as positive control; lanes 3 to 8, DNA amplified with N25-SP6, with 300, 150, 60, 30, 15, and 7.5 pg of recombinant DNA being used as template, respectively; lane 9, no sample; lanes 10 to 15, DNA amplified with N25-T7, with the same amount of DNA template as in the corresponding N25-SP6 reactions being used. The PCR product of lane 11 was cloned into plasmid pCRII for sequence analysis.

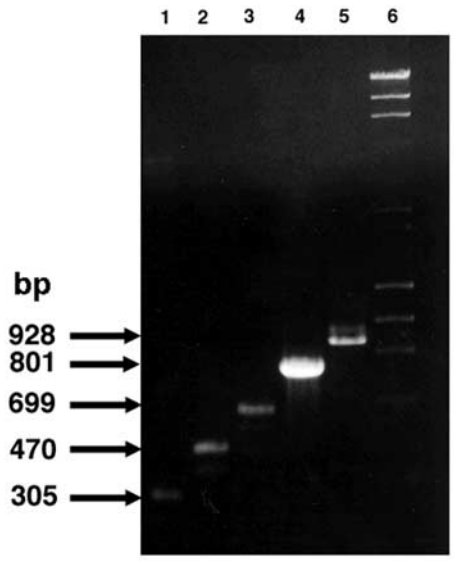

FIG. 8. PCR amplification of five *kerA* fragments in the λ library with primers N25-T7. Lanes 1 to 5 show different sizes of PCR products generated from PCR screening of the λ library. Lane 6 shows molecular weight markers.

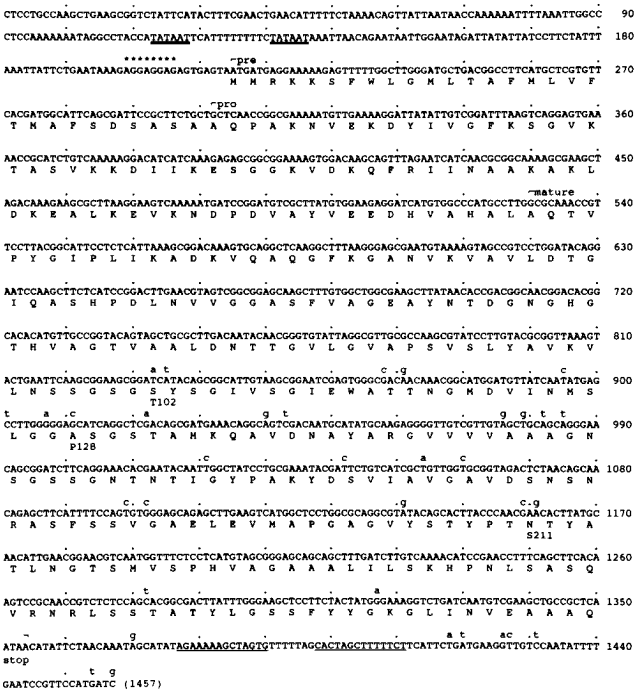

FIG. 9. Nucleotide and amino acid sequences of the keratinase gene. Two putative promoters (double underline), a possible ribosomal binding site (asterisks), and a transcriptional terminator sequence (single underline) are indicated. The nucleotides and amino acids that differ from those of the subtilisin Carlsberg sequence (7) are shown above the nucleotide sequence and under the amino acid sequence, respectively. The putative starting residues of the preprotein (pre), proprotein (pro), and mature protein (mature) are indicated.

a fixed primer in a series of PCRs with individual random primers. The selection of a sequence-specific fixed primer limits the frequency or opportunities of random primer annealing and extension. Amplification of the gene sequence between the fixed and a specific random primer can be monitored by a downstream (in downstream walking) or an upstream (in upstream walking) probe of the fixed primer.

The N-terminal amino acid sequences of keratinase and subtilisin Carlsberg are identical (Fig. 3). On the basis of the known gene sequence of the Carlsberg enzyme, two fixed primers, N10 and R10, were designed for downstream and upstream walking, respectively (Table 1). Primer N10 was derived from the sense strand, and R10 was derived from the antisense strand. Both N10 and R10 extensions can be detected by N25 hybridization. Moreover, new fixed primers for further downstream and upstream walking could be designed on the basis of the newly determined sequence. PCR walking provides a useful method for gene isolation from a given DNA sequence.

Similarly, sequence analysis was facilitated by PCR screening of the ligation mixture of an unfractionated PWD-1 genomic library. Primer N25, which was selected to target the genomic inserts, was paired with either the SP6 or T7 promoter sequence on the λ vector arms to perform PCR (Fig. 2). Although SP6 and T7 sequences are located at each side of the insert, one of them is suitable for the downstream gene isolation because of the orientation of the insertion.

Five keratinase gene fragments of different sizes were found in the λ library. The sequence results confirmed that all fragments start with the fixed primer N25 and end with the *Sau*3A1 restriction sites (5′-GATC-3′) downstream. A deletion set was established by this approach. Thus, PCR screening of the library not only afforded a method for gene isolation and sequencing but also established a rapid strategy to construct a deletion set of the target gene.

kerA is highly similar (97% identical) to the gene encoding subtilisin Carlsberg of *B. licheniformis* NCIMB 6816 (Fig. 9). Two potential promoter sequences (-TATAAT-) are located at positions 123 to 128 and 141 to 146. A potential ribosome binding site is located 8 bp 5′ to the translation start site (ATG). A potential transcriptional terminator is located 22 nucleotides 3′ of the translational stop codon TAA. The coding region of 1,137 nucleotides, encoding a protein of 379 amino acids organized into preprotein (29 residues), proprotein (76 residues), and mature protein regions (274 residues), corresponds exactly to that determined for subtilisin Carlsberg (7). The active site residues of the catalytic triad Asp-32, His-63, and Ser-220 are conserved.

Five amino acids (Ser-102, Ala-128, Ser-157, Asn-160, and Asn-211) in the mature enzyme differ from residues in subtilisin Carlsberg reported in two earlier publications. In 1985, Thr-102, Pro-128, and Ser-211 were reported in the deduced amino acid sequence based on the gene sequence (7). A 1968 paper (14) made the first report of the amino acid sequence of subtilisin Carlsberg. In the report of that sequence, those three amino acid residues were identical to those of the keratinase sequence obtained here, but the sequence varied at Asn-157 and Ser-160. It is possible that all three are natural variants of this serine protease of *B. licheniformis*. More recently, cloned *kerA* transformed a protease-deficient *Bacillus* sp. into a feather-degrading bacterium (unpublished data). This result further verified that *kerA* encodes the keratinase.

Northern blot analysis demonstrated that *kerA* RNA was detectable only in cells grown on feathers as the sole source of carbon and nitrogen. This suggests that *kerA*, which might associate with the expression of other genes, is expressed specifically for feather hydrolysis. It is not known whether *kerA* transcription or *kerA* mRNA stability is affected by the different growth conditions. Since keratin is an insoluble substrate, the regulation of *kerA* expression is of particular interest.

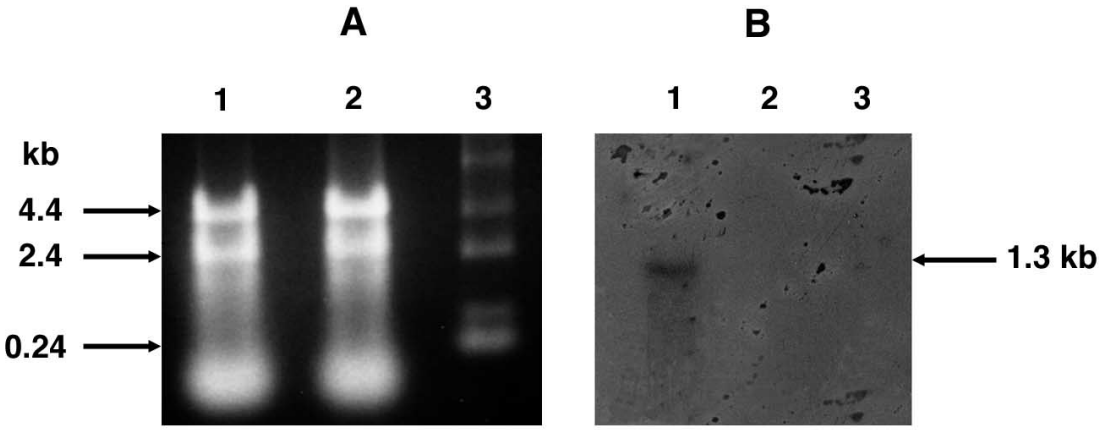

FIG. 10. Northern blot analysis of RNA from strain PWD-1. (A) Electrophoretic analysis of total RNA on 1.0% agarose gel with 6% formaldehyde. (B) Autoradiograph of the RNA after it was hybridized with the ^{32}P-labelled *kerA* fragment (683 bp). Lanes 1 and 2 are loaded with 30 μg of total RNA from cells grown on feather medium and nutrient broth, respectively; lane 3 is RNA markers.

ACKNOWLEDGMENTS

The research was supported by USDA-NRI competitive grant 93-37500-9247.

We thank Christopher S. Lancaster for his expert technical assistance and Steven Li, National Institute of Environmental Health Sciences, for protein sequence analysis.

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