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Molecular Cloning of *Serratia marcescens* Metalloprotease Gene into *Escherichia coli*

Kim, Ki Seok, Chang-Won Lee, Sang Yeol Lee[1], Byeong Ryong Lee[2] and Yong Chul Shin*

Department of Microbiology, [1]Department of Biochemistry, College of Natural Sciences, Gyeongsang National University, Chinju 660-701, Korea
[2]Kolon Research and Development Center, Kolon Industries, Inc., Kumi 730-030, Korea

Serratia marcescens Metalloprotease 유전자의 대장균에로의 클로닝

김기석 · 이창원 · 이상열[1] · 이병룡[2] · 신용철*

경상대학교 자연과학대학 미생물학과, [1]생화학과, [2]코오롱주식회사 기술연구소

Abstract — Molecular cloning of metalloprotease gene from *Serratia marcescens* ATCC 21074 into *Escherichia coli* JM109 was carried out. Chromosomal DNA of *S. marcescens* was completely digested with *Hin*dIII and southern hybridization with a synthetic oligonucleotide probe revealed that a 50 KD metalloprotease gene was contained in 4.0 Kb chromosomal DNA fragment. 4.0 Kb chromosomal DNA fragments eluted from agarose gel were ligated with pUC19 and transformed into *E. coli* JM109. Nine positive clones were obtained from about 1×10^3 transformants by colony hybridization. Their recombinant plasmids, pSP1 and pSP2 have same chromosomal DNA fragments in pUC19 in opposite-orientations. When cloned metalloprotease gene was expressed in *E. coli*, about 52 KD precursor protein of metalloprotease was detected by western blot analysis from *E. coli* harboring a recombinant plasmid pSP2. Plasmid pSP2 showed no protease activities in *E. coli* but overproduced the active metalloprotease in *S. marcescens* ATCC 27117.

Because gram-negative bacteria have additional outer membrane outside inner cytoplasmic membrane, they have unique secretion mechanisms for extracellular proteins through outer membrane into culture medium, distinct from those of gram-positive bacteria, plants, and animals having single cytoplasmic membrane. Among the gram-negative bacteria, there are many reports concerning on the secretion of extracellular proteins of *E. coli* such as α-hemolysin, foreign gene products, bacteriocins, and toxins (1-4). But these proteins are all originated from extrachromosomal plasmid DNAs, not from chromosomal DNA. It is generally accepted that *E. coli* has not true secretion mechanism of proteins encoded in chromosomal DNA through the outer membrane into culture medium (5, 6). So another gram-negative species is required to investigate the secretion of extracellular proteins. Until now, extracellular proteins of *Serratia marcescens* have been good model systems for this purpose (7-12). However, the secretion mechanism of extracellular proteins is still unclear.

S. marcescens is well known gram-negative bacterium which belongs to the family *Enterobacteriaceae*, like as *E. coli* and unusually, produces many kinds of extracellular enzymes into culture medium such as nuclease (7-9), chitinases (10), lipases (11), and proteases (12, 13). All the molecular biological techniques used in *E. coli* are well-applicable to *S.*

Key words: *Serratia marcescens,* cloning of 50 KD metalloprotease gene, expression of metalloprotease

*Corresponding author

marcescens (14-16). For these reasons, we chose *S. marcescens* as a model species and have studied on the extracellular chitinase (17), nuclease (18), and protease (19) of *S. marcescens*. Here, gene cloning of extracellular metalloprotease of *S. marcescens* ATCC 21074 was investigated in this article. Kodama *et al.* (20) isolated *S. marcescens* ATCC 21074, previously called as *Serratia* sp. E-15 from larval silkworms, secreting a potent proteolytic enzyme into culture medium, named as serratiopeptidase after its origin. Miyata *et al.* (21-23) purified and characterized this major protease from *S. marcescens* ATCC 21074. This protease was a metalloprotease requiring zinc atom for proteolytic activity and somewhat similar to thermolysin in substrate specificity. Lee *et al.* (24, 25) determined the amino acid sequences of the NH_2-terminal and COOH-terminal peptide of the metalloprotease. Recently, Nakahama *et al.* (26) cloned a metalloprotease gene from the *S. marcescens* ATCC 21074 and determined the nucleotide sequence. They suggested that this metalloprotease contains only pro-region but pre-region is not present. The metalloprotease was produced intracellularly as an inactive precursor form in *E. coli*, without processing of pro-region. Besides the metalloprotease, presence of a minor serine protease of *S. marcescens* was discovered and cloning and nucleotide sequencing of the gene were carried out (27). DNA sequence of serine protease gene revealed that it has pre- and pro-regions at 5′ end of mature protein sequence. Active serine protease was made and secreted into culture medium through the outer membrane in *E. coli*.

As a preliminary study for investigating the secretion mechanism and overproduction of metalloprotease by gene manipulation, we cloned metalloprotease gene of *S. marcescens* ATCC 21074 into *E. coli* and its expression was discussed in this paper.

Materials and Methods

Bacterial strains, plasmids, and culture conditions

Sarratia marcescens ATCC 21074, previously named as *Serratia* sp. E-15 (21) was used as a source of metalloprotease gene, *E. coli* JM109 as a host for recombinant plasmids, plasmids pUC19 and pKK 223-3 as a cloning vector and an expression vector, respectively. *E. coli* and *S. marcescens* were cultivated in LB media at 37℃ and 30℃, respectively. Bacterial cells were maintained on LB agar plate at 4℃ and transferred to fresh agar plate for every month. Skim milk agar plate was prepared by supplementing LB agar with 2% skim milk.

Chemicals and enzymes

Restricition endonucleases and other DNA modifying enzymes such as T4 DNA ligase, T4 polynucleotide kinase, calf intestinal alkaline phosphatase were purchased from New England Biolabs, or Promega. Nitrocellulose membrane was purchased from Schleicher & Schuell and γ-^{32}P-ATP from Amersham. Except the reagents indicated in otherwise, all other reagents were purchased from Sigma Chemical Co. in molecular biology grade.

Synthesis of ^{32}P-labelled oligonucleotide probe

A 22-mer oligonucleotide (5′-CGTCCCGGCCACTATGATTACC-3′) was designed from nucleotide sequence of *S. marcescens* metalloprotease gene (26) and synthesized using a DNA synthesizer (Beckman, U.S.A.) at Radio-Isotope Lab. in KAIST. The synthetic oligonucleotide was purified by 7 M urea polyacrylamide gel electrophoresis followed by Sephadex G-25 column chromatography. The oligonucleotide was labelled with ^{32}P by incubation with γ-^{32}P-ATP and T4 polynucleotide kinase. Labelled oligonucleotides were finally purified through Sephadex G-50 spum-column chromatography.

Southern blot analysis of *S. marcescens* chromosomal DNA

Chromosomal DNA purified from *S. marcescens* by the slightly modified method of Sato and Miura (28) was digested with *Hin*dIII. The chromosomal DNA/*Hin*dIII digest was electrophoresed on 0.8% agarose gel and transferred to nitrocellulose membrane by the technique described by Southern (29). The membrane was baked at 80℃ for 2 hr under vacuum and hybridized with ^{32}P-labelled oligonucleotide probe according to the laboratory manual

of Sambrook *et al.* (30).

Construction of *S. marcescens* genomic library

Chromosomal DNA was completely digested with *Hin*dIII and 4.0 Kb DNA fragments were eluted agarose gel by using Gene Clean Kit (Bio 101, U.S.A.). Plasmid pUC19 was isolated and purified according to laboratory manual of Sambrook *et al.* (30). The purified pUC19 was digested with *Hin*dIII and treated with calf intestinal alkaline phosphatase. The dephosphorylated, linearized pUC19 was ligated with 4.0 Kb chromosomal DNA/*Hin*dIII fragments by T4 DNA ligase. With the ligation mixture, *E. coli* JM109 was tranformed by the method of Cohen *et al.* (31).

Colony hybridization

To select transformants harboring metalloprotease gene, all the transformants were tooth-picked onto two LB agar plates containing 50 μg/m*l* of ampicillin and incubated overnight at 37℃. One of these two plates was stored at 4℃ as a master plate and the other was used for colony hybridization. Nitrocellulose membranes with bacterial colonies were removed from the agar plates and the bacteria were lysed by placing nitrocellulose membranes on 3 MM paper saturated with 10% SDS solution for 5 min, and their DNA was denatured with 0.5 N NaOH solution and neutralized with 1 M Tris-HCl buffer (pH 7.4) containing 1.5 M NaCl. After drying under vacuum oven, prehybridization, hybridization, stringent washing, and autoradiography were carried out in accordance with laboratory manual of Sambrook *et al.* (30).

Protease assay

Protease activity was measured by the slightly modified method of Braun and Schmitz (12). 0.1 m*l* of diluted enzyme was added to 0.6 m*l* of substrate solution containing 2.4% (w/v) azocasein in 50 mM potassium phosphate buffer (pH 7.5) and the mixture was incubated at 37℃ for 30 min. The enzyme reaction was stopped by adding 0.6 m*l* of 10% trichloroacetic acid and stood for 1 hr at room temperature. After centrifugation at 7,000 rpm for 10 min, 0.8 m*l* of supernatant was collected and added to 0.3 m*l* of 10 N NaOH. The absorbance was measured at 420 nm by spectrophotometer (Beckman, DU-65). One unit of enzyme activity was defined as the amount of enzyme required to increase absorbance 0.01 per min under the experimental conditions.

Preparation of rabbit anti-metalloprotease antiserum

For the preparation of anti-metalloprotease antiserum, metalloprotease was purified from *S. marcescens* ATCC 21074 as reported in our previous paper (19). The immunization of rabbit was carried out according to the procedures described by Johnstone and Thorpe (32). 200 μ*l* of the purified metalloprotease (0.23 mg/m*l*) was emulsified with mixture of 400 μ*l* of phosphate buffered saline (PBS) buffer (pH 7.2) and 900 μ*l* of adjuvent (Hoechst) and injected into a rabbit by the intramuscular injection. The rabbit was injected twice more with the same emulsified antigen solution for 2 weeks interval. One month later, the rabbit was boosted with 200 μ*l* of the purified metalloprotease and the rabbit was bled in 4 days and antiserum was prepared.

Western blot analysis

SDS-polyacrylamide gel (12%) was run and electroblotted onto nitrocellulose membrane at 60 V for 2 hr with Hoeffer Scientific Instruments (San Francisco, U.S.A.) in 20 mM Tris, 150 mM glycine buffer (pH 8.0) containing 20% methanol. Blots were blocked in PBS buffer containing 1% BSA for 40 min and incubated at 25℃ for 12 hr with rabbit anti-metalloprotease antiserum appropriately diluted with PBS buffer containing 1% BSA. Blots were washed twice in PBS buffer contaning 0.05% Triton X-100 for 10 min, and incubated at 25℃ for 3 hr with horse radish peroxidase-labelled goat anti-rabbit IgG antiserum (Kirkegaard & Perry Laboratories, Inc. Md. U.S.A.). After washing twice with PBS buffer containing 0.05% Triton X-100 for 10 min, bands on nitrocellulose membrane were visualized by adding substrate solution. Substrate solution was prepared by mixing 20 m*l* of 0.03% (w/v) 4-chloro-1-naphtol solution dissolved in ethanol, 36 μ*l* of 30% H_2O_2 solution, and 100 m*l* of 50 mM Tris-

HCl buffer (pH 7.6).

Results and Discussion

Southern blot analysis of *S. marcescens* chromosomal DNA

To elucidate DNA fragment containing metalloprotease gene in chromosomal DNA digest of *S. marcescens*, Southern blot analysis was carried out with a 22-mer synthetic oligonucleotide probe for metalloprotease gene. After electrophoresis of chromosomal DNA/*Hin*dIII digest on 0.8% agarose gel, DNA fragments were transferred to nitrocellulose membrane and hybridized with ^{32}P-labelled oligonucleotide probe. Autoradiogram is shown in Fig. 1. The probe hybridized to a single band sized about 4.0 Kb. This 4.0 Kb DNA fragment of chromosomal DNA/*Hin*dIII digest was expected to contain metalloprotease gene. Hence, 4.0 Kb DNA fragments were eluted and used to clone the metalloprotease gene.

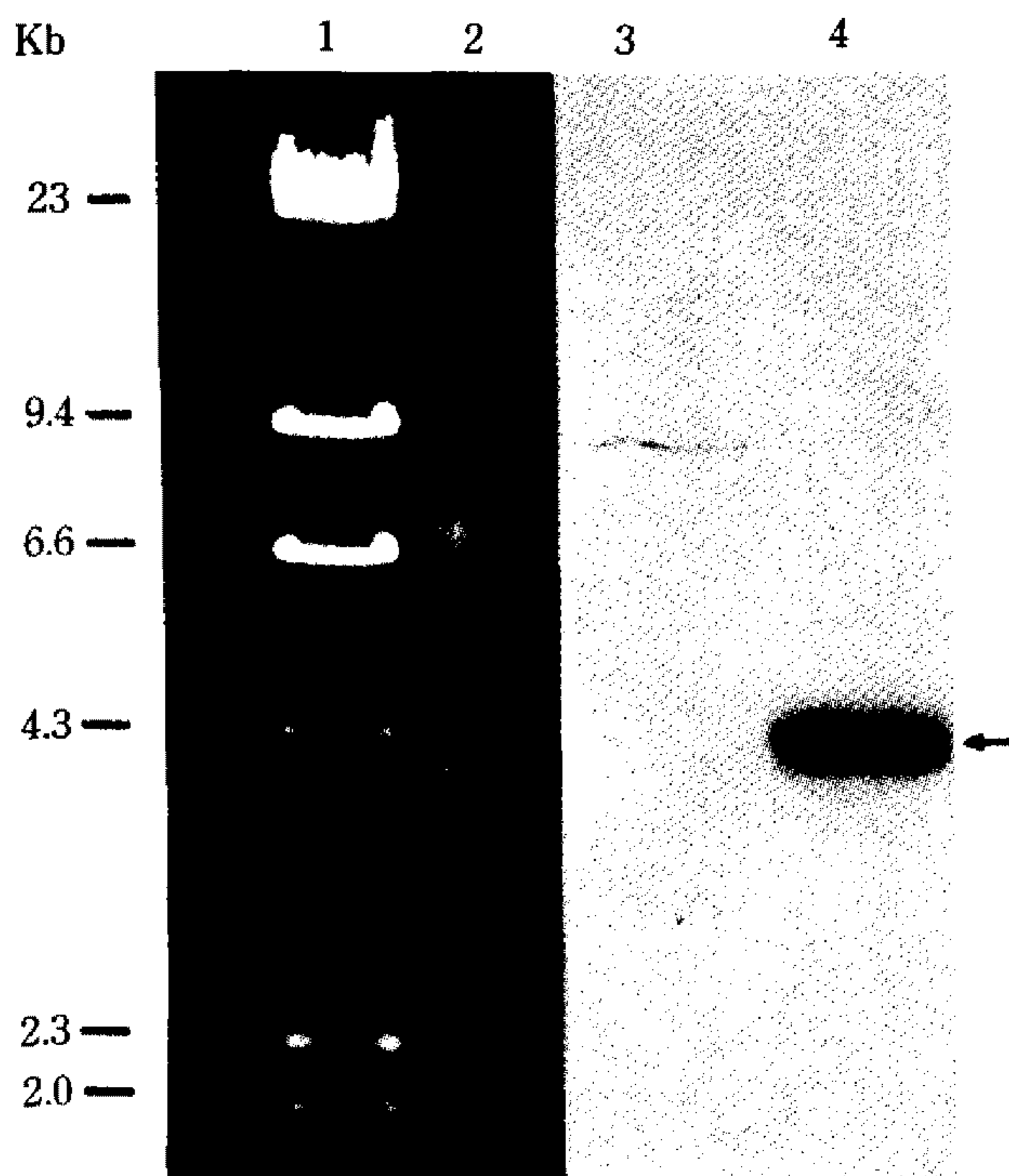

Fig. 1. Southern blot analysis of *S. marcescens* chromosomal DNA/*Hin*dIII digest with a ^{32}P-labelled, synthetic oligonucleotide probe for metalloprotease gene.
S. marcescens chromosomal DNA was completely digested with *Hin*dIII and electrophoresed on 0.8% agarose gel. Agarose gel was stained with ethidium bromide and photographed (lane 1 and 2). DNA fragments on agarose gel were blotted to nitrocellulose membrane and hybridized with a ^{32}P-labelled, synthetic oligonucleotide probe for *S. marcescens* metalloprotease gene and autoradiographed (lane 3 and 4). Lane 1 and 3, λ/*Hin*dIII digest; 2 and 4, *S. marcescens* chromosomal DNA/*Hin*dIII digest. Arrow indicates 4.0 Kb DNA fragment encoding metalloprotease gene of *S. marcescens*.

Cloning of metalloprotease gene

Chromosomal DNA of *S. marcescens* was completely digested with *Hin*dIII and electrophoresed on 0.8% agarose gel. 4.0 Kb DNA fragments were eluted and purified with Gene Clean kit. Plasmid pUC 19 was digested with *Hin*dIII, dephosphorylated with calf intestinal phosphatase, and electrophoresed on 0.8% agarose gel. The linearized pUC19 was also eluted and purified with Gene Clean kit. 4.0 Kb chromosomal DNA fraction was ligated with the dephosphorylated, linearized pUC19 by T4 DNA ligase. Ligation mixture was used to transform *E. coli* JM109 and transformants were selected on LB agar plate containing 50 μg/m*l* of ampicillin. When the transformants were tooth-picked onto LB-ampicillin plate containing 10 μg/m*l* X-gal, about 70% of them showed white colonies by insertional inactivation of α-complementation. By using ^{32}P-labelled

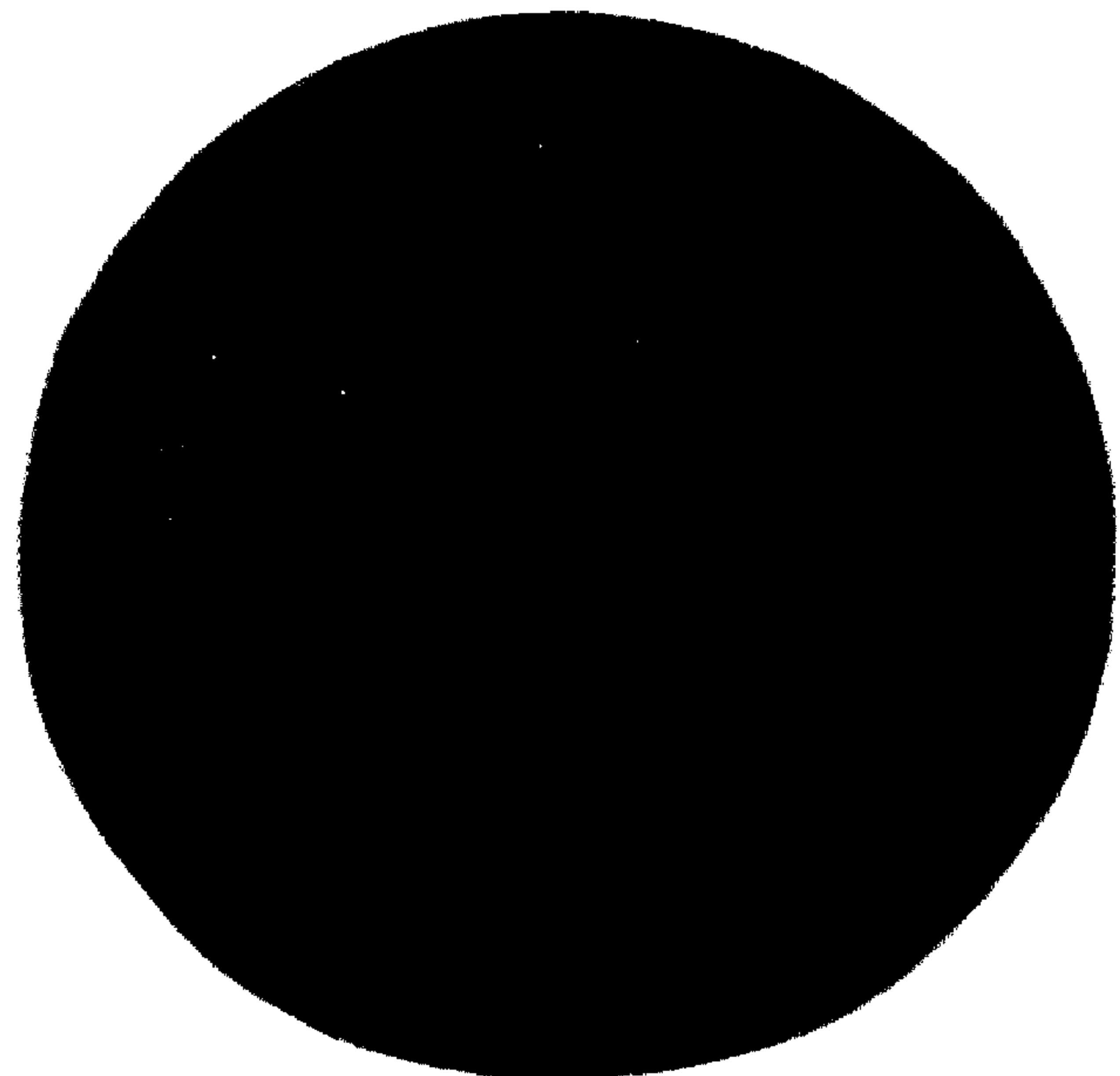

Fig. 2. Colony hybridization of transformants showing the positive signals.
4.0 Kb chromosomal DNA fragments were ligated into pUC19 and transformed into *E. coli* JM109. Colony hybridization of transformants was carried out according to the method described in Materials and Methods.

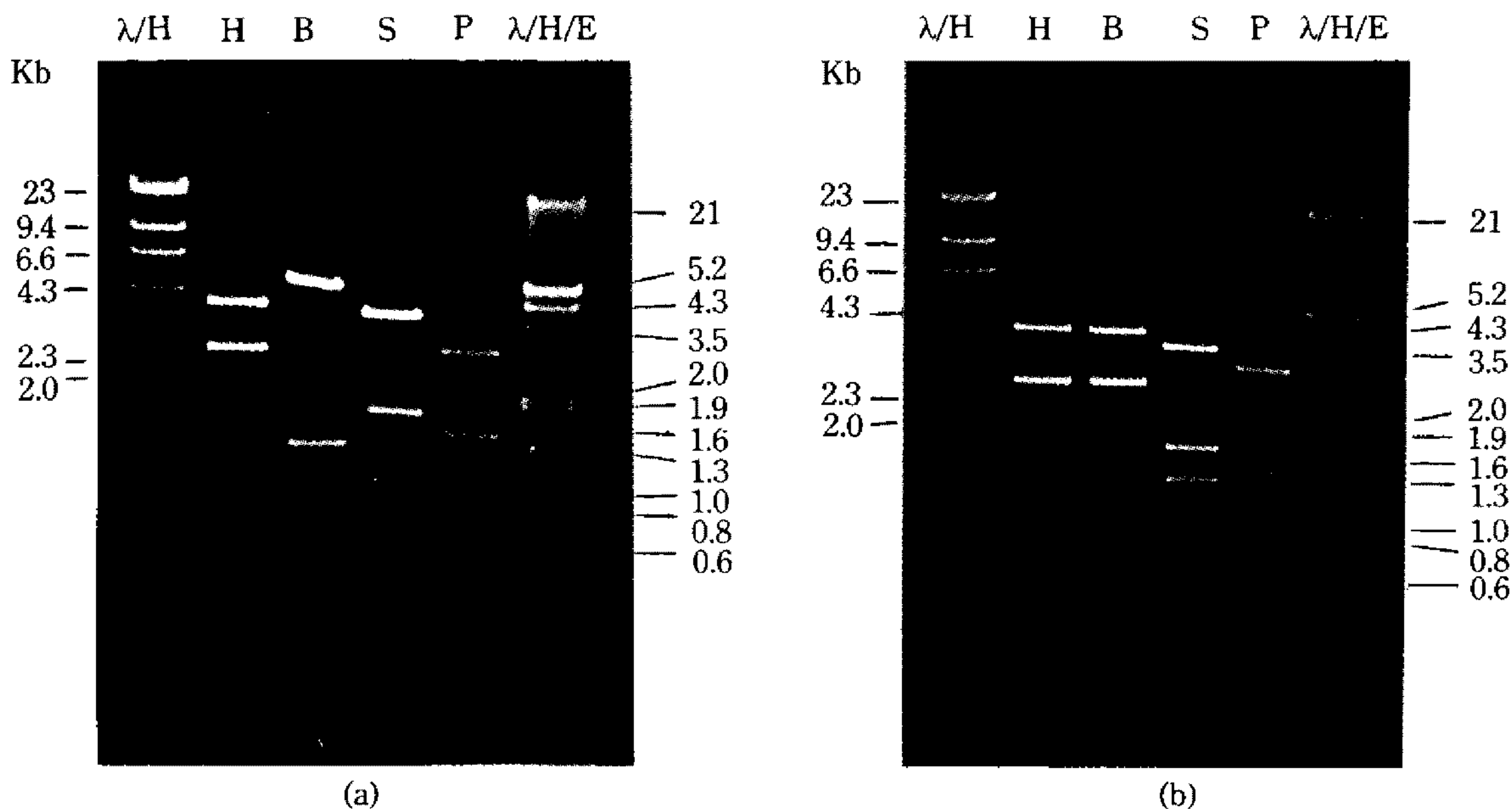

Fig. 3. Single restriction endonuclease digestions of recombinant plasmid pSP1 and pSP2.
Plasmid pSP1(a) and pSP2(b) were completely digested with *Hin*dIII(H), *Bam*HI(B), *Sal*I(S), and *Pst*I(P). λ/H and λ/H/E represent *Hin*dIII and *Hin*dIII/*Eco*RI digests of bacteriophage λ DNA.

oligonucleotide as a probe, tooth-picked colonies were screened by colony hybridization. From about 1×10^3 transformants examined, 9 positive clones were identified and the representative result is shown in Fig. 2. Recombinant plasmid DNAs were isolated from 9 positive clones by alkaline lysis method (30) and digested with *Hin*dIII or *Bam*HI. All *Hin*dIII digests of recombinant plasmids showed the same restriction fragment pattern, 4.0 Kb insert DNA and 2.7 Kb pUC19 fragments. But *Bam*HI digests showed two different types of restriction fragment pattern. One type of recombinant plasmids contained 1.4 and 5.3 Kb DNA fragments and the other contained 2.7 and 4.0 Kb DNA fragments. The former was named as a recombinant plasmid pSP1 and the latter was named as a recombinant plasmid pSP2.

Physical maps of pSP1 and pSP2

Plasmids pSP1 and pSP2 were digested with several restriction endonucleases such as *Hin*dIII, *Bam*HI, *Sal*I, and *Pst*I. Single and double digestions were performed and restriction enzyme digests were analyzed by electrophoresis on 1.0% agarose gel. The results are shown in Fig. 3 and 4. By using λ/*Hin*dIII and λ/*Hin*dIII/*Eco*RI digests as standard size markers, physical mapping of 4.0 Kb insert DNA fragment was carried out. Physical maps of pSP 1 and pSP2 shown in Fig. 5 indicate that recombinant plasmid pSP1 and pSP2 have the same insert chromosomal DNA but have the opposite orientation in pUC19.

Expression of pSP1 and pSP2 in *E. coli*

When recombinant plasmid pSP1 and pSP2 were expressed in *E. coli* JM109 by addition of 1 mM isopropyl thiogalactoside (IPTG), no detectable protease activity was found in either extracellular or intracellular fractions. When enzyme fractions were analyzed by SDS-polyacrylamide gel electrophoresis, any differences in electrophoretic pattern were not found comparing with that of *E. coli* JM109(pUC 19) used as a control. To confirm whether the gene products of pSP1 and pSP2 are metalloprotease or not, western blot analysis was carried out. As shown in Fig. 6, rabbit anti-metalloprotease antiserum did not blot to protein bands of *E. coli* JM109 (pUC19) and *E. coli* JM109(pSP1) but only blotted to protein bands of *E. coli* JM109 (pSP2) and yielded a single major band. Blotted protein band of *E. coli* JM109(pSP2) has the molecular weight of about 52 KD, which is slightly higher than 50 KD

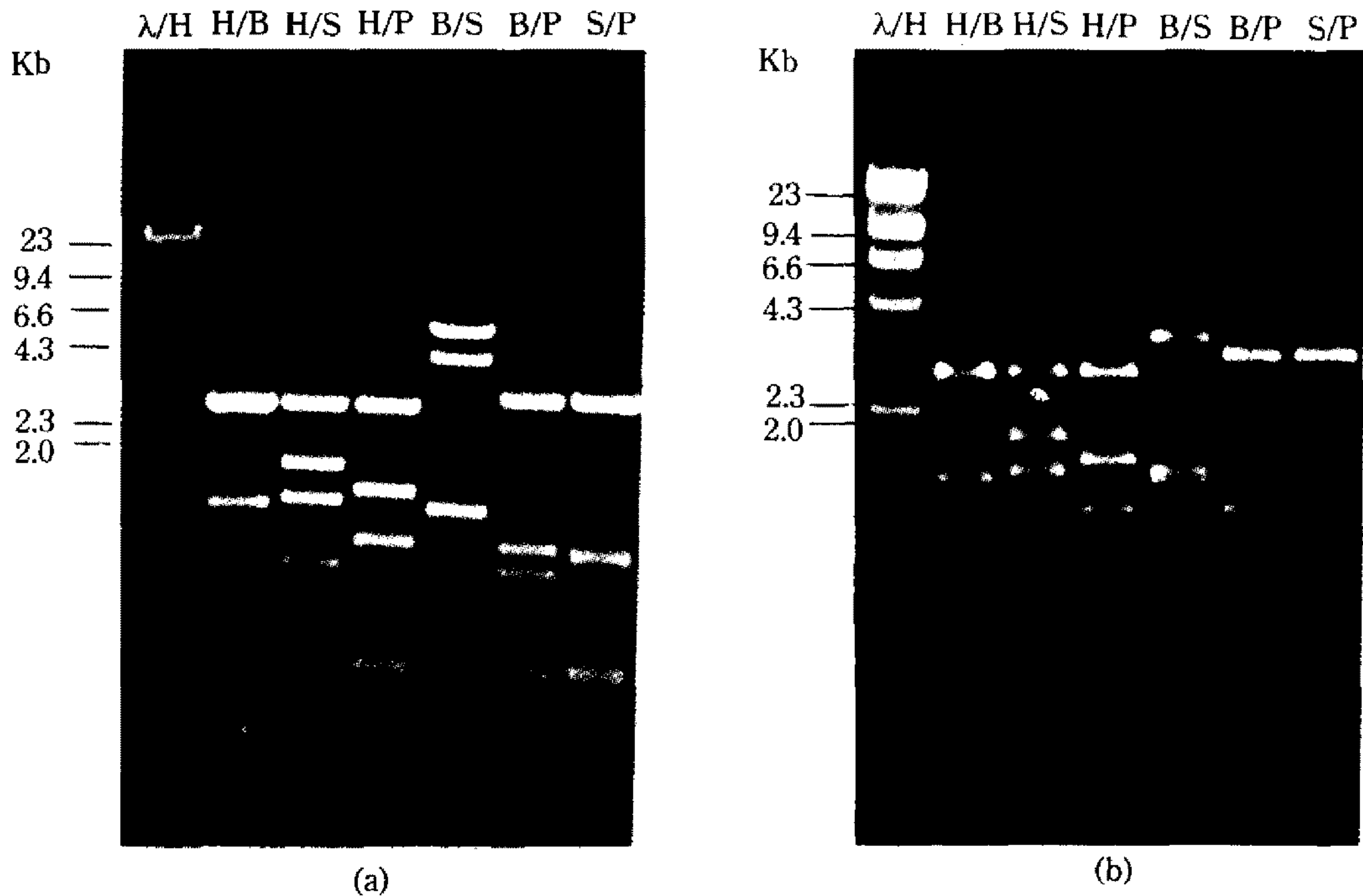

Fig. 4. Double restriction endonuclease digestions of recombinant plasmid pSP1 and pSP2.
Plasmid pSP1(a) and pSP2(b) were completely double-digested. The symbols of restriction enzymes are the same described in Fig. 3.

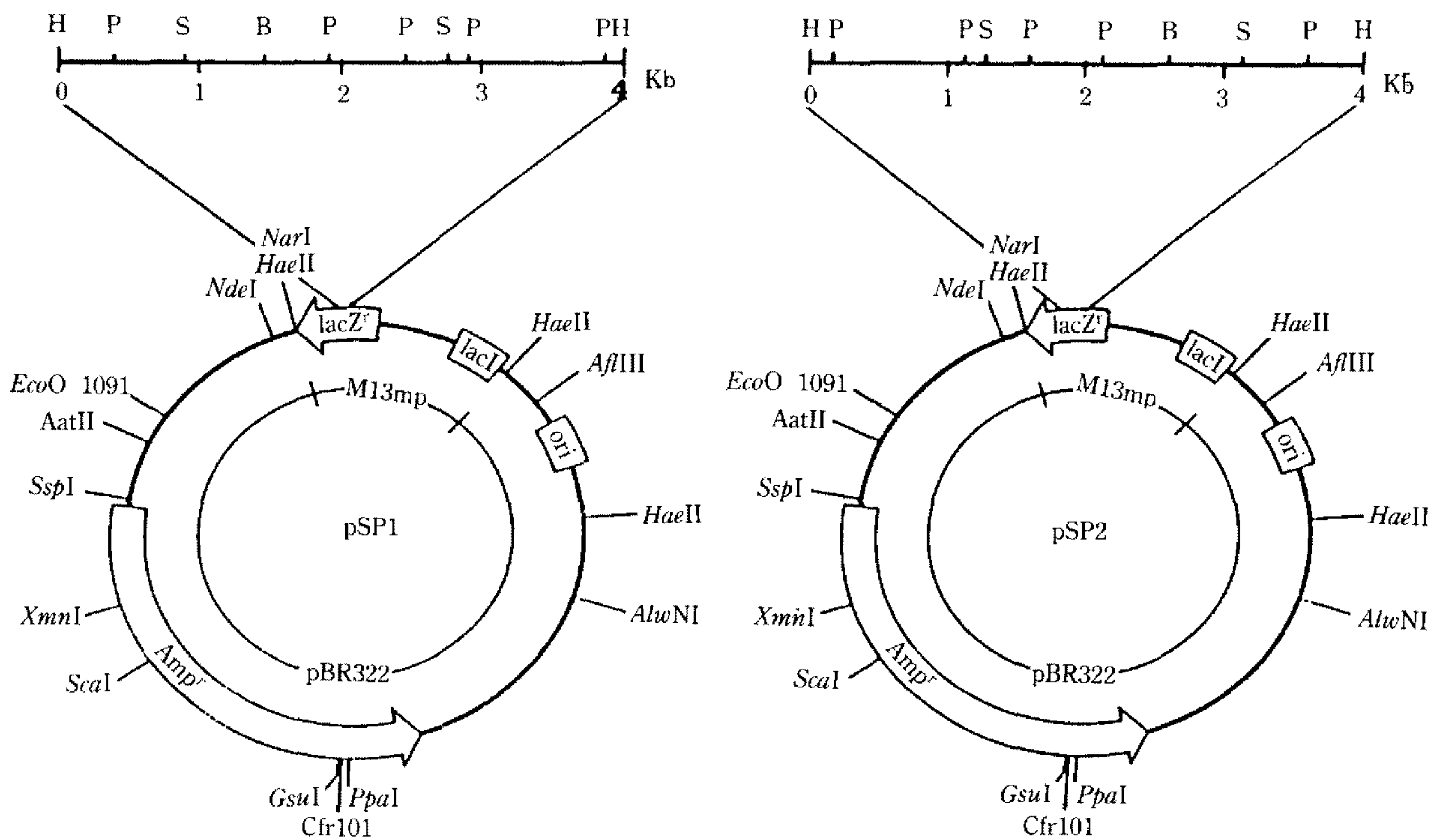

Fig. 5. Restriction endonuclease maps of recombinant plasmid pSP1 and pSP2.
The symbols of restriction endonucleases are the same described in Fig. 3.

active metalloprotease of *S. marcescens*. The 52 KD protein was considered as a precursor protein of *S. marcescens* metalloprotease, having additional pro-sequence to mature protein, as reported by Nakahama *et al.* (26). From DNA sequencing of metalloprotease gene, they suggested that metallopro-

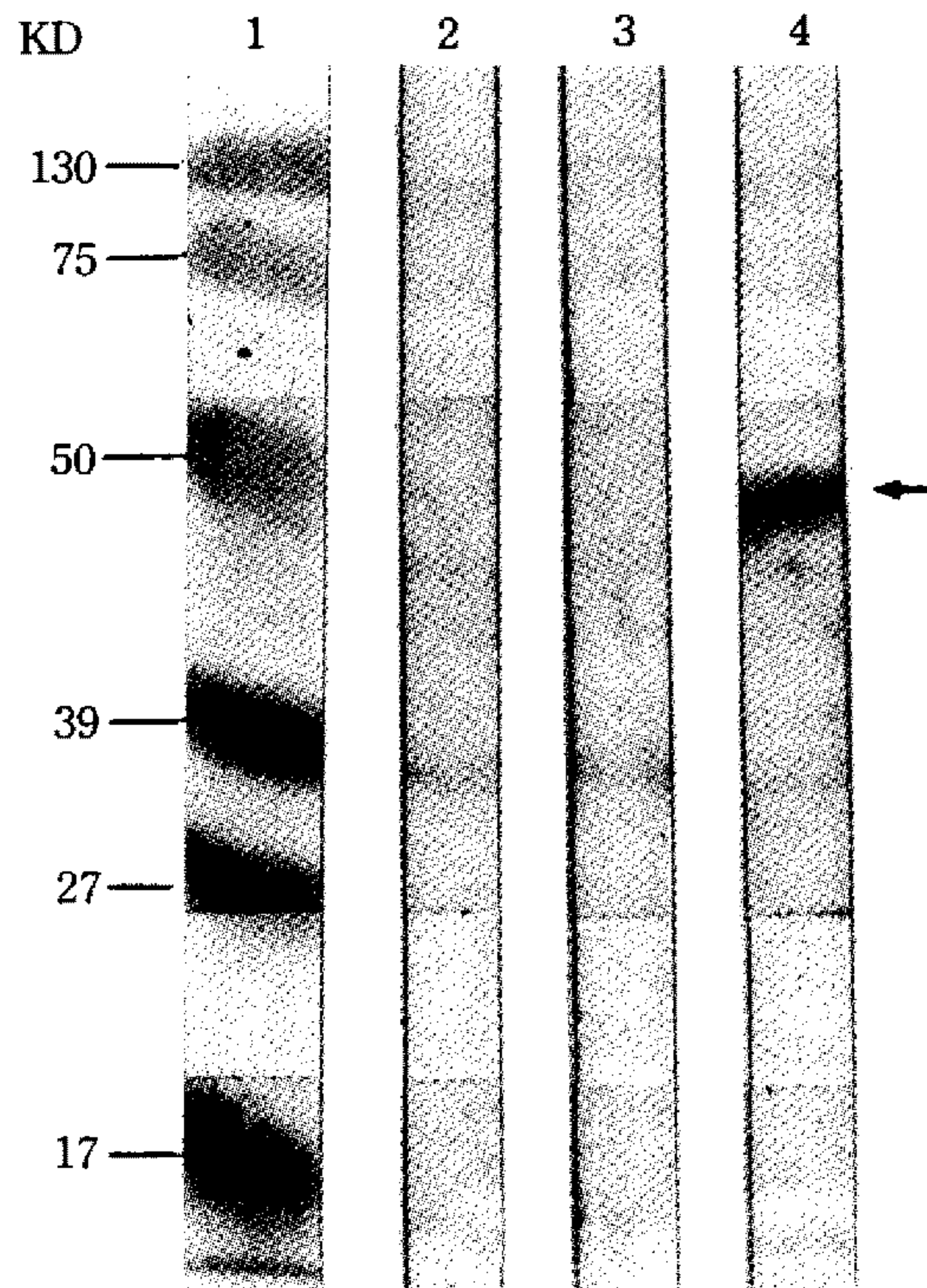

Fig. 6. Western blot analysis of the proteins of *E. coli*.
Three *E. coli* strains, *E. coli* JM109(pUC19), *E. coli* JM 109 (pSP1), and *E. coli* JM109(pSP2) were cultivated in shaking incubator at 37℃ for 15 hr and final 1 mM IPTG was added to their culture broths and incubated further 6 hr. Cells harvested were disrupted by sonication and the supernatants were electrophoresed on 12 % SDS-polyacrylamide gel. Western blot analysis was carried out according to the method described in Materials and Methods. Lane 1, size marker proteins; 2, proteins from *E. coli* JM109(pUC19); 3, proteins from *E. coli* JM109(pSP1); 4, proteins from *E. coli* JM109 (pSP2).

tease gene of *S. marcescens* has not signal sequence (pre-sequence) but has pro-sequence before the mature protein sequence.

For investigating the expression of metalloprotease gene, we constructed an expression plasmid pKSP2 by ligating *Hin*dIII DNA fragments of pSP2 with pKK223-3. Plasmid pKSP2 produced a large amount of precursor protein in *E. coli*, about 36% of total cellular proteins (data not shown). But it did not show any protease activity at all in *E. coli*. When pSP2 and pKSP2 were introduced into *S. marcescens* ATCC 27117, pSP2 showed increased protease activities on skim milk agar plate (Fig. 7). But the expression vector pKSP2 did not show protease activities on skim milk agar plate. Growth of *S. marcescens* harboring pKSP2 was retarded and

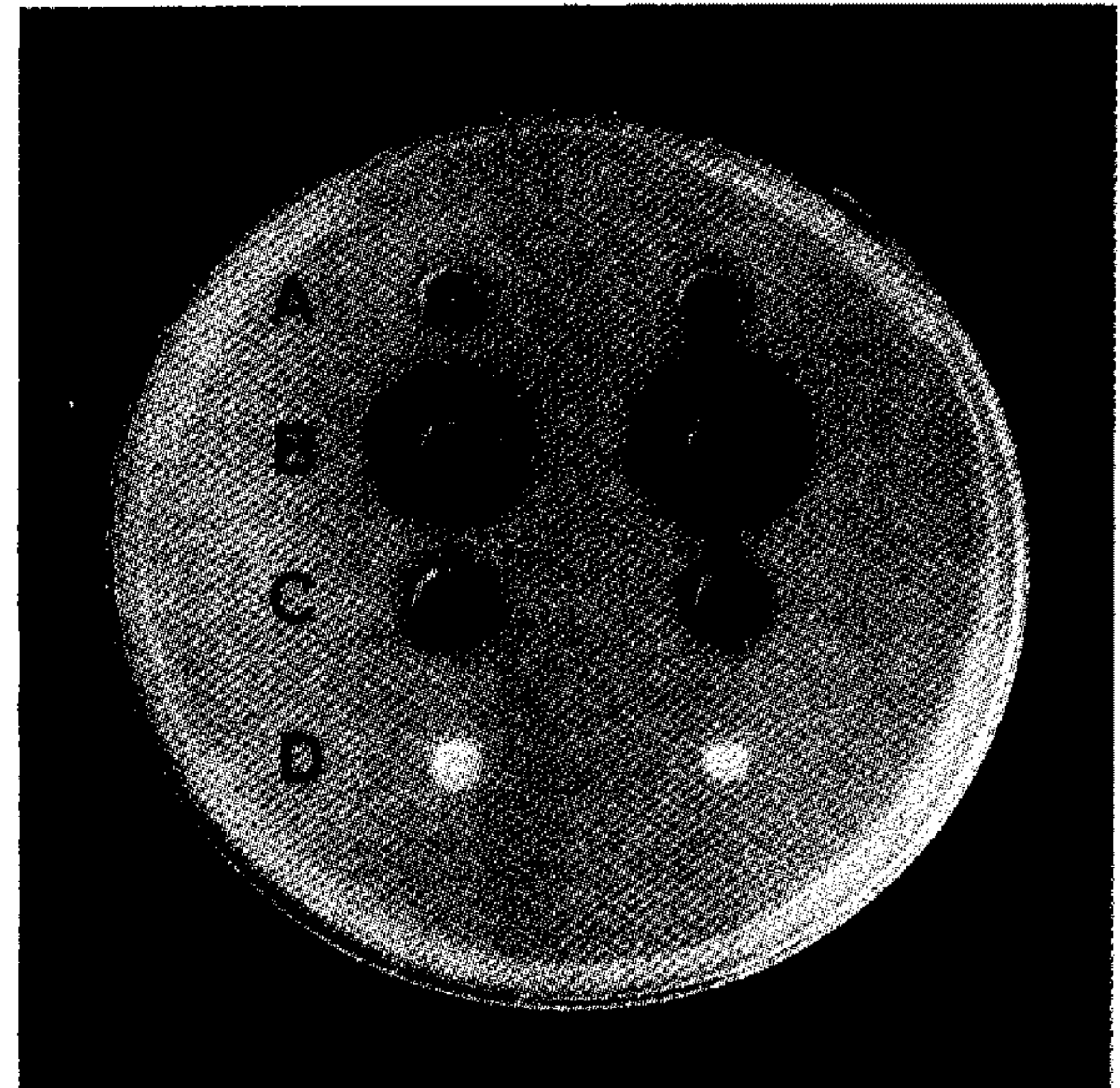

Fig. 7. Protease activities of *S. marcescens* transformants on skim milk agar plate.
S. marcescens ATCC 27117 was transformed with plasmid pUC19(A), pSP2(B), pKK223-3(C), pKSP2(D).

the red pigment observed in normal cells was not produced. *S. marcescens* containing pKSP2 seemed to be sick, suggesting that pKSP2 give harmful effects on *S. marcescens*. However, further researches are required to elucidate the activation and secretion of *S. marcescens* metalloprotease. We are now studying on the expression of metalloprotease gene in *E. coli* and *S. marcescens*.

Acknowledgment

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요 약

Serratia marcescens ATCC 21074 균주가 세포밖으로 분비하는 metalloprotease 유전자를 대장균으로 클로닝하고 그 발현을 살펴보았다. *Serratia marcescens* ATCC 21074 균주의 염색체 DNA를 제한효소 *Hin*dIII로 절단하고 아가로스 전기영동 후 ^{32}P로 표지된 합성 oligonucleotide를 사용하여 southern hybridization한 결과 4.0 Kb의 DNA 절편에 metalloprotease가 존재함을 알 수 있었다. 4.0 Kb 염색체 DNA 절편을 분리하여 pUC19에 연결한 후 대장균으로

transformation하였다. Transformants 중에서 metalloprotease 유전자를 가진 transformants를 colony hybridization 방법으로 선발하였다. 선발된 9개 클론들로부터 재조합 플라스미드를 분리하여 제한효소지도를 작성한 결과 pUC19에 삽입된 DNA는 모두 같은 DNA 절편으로서 삽입된 방향만 반대인 pSP1과 pSP2임을 알 수 있었다. Western blot analysis 결과 pSP2를 함유한 대장균에서 약 52 KD의 단백질이 만들어졌으나 protease 활성은 없었다. 이러한 결과로 보아 pSP2를 함유한 대장균은 metalloprotease의 전구체 단백질을 발현하는 것으로 사료되었다. 플라스미드 pSP2를 *S. marcescens* ATCC 27117에서 발현시켰을 때 활성이 있는 metalloprotease가 대량 발현됨을 확인하였다.

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