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In Vivo Monitoring System for Structure-Function Relationship Analysis of the Antibacterial Peptide Apidaecin

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A unique antibacterial peptide derivative found in immune honeybee lymph, apidaecin 1b (AP1), was randomly mutagenized and characterized by a newly established system to analyze in vivo its structure-function relationship. Initially, a high-level expression host-vector system for AP1 in *Escherichia coli* was constructed by creating a fusion protein with the highly stable *Streptomyces* subtilisin inhibitor (SSI) molecule. Expression of the SSI-AP1 fusion protein was found to depend on the concentration of the transcriptional inducer isopropyl-β-D-thio-galactopyranoside (IPTG) and to parallel the degree of growth inhibition of the transformant cells. Subsequently, apidaecin derivatives produced by localized random mutagenesis were screened with this IPTG concentration-controlled in vivo system by monitoring the growth inhibition patterns of the transformant cells. One mutant apidaecin (P9L) that had reduced activity was purified and isolated from the periplasmic fraction of an *E. coli* transformant. Its antibacterial activity was reduced to one-third of that of wild-type apidaecin. When considered together with the other mutations, it was concluded that several Pro residues, including that at the ninth position, are responsible for expression of the antibacterial action of apidaecin.

Various peptides with antibacterial actions have been shown to constitute part of the inducible immune response in several insect species. In fact, there is an increasing body of evidence that insects use a wide variety of peptides to resist bacterial infection. For example, antibacterial peptides unrelated to already well characterized insect peptides such as melittin (8) and cecropins (9) have been isolated from honeybee hemolymph. Such a novel type of antibacterial peptide, termed apidaecin (AP), with three different isoforms, was discovered by Casteels et al. (3) in the lymph fluid of the honeybee (*Apis mellifera*). AP is unique in that it consists of 18 amino acids, including three arginine and six proline residues, very stable at high temperature and low pH, and exhibits bacteriostatic action specifically toward gram-negative bacteria such as *Escherichia coli*. However, the antibacterial action has not yet been investigated in detail. Our final goal is to clarify the mode of action of AP and to improve it by genetic manipulation for practical use.

We have already established an efficient system for secretory production of *Streptomyces* subtilisin inhibitor (SSI) (13, 14) and fusion proteins of it with the *Enterococcus faecalis* sex pheromone cAD1 (18) and AP Ib (AP1) (11, 19), using a *Streptomyces* sp. as the vector host. Also, we have demonstrated that not only *E. coli* but also a *Bacillus* strain with weaker protease productivity are sensitive to AP (11).

In this study, to investigate the structure-function relationship of AP through random mutation, we attempted to establish an in vivo system for monitoring the antibacterial activity of the altered AP molecule. The system developed here is characteristic in the sense that *E. coli*, intrinsically sensitive to the attack of the AP molecule, is used as the recipient. It was considered that comparison of several mutant peptides obtained by using this system would enable us to find the determinant residue(s) which gives AP its functional activity. In particular, we describe here the isolation and characterization of one selected mutant, AP1(P9L), with a CCG→CTG mutation corresponding to an amino acid change of Pro→Leu at position 9. To our knowledge, this is the first report of a unique method for genetic analysis of an antibacterial peptide using an in vivo monitoring system combined with random mutagenesis.

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MATERIALS AND METHODS

Materials, bacterial strain, and genetic manipulation. Restriction endonucleases and DNA-modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan), Boehringer (Mannheim, Germany), and Nippon Gene (Tokyo, Japan). All other chemicals were of analytical grade for biochemical use. *E. coli* JM109 (Amersham Co., Ltd.) was used as the host for transformation and gene expression. Preparation of plasmid DNA from *E. coli* cells and transformation of *E. coli* were carried out by standard procedures (16).

Construction of a secretory expression vector for the fusion gene. The detailed scheme for construction of an SSI-AP1 fusion expression vector, termed pOSΔB-AP1, is presented in Fig. 1. pIN-III-*ompA* (7), provided by Dr. Inouye, University of Medicine and Dentistry of New Jersey, is an efficient secretion vector that contains the signal sequence for the OmpA protein of *E. coli* and allows fusion of a foreign protein to the signal sequence. The expression vector for the SSI gene, pOS1, was previously constructed by combining the OmpA signal sequence and SSI mature sequence (20). For the mutational treatment of the AP1 gene, the *Bam*HI site of this plasmid was disrupted by treatment with T4 DNA polymerase to generate a modified plasmid, termed pOΔB. pSI84S1 has a mutationally introduced *Eco*RI restriction site at a position between the signal sequence and the part of the SSI gene encoding the mature part of the protein to establish fusion between the OmpA signal sequence and the SSI mature sequence (20). pSI205ΔEAP1M contains a methionine codon (ATG) just in front of the AP1 gene, introduced by site-directed mutagenesis

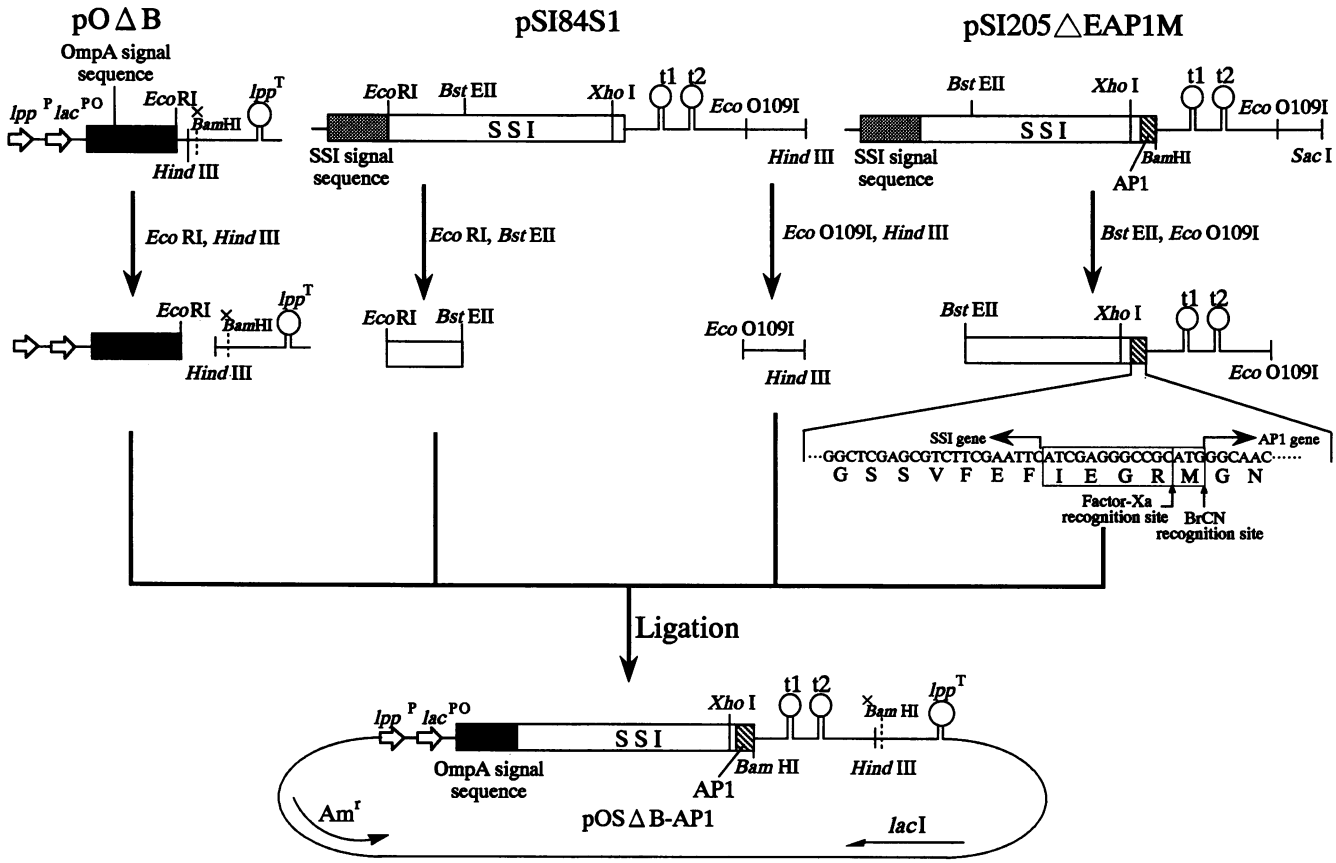

FIG. 1. Construction of a plasmid, pOSΔB-AP1, for secretory expression of SSI-AP1 in *E. coli*. A full explanation is given in Materials and Methods. The heavily and lightly stippled, open-boxed, and hatched regions represent the OmpA and SSI signal sequences and the regions encoding the mature parts of SSI and AP1, respectively. lpp^P, *lpp* promoter; lpp^T, *lpp* terminator; lac^{PO}, *lac* promoter-operator; t1 and t2, putative SSI terminators 1 and 2; Am^r, ampicillin resistance gene; *lacI*, lactose repressor gene; ×, disruption of the *Bam*HI site.

(12). Therefore, the SSI-AP1 fusion gene product is chemically cleavable with BrCN to isolate the AP1 molecule. Combinational ligation of DNA fragments derived from these three plasmids was performed at the same time to generate pOSΔB-AP1.

Detection of expression of recombinant SSI-AP1 and SSI-mutated AP1. *E. coli* JM109 strains harboring pOS1, pOSΔB-AP1, and pOSΔB-AP9L were cultivated in 5 ml of Luria broth (LB) (7) medium supplemented with ampicillin (50 μg/ml) for 16 h at 37°C in the absence or presence of 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) added at various times. In order to analyze the location of the gene products, the supernatant fraction was harvested from the culture, and the periplasmic fraction was extracted from the cell pellet by the method reported previously (17). The cytoplasmic fraction was also prepared from the cell pellet by sonication and centrifugation. Detection of the gene products localized in the three fractions, cytoplasm, periplasm, and culture supernatant, was carried out by sodium dodecyl sulfate–18.8% polyacrylamide gel electrophoresis (SDS-PAGE) (10) and immunoblot analysis (17).

In vitro random mutagenesis. The *Xho*I-*Bam*HI DNA fragment including the whole coding region of the AP1 gene (represented in Fig. 1) was purified electrophoretically from a 0.8% agarose gel and then treated with 0.8 M hydroxylamine at 65°C for 2 h in 0.1 M phosphate buffer (pH 6.0) containing 1 mM EDTA, as described previously (21). The mutagen-treated DNA fragment was dialyzed against 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, precipitated with ethanol, and redissolved in the same buffer for dialysis.

Isolation and purification of mutant apidaecin (P9L). The SSI-mutated AP1(P9L) fusion protein was osmotically extracted from the periplasmic space of transformant cells and purified by two steps of chromatography, as described previously (17, 20). An *E. coli* JM109 transformant harboring the expression plasmid pOSΔB-AP9L was cultivated in 500 ml of LB medium containing ampicillin (50 μg/ml) with IPTG (1 mM) at 37°C for about 20 h. The supernatant fluid, extracted by osmotic shock from the culture, was precipitated with ammonium sulfate (80% saturation) and subjected to ion-exchange chromatography on a DEAE-cellulose column, followed by reverse-phase high-pressure liquid chromatography (RP-HPLC). The purification of the SSI-mutated AP1(P9L) through each chromatography step was monitored by SDS-PAGE, and the micro-Ouchterlony method (15) was also used to monitor it immunologically with anti-SSI antiserum. Chemical digestion with CNBr was used to isolate the mutated AP1 molecule from the SSI-AP1 fusion protein as described before (18). The sample in the reaction mixture was diluted with water, lyophilized, and subjected to RP-HPLC.

Analytical procedures. Analysis of the mutated positions in the AP1 gene was carried out by the dideoxynucleotide chain

termination method with a *BcaBEST* sequencing kit purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). The sequencing primer (SP3; 5′-GGCAGGGCAAGCGGGTC-3′), complementary to the upstream sequence close to the stop codon of the SSI gene, was synthesized by the solid-phase phosphoamidite method with an Applied Biosystems 381A DNA synthesizer (18). Antiserum containing anti-SSI polyclonal antibody was prepared by inoculation of rabbits with purified SSI emulsified with Freund's complete adjuvant. Double immunodiffusion and immunoblot analysis was carried out in 1.2% agarose gel in 10 mM Tris-HCl (pH 7.6) containing 0.1 M NaCl, 20 mM EDTA, 0.2% NaN_3, 2% polyethylene glycol 6000, and 2% dextran T-500, as described previously (13, 17).

Protein samples were routinely subjected to SDS-PAGE analysis. The concentrations of SSI-AP1 and SSI-mutated AP1 fusion proteins were estimated by a modified Lowry method (1) with authentic SSI as a standard. Automated amino acid sequence analysis of the gene product was performed with an Applied Biosystems model 476A protein sequencer. The antibacterial activities of wild-type and mutated AP1 were measured by a microtitration assay with *E. coli* JM109 cells in wells of multititer plates, as reported before (19). The assay was performed by adding each purified sample to 100 μl of 10% nutrient broth (NB) medium inoculated with 3×10^4 cells of the test strain, *E. coli* JM109, followed by overnight culture at 37°C under static conditions in a multititer plate with 96 wells. Experimental reproducibility was confirmed by two trials.

RESULTS

In vivo monitoring system for AP activity. To genetically analyze the structure-function relationship of the AP1 molecule, we attempted to make use of the fact that *E. coli* is intrinsically sensitive to AP1. There are two opposite problems, however; one is the supposed fragility of AP1 molecule, and the other is the difficulty of obtaining an AP1-producing *E. coli* transformant because of a kind of suicide effect.

As for the former problem, it is generally observed that a soluble (especially small) polypeptide is susceptible to proteolytic decomposition inside or outside the cell, and thus the yield of a recombinant peptide is reduced. This might also be the case with AP1. We had previously succeeded in producing AP1 stably as a fusion protein with the SSI molecule, which acts as a protector, using the *Streptomyces lividans* host-secretory vector system, and found that the protein had bifunctional activity, full subtilisin-inhibitory activity and antibacterial activity (19). Therefore, we tried to solve the former problem by producing an SSI-AP1 fusion protein (still active as AP1) in *E. coli.*

To solve the latter problem, we adopted an efficiently controlled expression system with the pIN-III-*ompA* vector to realize the suicide effect only when it is necessary. Thus, we constructed a secretory production system for the SSI-AP1 fusion protein in *E. coli* (20) according to the scheme shown in Fig. 1. To investigate the influence of SSI-AP1 production on the growth of *E. coli*, the resulting plasmid, pOSΔB-AP1, was introduced into *E. coli* JM109. Figure 2B shows the growth curves of the transformant carrying pOSΔB-AP1. Growth inhibition was clearly observed only when the transcriptional inducer of the SSI-AP1 fusion gene, IPTG, was added, and the degree of inhibition was stronger when IPTG was added earlier. On the other hand, the control strain producing SSI efficiently with plasmid pOS1 showed almost no growth inhibition (Fig. 2A). These results strongly suggest that the growth inhibition of the host cell was due to the antibacterial activity of the SSI-AP1 produced (the suicide effect).

In order to investigate the localization of the SSI-AP1 fusion gene products, cytoplasmic, periplasmic, and extracellular fractions of the cells induced with IPTG (at an optical density of 0.5) and cultivated for 6 h to reach the stationary phase were subjected to SDS-PAGE and immunoblot analysis. An SSI-AP1-specific band with apparently the same mobility as that of recombinant wild-type SSI-AP1 produced by *S. lividans* (11) could be clearly identified in all three fractions (Fig. 3). The production pattern of SSI-AP1 in each fraction was detected by a method similar to that used for SSI (20), although their quantities could not be estimated. Also, several bands probably corresponding to degraded SSI-AP1 species were detected in the lower zone of the SSI-AP1-specific band. This suggested that the growth inhibition of *E. coli* mentioned above was caused by SSI-AP1 itself and/or by degraded SSI-AP1 species.

On the basis of these findings, we attempted to establish an in vivo monitoring system for the screening of mutant AP1 molecules with different activities using *E. coli*, which is intrinsically sensitive to AP1. First, the IPTG concentration dependency of growth inhibition caused by SSI-AP1 gene expression was examined. Since IPTG is a well-known switching material for the *lac* operon, controlling the transcription of the SSI-AP1 fusion gene, its use would be convenient and quantitative in the SSI-AP1 gene expression experiment. As shown in Fig. 4, a good correlation between IPTG concentration and transformant colony size was observed. This result suggested that the amino acid residue(s) responsible for expression of the antibacterial activity of AP1 might be determined. Namely, if mutant AP1 can grow on a plate supplemented with IPTG at a concentration (0.03 mM in this system) sufficient to inhibit the growth of wild-type *E. coli*, then the mutation might be considered to be related to the antibacterial action of AP1, and such a mutant could be positively selected. Therefore, we next introduced in vitro random mutations into the *Xho*I-*Bam*HI DNA fragment containing the full-length gene encoding AP1 by using hydroxylamine as a chemical mutagen. The mutagenized DNA fragments, including heterogeneous mutant AP1 genes, were religated at the *Xho*I and *Bam*HI sites with the original plasmid DNA (pOSΔB-AP1), including the *lac* promoter, and then introduced into *E. coli*. The resulting ampicillin-resistant transformant colonies were formed at room temperature on a plate containing IPTG (at a rather stringent concentration of 0.04 mM) and ampicillin.

The frequency of appearance of colonies on a plate containing 0.04 mM IPTG relative to that on a plate without IPTG was calculated to be about 7%. For identification of the mutation point(s) of each arbitrarily selected mutant AP1 with reduced activity, we sequenced mutated AP1 genes prepared from five transformant strains. The results of the sequence analysis are summarized in Fig. 5. The five transformants included two identical single mutations (Pro-9 to Leu [P9L]) and three double mutations (P14S and R17C, P11L and R17C, and P5L and R12Q). Interestingly, the mutation positions were significantly concentrated at Pro residues.

We also explored the effect of expression of each mutant AP1 gene on the growth of *E. coli*. As shown in Fig. 6, one representative mutant, AP1(P9L), had a lower inhibitory effect than wild-type AP1.

Purification and characterization of mutant AP. In a small-scale preliminary expression experiment done with the above-mentioned *E. coli*-pOSΔB-AP9L system, we observed almost the same patterns of production in the cytoplasm, periplasm, and culture medium as with the wild-type *E. coli*-pOSΔB-AP1 (Fig. 3). The production level was higher than that of *E. coli* carrying the wild-type AP1 fusion gene (data not shown). This suggested that the decrease in the antibacterial activity of the

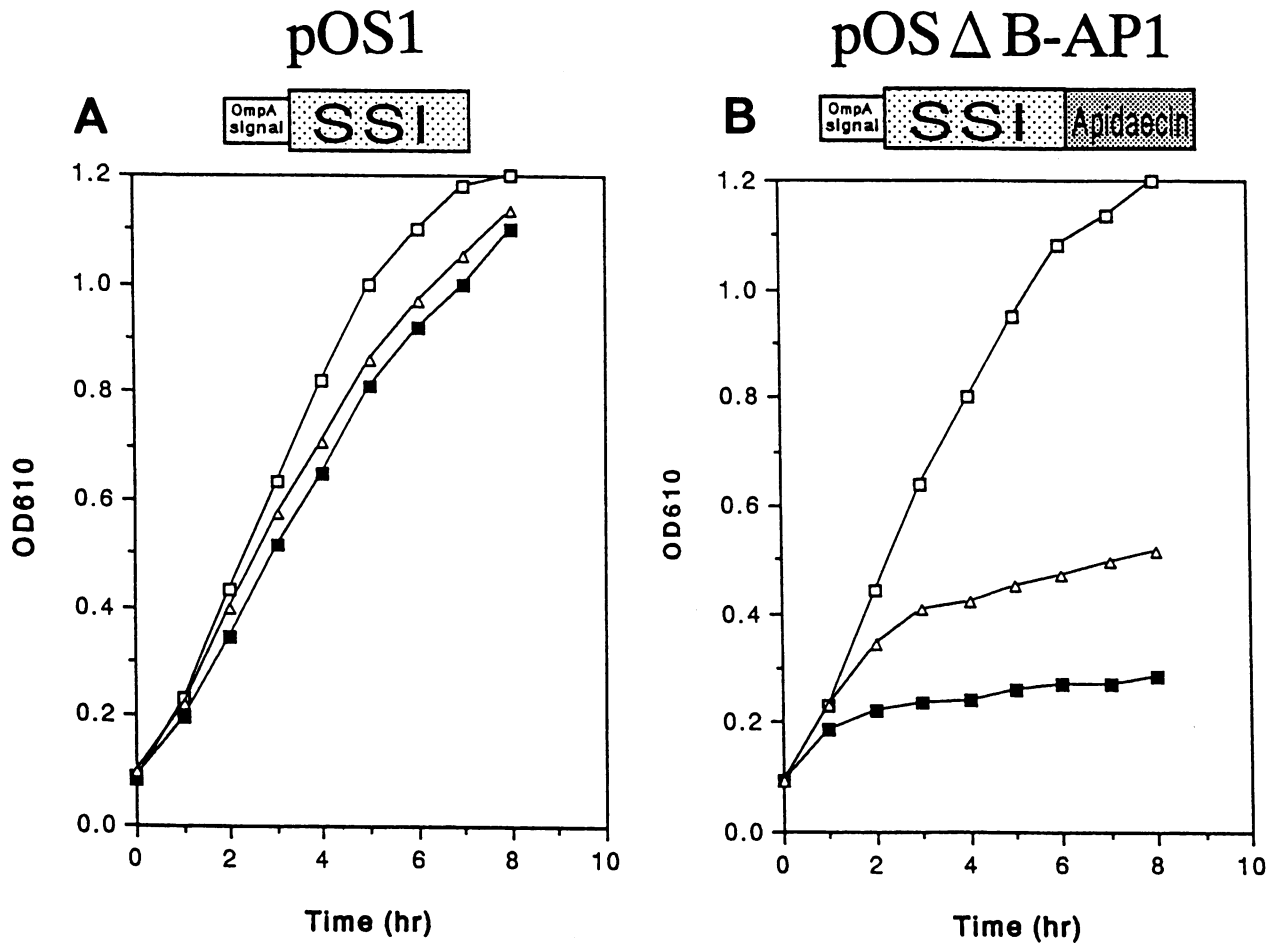

FIG. 2. Growth patterns of transformants carrying pOS1 and pOSΔB-AP1. The effects of SSI (A) and SSI-AP1 (B) gene expression by IPTG induction at various cultivation times on the growth of *E. coli* JM109 carrying pOS1 or pOSΔB-AP1 were monitored spectrophotometrically. Experimental details are given in Materials and Methods. □, without IPTG; ■, with 1 mM IPTG added at 0 h; △, with 1 mM IPTG added when the optical density at 610 nm reached 0.2.

mutated AP1 brought about the increase in its production level. Therefore, we expected that the SSI-mutant AP(P9L) fusion protein might be isolated directly from the periplasm of the transformant cells. In order to test whether the reduction of growth inhibition observed in *E. coli* producing SSI-mutant AP1(P9L) was indeed due to the replacement of Pro by Leu at the ninth residue of the AP1 molecule, we then attempted to isolate and characterize the P9L mutant peptide.

The amount of the SSI-AP1(P9L) fusion protein produced was 32.2% of that of SSI alone produced by the same system (20). Figure 7 shows the SDS-PAGE pattern of the fusion protein at each step of the purification procedure, beginning with the culture supernatant and followed by ammonium sulfate precipitation (recovered sample, about 20 mg; Fig. 7, lane 3), ion-exchange chromatography (recovered, about 1 mg from the top peak fraction [lane 4] and about 10 mg from all the fractions [lane 5]), and RP-HPLC (recovered, 7.2 mg; lane 6). In lane 5 of Fig. 7, minor bands of slightly lower molecular weight were observed. Since these proteins showed a positive reaction with anti-SSI antiserum in immunoblot analysis (data not shown), it seems that the fusion protein was slightly degraded by the periplasmic protease(s) of the host cell.

The isolated fusion protein was cleaved with CNBr to release the mutant AP1 from its fusion partner, SSI. On

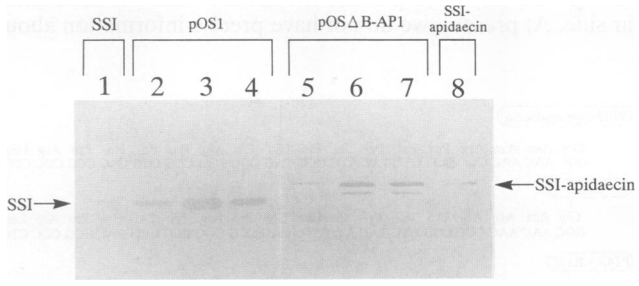

FIG. 3. Immunoblot analysis. Each transformant cell was cultivated in 5 ml of LB medium containing 1 mM IPTG and 50 μg of ampicillin per ml at 30°C for 16 h. Lane 1, authentic SSI (100 ng); lane 2, cytoplasmic fraction of JM109 carrying pOS1 (2.5% of total sample volume); lane 3, periplasmic fraction of JM109 carrying pOS1 (2.5% sample); lane 4, medium fraction of JM109 carrying pOS1 (2.5% sample); lane 5, cytoplasmic fraction of JM109 carrying pOSΔB-AP1 (10% sample); lane 6, periplasmic fraction of JM109 carrying pOSΔB-AP1 (10% sample); lane 7, medium fraction of JM109 carrying pOSΔB-AP1 (10% sample); lane 8, pure SSI-AP1 produced by *S. lividans* 66 (1.75 μg).

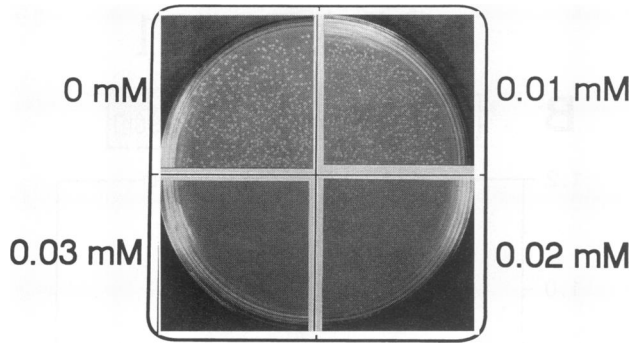

FIG. 4. IPTG concentration dependency in colony formation by transformed cells expressing SSI-AP1. After adequate dilution of suspended cells, transformant colonies (JM109/pOSΔB-AP1) were incubated until they became visible on each plate (in the absence of or supplemented with 0.01, 0.02, or 0.03 mM IPTG) at 37°C for 15 h.

RP-HPLC (Fig. 8), the mutant AP1 had a slightly longer retention time than the wild-type AP1. This may be due to the mutational alteration of the Pro residue to a more hydrophobic one. Sequencing analysis of the recombinant AP1(P9L) purified by RP-HPLC revealed the reasonable result of a Pro→Leu alteration.

Finally, purified mutant AP1(P9L) was examined for its antibacterial activity against *E. coli* JM109 (data not shown). The mutation from Pro to Leu at the ninth position in the AP1 molecule was found to reduce the antibacterial activity by one-third.

DISCUSSION

In this study, we established an in vivo system for the screening of a variety of AP1 evolvants with altered antibacterial properties, in which inducer-dependent growth inhibition was used for *E. coli* carrying a high-level secretory expression vector producing AP1 as a fusion protein with SSI. The cell envelope of *E. coli* has a complex structure, composed of three morphologically distinct layers: a cytoplasmic membrane, a rigid peptidoglycan layer external to the cytoplasmic membrane, and an outer membrane at the surface of the cell. In this system, SSI-AP1 and SSI-mutant AP1 proteins can pass through the inner cytoplasmic membrane from the intracellular side. At present, we do not have precise information about

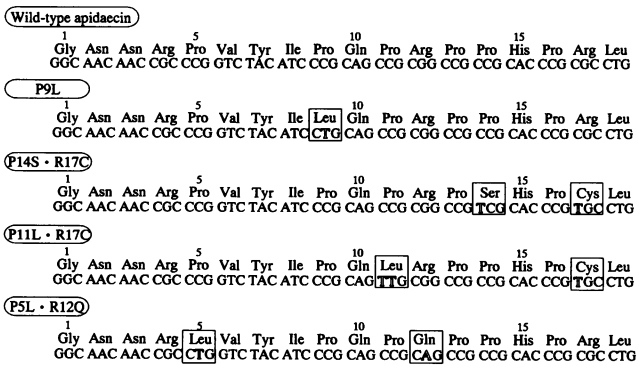

FIG. 5. Identification of mutation(s) in mutated AP1 genes. Open letters, mutated nucleotides; mutated codons and consequently altered amino acid residues are boxed.

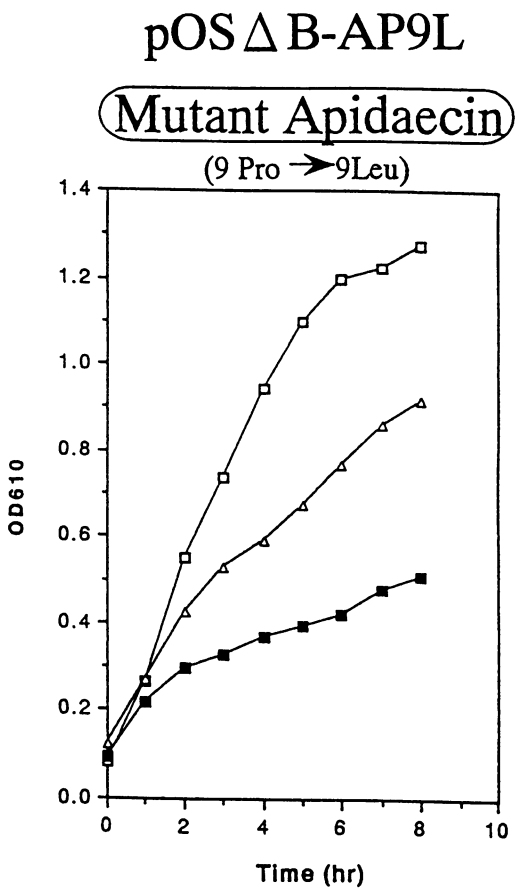

FIG. 6. Growth pattern of transformant carrying pOSΔB-AP9L. The effect of SSI-AP1(P9L) gene expression after IPTG induction at various cultivation times on the growth of *E. coli* JM109 carrying pOSΔB-AP9L was monitored spectrophotometrically under the conditions described in the legend to Fig. 2. Symbols are the same as in Fig. 2.

where and how inhibition occurs. It has been reported that hymenoptaecin, a larger (93 amino acids), cationic and Pro-rich polypeptide found in AP1-producing honeybees, exerts a lethal effect on *E. coli* through sequential permeabilization of

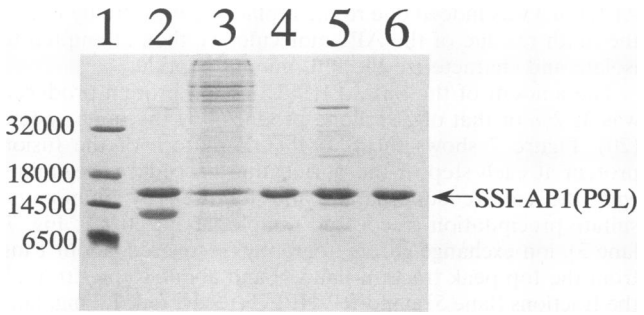

FIG. 7. SDS-PAGE analysis at each step of the purification of recombinant SSI-AP1(P9L). Lane 1, molecular size markers (in daltons); lane 2, mixture of authentic SSI and purified recombinant SSI-AP1; lane 3, ammonium sulfate (80% saturation)-precipitated culture filtrate of *E. coli* harboring pOSΔB-AP9L; lanes 4 and 5, samples purified by ion-exchange (DEAE) chromatography (4, top peak fraction; 5, all fractions containing SSI-AP1 protein); lane 6, sample purified by RP-HPLC.

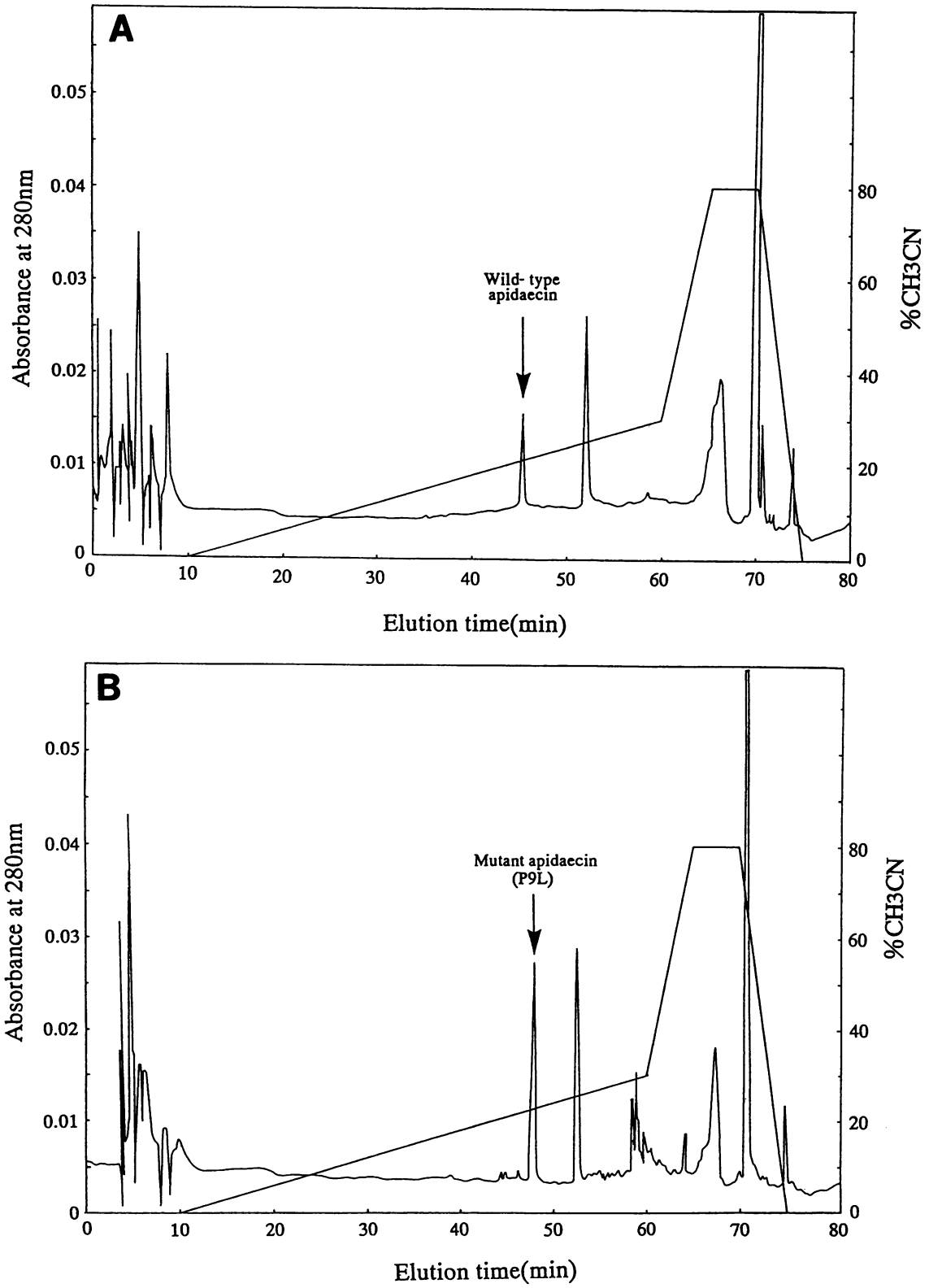

FIG. 8. HPLC profiles of SSI-AP1 and SSI-AP1(P9L) digested with CNBr. Each fusion sample treated with CNBr was applied to an RP-HPLC (C18) column. Arrows indicate peaks having (A) full wild-type AP1 antibacterial activity and (B) the predicted peptide sequence of AP1(P9L).

the outer and inner membranes (2). In contrast, Casteels and Tempst reported that AP1 lacks membrane permeability and functions through a nonpore-forming mechanism (5). These findings, if true, suggest that an unknown target molecule(s) against AP1 attack would be present on the cell surface and that the antibacterial effect of SSI-AP1 molecules excreted into the medium would lead to the growth inhibition of host cells by affecting some target molecule(s).

Since the change in growth inhibition pattern corresponded well with that in antibacterial activity, the in vivo system presented in this study has proved useful for easy monitoring of various antibacterial activities of randomly mutated AP1 molecules and should be used as a general tool to study the determinant residue(s) for the action of various antibacterial peptides. Furthermore, and more intriguingly, this system might be applicable to genetic improvement of these peptides for practical use. Namely, evolved AP1s with increased antibacterial activity would be gained when transformant colonies smaller than wild-type AP1 gene-carrying transformant colonies occur on the IPTG concentration-controlled growth plate. Work in this direction is now in progress.

The mutant AP1(P9L) was found to have one-third the antibacterial activity of the wild-type AP1, and this decrease in activity is a common feature for other mutants with Pro substitutions at positions 5, 9, and 14. This finding suggests that the Pro residues abundantly present in the AP1 molecule play an important role in its mode of action. Similar Pro-rich sequence motifs have also been found in abaecin (from bee) (4) and diptericins (from flies) (22). Particularly, Casteels-Josson et al., on the basis of cDNA sequence analysis, suggested that a fourth AP1 isoform with Ser at position 9 is actively silent (6). However, we cannot exclude the possibility that the presence of many Pro residues (33%) and the use of hydroxylamine with C→T converting activity render Pro codons hot spots for mutation. Additionally, we still have to clarify the contribution of the individual mutations of doubly mutated AP1 molecules. When we added a methionine codon (ATG) just in front of the AP1 gene by site-directed mutagenesis, an artificially generated mutant (I8T) was obtained. The pure AP1(I8T) produced by the *S. lividans* host-vector system (11) exhibited almost the same antibacterial activity as wild-type AP1 (data not shown). Identification of the silent mutation produced by our screening system would also provide further useful information on the structure-function relationship of AP1.

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