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Cloning of the Genes Coding for Extracellular Proteases from Alkalophilic *Xanthomonas* sp. JK311

Young Hun Kim, Ji Yeon Jang, Yeehn Yeeh[1], Yong Ho Kim[2], and Sang Hae Kim*

Department of Biology, [1]Institute of Basic Science, and
[2]Department of Medical Laboratory Science, Inje University, Kimhae 621-749, Korea

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The alkalophilic bacterium, *Xanthomonas* sp. JK311, producing extracellular proteases, was isolated from soil. *Xanthomonas* sp. JK311 produced five extracellular proteases that are all metalloproteases. Four of them were resistant against 1% SDS. Chromosomal DNA of the *Xanthomonas* sp. JK311 was digested with *Bam*HI and cloned into pUC19. Among *E. coli* strain HB101 transformants, a clone secreting the proteases was screened through halo formation on skim-milk agar plate and by Southern blot analysis. It had the recombinant plasmid pXEP-1 containing the 7.5 kb-*Bam*HI DNA fragment and produced three extracellular proteases. Their protease properties corresponded to those of *Xanthomonas* sp. JK311.

Key words: *Xanthomonas* sp. JK311, alkaline proteases, SDS-resistance, protease gene cloning

Microbial proteases are very important enzymes that are now widely used in food, leather, detergent, and pharmaceuticals. The industrial importance of these proteases has influenced many workers to study the gene structure, regulation of gene expression and excretion processes of the proteases. Microbial extracellular proteases are produced by various microorganisms including genus *Bacillus* (15, 18), *Serratia* (8, 19), *Vibrio* (3, 4, 5), and *Pseudomonas* (6). Those from Gram-positive bacteria have been extensively studied mainly due to their quantity production, and information on the expression, processing, and industrial application have been markedly accumulated. On the other hand, in Gram-negative bacteria, their toxicity and poor ability to secrete proteins have limited studies mainly to the genetic level, and comparatively little is known of them. Among them, the major extracellular protease of *Serratia marcescens*, which is widely used as an anti-inflammatory agent, has been characterized by a number of groups (10, 12). Recently, there are numerous applications for enzymes or pharmacologically active chemicals that are stable at high pH. Alkaline proteases secreted by both neutrophilic and alkalophilic microorganisms are of interest because they represent a major source of commercially produced proteolytic enzymes (1, 9). Most of these enzymes are classified as serine protease by their catalytic mechanisms and exhibit optimal activity at pH of 9 to 11. In general, these enzymes have molecular weights ranging from 20,000 to 30,000 and have characteristically high isoelectric points (9, 11). Most of the truly alkalophilic microorganisms have either been isolated from specific, enriched environments such as indigo dye balls (16) or alkaline lakes (14, 17), or they have been isolated upon suitable enrichment culturing of soil. Notably, the alkalophilic *Actinomycetes* and many of the alkalophilic *Bacillus* species have been isolated from soils that are not particularly alkaline. In Gram-negative bacteria, *Pseudomonas aeruginosa* is known to secrete two proteases, elastase and alkaline protease. *Vibrio alginolyticus* secretes several alkaline serine proteases (2, 5). In this study, we found that the alkalophilic bacteria *Xanthomonas* sp. JK311 screened from soil produced the extracellular alkaline proteases and that the protease genes were expressed in *E. coli* HB101.

* To whom correspondence should be addressed.

Materials and Methods

Media and culture condition

Isolates and *E. coli* were grown aerobically in LB medium at 30℃ and 37℃. Isolates were routinely cultivated on LSC agar medium containing 10 g of Bacto tryptone, 5 g of yeast extract, 10 g of sodium chloride, 15 g of Bacto-agar (per liter), 2% (w/v) of sodium carbonate, and 1.5% (w/v) of skim milk. *E. coli* transformants were sc-

reened based on the halo formation on LB agar medium containing 50 μg/ml of ampicillin and 3% skim milk after 12 hr cultivation at 37℃.

Strain isolation and characterization

Xanthomonas sp. JK311 was isolated from soil. A soil sample was suspended in 3.0 ml of distilled water by vigorous vortexing and the suspension was plated directly onto alkaline LSC-agar medium (pH 9.0) and incubated for 24 hr at 37℃. Individual colonies were picked and purified by streaking four times onto alkaline LSC-agar plates. The purified isolates were analysed by microscope and Gram-staining. The other physiological properties were analysed by the API 20E kit and ATB system.

Protease and protease inhibitor assay

Proteolytic activity was measured by the hydrolysis of azocasein (19). The standard assay mixture (90.5 ml) contained 0.1% azocasein, 50 mM sodium carbonate buffer (pH 9.5) and appropriately diluted enzyme samples. The reaction was terminated after 30 min by the addition of an equal volume of 15% trichloroacetic acid and then cooled on ice for 5 min. The precipitate formed was removed by centrifugation for 3 min at 12,000×*g* in a microcentrifuge. Supernatant absorbance was measured at 440 nm. One unit of activity is defined as the amount of protein which produces a change of 0.1 absorbance unit under the assay conditions.

To test their effects on protease activity, the following protease inhibitors were added to the gelatin gels during incubation in Triton X-100 and Tris-HCl buffer: PMSF (0.5 to 2 mM), EDTA (2 to 8 mM), mercaptoethanol (20 mM), DTT (20 mM), and histidine (0.5%).

Electrophoresis

The gelatin SDS-PAGE in slab gels containing SDS and gelatin as a copolymerized substrate was used for the detection of protease activity. Cultures were sedimented by centrifugation and 1.0 ml supernatant samples were mixed with 0.1 ml of SDS (25% w/v) and 0.1 ml of glycerol, and incubated at 37℃ for 30 min. After electrophoresis, the gels were washed in Triton X-100 for 1 hr at 4℃ to remove the SDS and restore enzyme activity. The gels were incubated in 0.1 M Tris-HCl buffer (pH 8.0) for 3 hr at 37℃, and stained with 1% (w/v) amido black (Sigma Co.).

Southern blot analysis

Southern blotting was performed as described by Southern (13) with slight modifications. DNA used as a hybridization probe was labeled by random primed incorporation of digoxigenin (DIG)-labeled deoxyuridine-triphosphate (Boehringer Mannheim Biochemica). After vacuum blotting, the nitrocellulose filter paper was incubated at 68℃ for 18 hr with the DIG-labeled probe. The hybridized nitrocellulose filter was detected by enzyme-linked immunoassay using an antibody-conjugate and enzyme-catalyzed color reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium salt (NBT).

Results and Discussion

Isolation and identification of the alkalophilic bacteria producing extracellular proteases

To isolate the alkalophilic bacteria producing an extracellular alkaline protease, we screened the microorganisms from soil through plating on skim-milk agar plate (Fig. 1). The isolate forming the halo on the plate was identified as a motile, rod shaped, Gram-negative bacterium by microscope and Gram-staining. The characteristics of the isolate JK311 were analysed by the API 20E kit and the ATB system and are listed in Table 1. On the basis of these results, the isolate was identified as *Xanthomonas* sp. belonging to the family *Pseudomonadaceae*, and was designated as *Xanthomonas* sp. JK311.

Effect of pH and temperature on the growth of the *Xanthomonas* sp. JK311 and on the production of proteases

To determine the effects of culture pH on growth rate and protease production, *Xanthomonas* sp. JK311

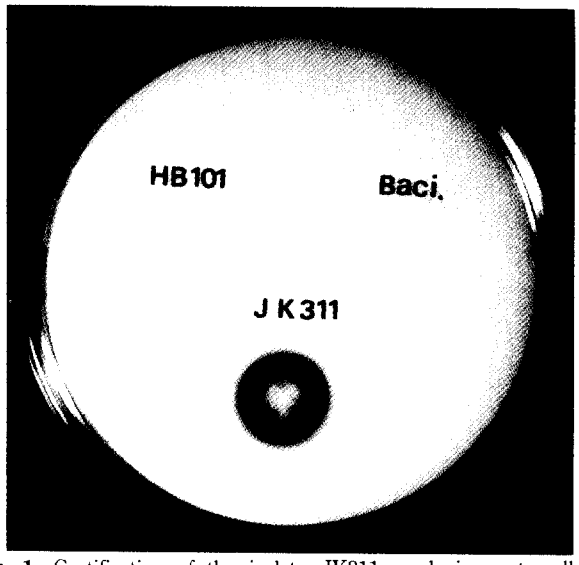

Fig. 1. Certification of the isolate, JK311 producing extracellular proteases. The clone was toothpicked and incubated at 37℃ for 18 hr. The control cells, *E. coli* HB101 (HB101) and *Bacillus subtilus* (Baci.) did not show halo, whereas a large halo appeared around JK311 strain.

Table 1. Characteristics of *Xanthomonas* sp. strain JK311.

Test	Result
General properties	
Cell morphology	rod
Motility	high
Gram staining	−
API 20E	
ONPG (β-galactosidase)	−
Arginine dihydrolase	−
Lysine decarboxylase	−
Ornithine decarboxylase	−
Citrate utilization	−
H_2S production	−
Urease production	−
Tryptophane deaminase	−
Indole production	−
VP reaction	−
Gelatin liquefaction	+
Cytochrome C oxidase	+
Acidification of	
Glucose	−
Mannitol	−
m-inositol	−
D-sorbitol	−
L-rhamnose	−
Sucrose	−
Melibiose	−
Amygdalin	−
L-arabinose	−
ATB system	
Rhamnose	−
N-acetyl-glucosamine	−
D-ribose	−
Inositol	−
D-saccharose	+
Maltose	+
Itaconate	−
Suberate	−
Malonate	−
Acetate	−
D-lactate	−
L-alanine	+
Mannitol	−
Salicin	−
D-melibiose	−
L-fucose	−
L-arabinose	−
Propionate	−
Caprate	−
Histidine	−
2-aceto-gluconate	−
3-hydroxy-butyrate	−
L-proline	−
Glycogen	−
3-hydroxybenzonate	−
L-serine	−
Esculine	−
Oxidase	+
Nitrate reduction	+
DNase	+

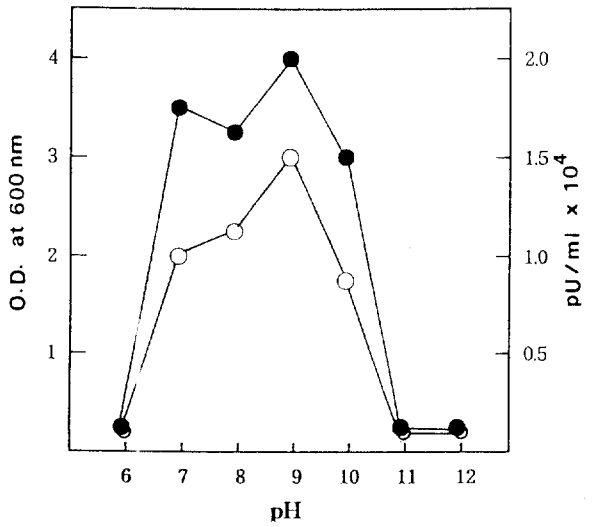

Fig. 2. Effect of pH on growth (●) of *Xanthomonas* sp. JK311 and on productivity (○) of proteases. Cells were grown at 37℃. The pH of the media (LB) was maintained either by 50 mM sodium phosphate buffers (pH 6.0 and 7.0), 50 mM Tris-HCl (pH 8.0), 50 mM sodium carbonate buffer (pH 9.0 and pH 10), or 50 mM CAPS buffer (pH 11 and pH 12).

was cultured at various pH and the growth rate of cells and the production of proteases were determined (Fig. 2). The isolate grew well in the pH range of 7 to 10, indicating that the strain belongs to a facultative alkalophilic bacteria. The cell growth rate was maximum at pH 9.5. The production of the total protease activity was also nearly constant at that pH range, suggesting that more than one alkaline protease may be produced in this strain. As the effect of temperature on the growth and protease productivity of *Xanthomonas* sp. JK311 was investigated, both cell density and protease productivity were a little higher at 37℃ than at 30℃. Production of proteases started when growth reached late log phase, and total protease activity reached maximum at stationary phase.

Analysis of characteristics of the extracellular proteases by gelatin-PAGE

The extracellular proteases produced by *Xanthomonas* sp. JK311 were examined by gelatin-PAGE. At least five bands (Prt 1~Prt 5) of protease activity were detected (Fig. 3, A). To identify the presence of a protease having a basic isoelectric point, the electrophoresis was carried out at reverse polarity. However, no protease activity was detected in this condition (data not shown). When the effects of inhibitors on protease activity were investigated, all proteases were inhibited by 5 mM EDTA but not by 2 mM PMSF (Fig. 3, B). This result suggests that all extracellular proteases from *Xanthomonas* sp. JK

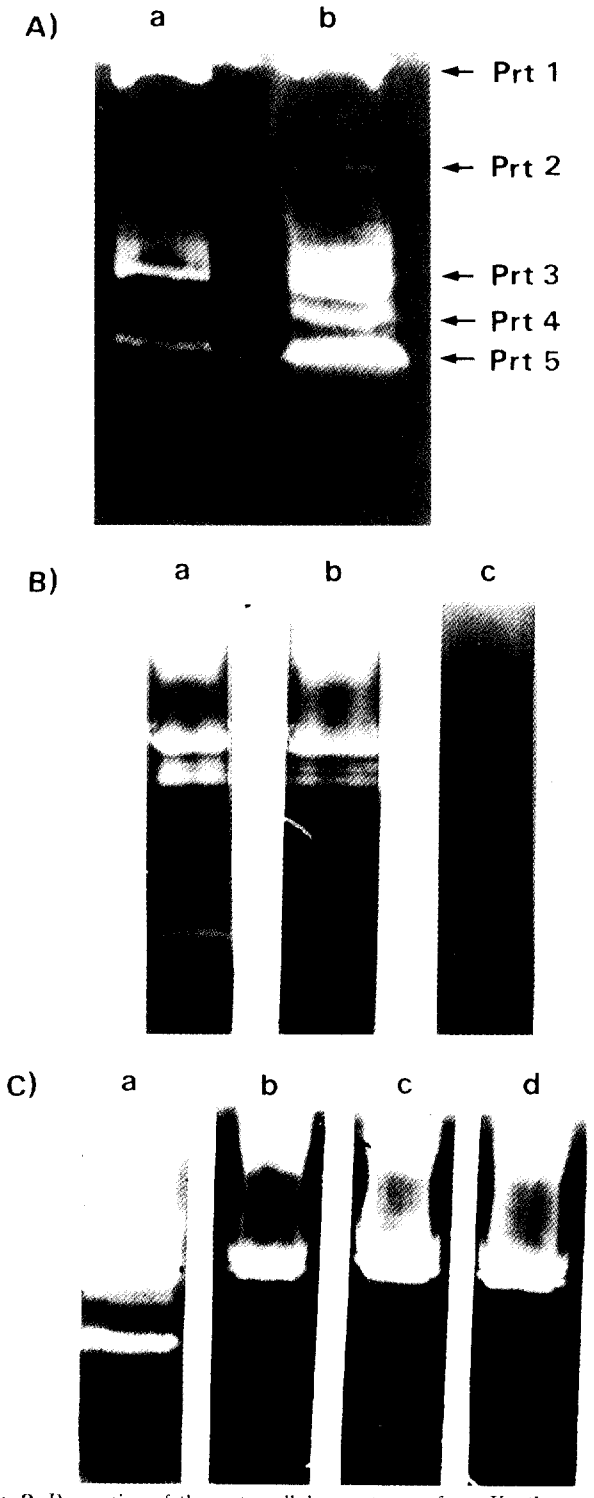

Fig. 3. Properties of the extracellular proteases from *Xanthomonas* sp. JK311. Cells were cultured in LSC-medium (pH 9.5) at 37°C. A; Extracellular proteases of the cultured medium secreted from *Xanthomonas* sp. JK311 on the gelatin SDS-PAGE. Lane a, no concentration of the sample. Lane b, 20-fold concentration. B; Effect of PMSF and EDTA on the activity of proteases. a, untreated control sample; b, preparation treated with 2 mM PMSF; c, 5 mM EDTA. C; Effect of SDS on the activity of proteases. a, untreated control sample; preparation treated with SDS at 0.2% (b), 0.5% (c), and 1% (d).

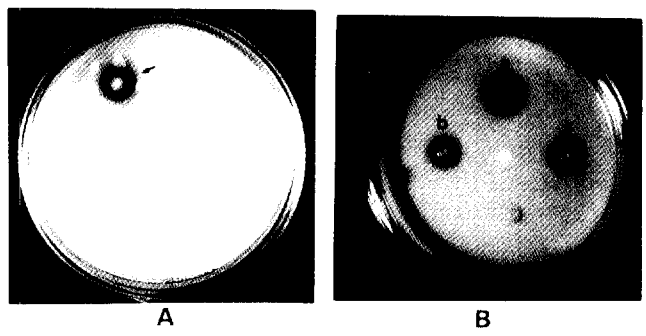

Fig. 4. Screening of *E. coli* transformants on the skim-milk agar plate. A; The transformant represented as arrow was selected as a protease producing clone. B; *Xanthomonas* sp. JK311 (a), *E. coli* transformant containing the plasmid, pXEP-1 (b) and pXEP-2 (c).

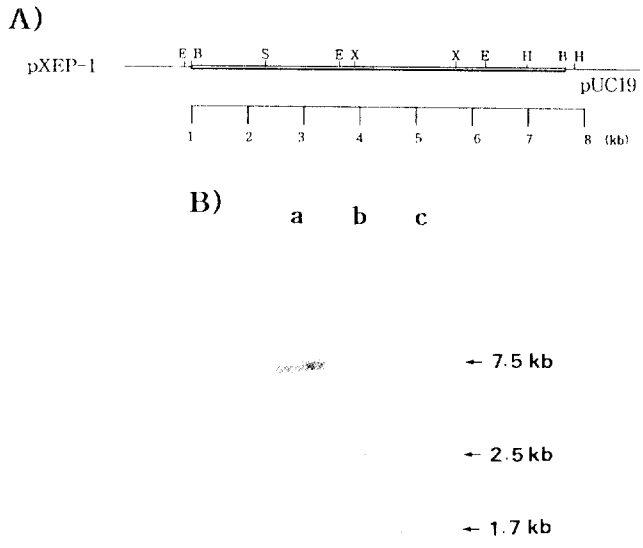

Fig. 5. Restriction map and characterization of the pXEP-1. A) Restriction map of plasmid. The thin line indicates the part of pUC 19 vector and the thick line indicates the cloned protease gene of *Xanthomonas* sp. JK311. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sma*I; X, *Xho*I. B) Southern blot analysis of *Xanthomonas* sp. JK311 chromosomal DNA by using the cloned protease gene as probe. The chromosomal DNA was digested with *Bam*HI (a), *Eco*RI (b), and *Hin*dIII (c).

311 are metalloproteases. In order to investigate SDS-resistance of proteases, the gelatin gel was treated with SDS during soaking in Triton X-100 and incubation in 0.1 M Tris-HCl buffer. Four proteases except protease 5 (Prt 5) were resistant to SDS up to 1% (Fig. 3, C). This property of SDS-resistancy is very exceptional in microbial proteases except for the fungus *Tritirachium* proteinase K which is widely used for protein removal in genomic DNA isolation (7) and the *V. allginoliticus* exoprotease A (5).

Fig. 6. Comparison of the extracellular proteases produced from the *E. coli* transformant [pXEP-1] with those from *Xanthomonas* sp. JK311 by gelatin SDS-PAGE. Lane a shows the extracellular proteases from *Xanthomonas* sp. JK311 and lane b, those from *E. coli* HB101 [pXEP-1], and lane c, those from *E. coli* HB101 [pXEP-2].

Cloning and expression of the protease gene of *Xanthomonas* sp. JK311 in *E. coli* strain HB101

In order to clone the genes encoding the extracellular proteases of *Xanthomonas* sp. JK311, bacterial chromosomal DNA was isolated and cut completely with *Bam*HI. The digested DNA fragments were inserted into the corresponding site of pUC19, and *E. coli* HB101 was transformed with the ligated DNAs. Among about 3,000 transformants, one clone showed a clear zone around the colony on LB agar medium containing 1.5% skim-milk and 50 μg/ml of ampicillin (Fig. 4). The plasmid DNA was isolated from the selected transformant, designated as pXEP-1, and digested with several restriction enzymes to map their restriction sites within the cloned fragment (Fig. 5, A). The size of the cloned DNA fragment was about 7.5 kb. To identify the foreign gene fragment originating from *Xanthomonas* sp. JK311, the cloned DNA fragment from pXEP-1 was electro-eluted and labelled with DIG by random priming. As a result of Southern blot analysis, bands specific to the probe DNA were detected to correspond with the restriction mapping of the cloned plasmid (Fig. 5, B). On the basis of the restriction map, plasmids deleted at both ends of the cloned DNA fragment were constructed (*Sma*I or *Hin*dIII deletion from pXEP-1) and their protease production was investigated. However, no halo formation on skim-milk agar plate was detected in both cases (data not shown). It is not clear whether these deletions inhibit protease gene expression or damage the sequence involved in secretion process of the proteases. On the other hand, the plasmid, pXEP-2, which was constructed to be an anti-orientation of the cloned gene fragment against the pXEP-1, produced the extracellular proteases in *E. coli* HB101. This suggests that the cloned gene express the protease using its own promoter. The extracellular protease produced from *E. coli* [pXEP-1 and pXEP-2] was compared with that from *Xanthomonas* sp. JK311 by gelatin-PAGE (Fig. 6). The result showed that three bands of protease activity were detected and they corresponded to those of *Xanthomonas* sp. JK311 protease Prt 3, Prt 4, and Prt 5. Also, effects of the protease inhibitors and SDS on these proteases were similar to those on *Xanthomonas* sp. JK311.

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References

1. **Aunstrup, K.,** 1979. Production, isolation, and economics of extracellular enzymes. *Appl. Biochem. & Bioengin.* **2**, 27-68.
2. **Deane, S.M., J.R. Mahara, F.T. Robb, and D.R. Wood,** 1987. Cloning, expression and release of a *Vibrio alginolyticus* SDS-resistant Ca^{2+}-dependent exoprotease in *Escherichia coli*. *J. Gen. Microbiol.* **133**, 2295-2302.
3. **Deane, S.M., F.T. Robb, and D.R. Wood,** 1986. Isolation and characterization of a *Vibrio alginolyticus* mutant that overproduces extracellular proteases. *J. Gen. Microbiol.* **132**, 893-898.
4. **Deane, S.M., F.T. Robb, and D.R. Wood,** 1987. Production and activation of a SDS-resistant alkaline serine exoprotease of *V. alginolyticus*. *J. Gen. Microbiol.* **133**, 391-398.
5. **Deane, S.M., F.T. Robb, S.M. Robb, and D.R. Wood,** 1989. Nucleotide sequence of the *Vibrio alginolyticus* calcium-dependent, detergent-resistant alkaline serine exoprotease A. *Gene* **76**, 281-288.
6. **Fukushima, J., S. Yamamoto, K. Morihara, Y. Atsumi, H. Takeuchi, S. Kawamoto, and K. Okuda,** 1989. Structural gene and complete amino acid sequence of *Pseudomonas aeruginosa* IFO 3455 elastase. *J. Bacteriol.* **171**, 1698-1704.
7. **Jany, K.D., G. Lederer, and B. Mayer,** 1986. Amino acid sequence of proteinase K from the mold *Tritirachium album Limber*. *FEBS Lett.* **199**, 139-144.
8. **Kwon, Y.T., H.H. Lee, and H.M. Rho,** 1993. Cloning, expression and sequencing of the minor protease encoding

gene from *Serratia marcescens* ATCC 21074. *Gene* **125**, 75-80.

9. **Markland, F.S. and E.L. Smith,** 1971. Subtilisins: Primary structure and physical properties, p. 561-608. *In* P.D. Boyer (ed.), The enzymes. Academic press, Inc., New York.
10. **Miyata, K., K. Maejima, K. Tomoda, and M. Isono,** 1970. *Serratia* protease: Purification and general properties of the enzyme. *Agric. Biol. Chem.* **34**, 310-318.
11. **Priest, F.G.,** 1977. Extracellular enzyme synthesis in the genus *Bacillus*. *Bacteriol. Rev.* **41**, 711-753.
12. **Schmitz, G. and V. Braun,** 1985. Cell bound and secreted protease of *Serratia marcescens*. *J. Bacteriol.* **161**, 1002-1009.
13. **Southern, E.M.,** 1975. Detection of specific sequences among DNA fragment separated by gel electrophoresis. *J. Mol. Biol.* **98**, 503-517.
14. **Souza, K.A., P.H. Deal, H.M. Mack, and C.E. Turnbill,** 1974. Growth and reproduction of microorganisms under extremely alkaline conditions. *Appl. Microbiol.* **28**, 1066-1068.
15. **Takagi, M., T. Imanaka, and S. Aide,** 1985. Nucleotide sequence and promoter region for the neutral protease gene from *Bacillus stearothermophilus*. *J. Bacteriol.* **163**, 824-831.
16. **Takahara, M., D.W. Hibleer, P.J. Barr, J.A. Gerlt, and M. Inouye,** 1985. The OmpA signal peptide directed secretion of staphylococcal nuclease A by *Escherichia coli*. *J. Biol. Chem.* **260**, 2670-2674.
17. **Tindall, B.J.,** 1988. Prokaryotic life in the alkaline, saline, athalassic environment p. 31-67. *In* F. Rodriguez-valera (ed.), Halophilic Bacteria, CRC, 1. Boca Raton, Fla.
18. **Yang, M.Y., E. Ferrari, and D.J. Henner,** 1984. Cloning of the neutral protease gene of *Bacillus subtilis* and the use of the cloned gene to create an *in vitro* derived deletion mutation *J. Bacteriol.* **160**, 15-21.
19. **Yanagida, N., T. Uozumi, and T. Beppu,** 1986. Specific excretion of *Serratia marcescens* protease through the outer-membrane of *Escherichia coli*. *J. Bacteriol.* **166**, 937-944.