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# Alanine-Scanning Mutagenesis of HM-1 Killer Toxin and the Essential Residues for Killing Activity

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**Each of the aromatic, acidic and basic amino acid residues in HM-1 were separately substituted with alanine by site-directed mutagenesis. The mutant genes were successfully expressed in HM-1 resistant *Saccharomyces cerevisiae*. HM-1 gene analogues corresponding to the aromatic substitutions resulted in lower production of HM-1 analogues. In the case of the acidic amino acid residue and basic amino acid residue substitutions, some analogues were produced in the same amount as and exhibited similar killing activity to that of the wild type HM-1. But the H35A HM-1 analogue had completely lost the killing activity, and D44A, K21A, K46A, R82A, R85A and R86A HM-1 showed highly decreased killing activities. These results strongly indicate the importance of histidine-35, aspartic acid-44, lysine-21, lysine-46, and C-terminal arginine residues in HM-1 for the killing activity.**

**Key words: alanine scanning, HM-1, killer toxin, site-directed mutagenesis.**

Abbreviations: HM-1, HM-1 killer toxin; ELISA, enzyme-linked immunosorbent assay; HYI, HYI killer toxin.

HM-1 is produced by the yeast *Williopsis saturnus* var. *mrakii* IFO 0895 (*1*, *2*). HM-1 kills several yeast strains effectively and belongs to the K9-type killer toxin group. HM-1 consists of 88 amino acids, and is very stable against heat treatment and at the pH range of 2–11 (*3*). When sensitive yeasts were treated with HM-1, discharging of the cell contents at the budding position was observed on phase-contrast and scanning electron microscopy (*4*). The yeast killing mode of HM-1 is similar to that of anti-fungal compounds aculeacin A and papulacandin B (*5*, *6*). On the other hand, HM-1 sensitive yeast spheroplast cells are not killed by HM-1 even after prolonged incubation for 24 h. HM-1 treated spheroplasts became enlarged, retaining their round shape without multiplication (*7*). It is believed that HM-1 binds weakly to the cell wall at first, followed by binding to a putative receptor on the cell membrane, and it finally inhibits β-1,3-glucan synthesis (*8*–*12*). In this study, to identify the important amino acid residues for the yeast killing action of HM-1, we performed site-directed mutagenesis, and found the importance of the specific features of histidine-35, aspartic acid-44, lysine-21, lysine-46, and C-terminal arginine residues of HM-1.

## MATERIALS AND METHODS

*Materials*—HM-1, the HM-1 gene, plasmid YEp51, which contains the *Gal10* promotor, the HM-1 resistant strain of *Saccharomyces cerevisiae* BJ1824 *rhk1*Δ::*URA3,* and other strains were obtained as reported previously (*13*, *14*). The YPD medium consisted of 1% yeast extract, and 2% each of peptone and glucose. Rabbit polyclonal and mouse monoclonal antibodies against HM-1 were prepared using purified HM-1 by Nippon Bio-Test Laboratories Inc. (Tokyo). The LA PCR *in vitro* mutagenesis kit was obtained from Takara Shuzo Co., Ltd. (Tokyo) (*15*), and the oligonucleotide primers used in this study are shown in Table 1.

Table 1. **Primers used for alanine-scanning mutagenesis.**

| Mutant | Primer |
|---|---|
| F79A | 5′-AGCGAATGTAAGCCCCTGTGTTG-3′ |
| W20A | 5′-AGTTCTGCTTAGCATCACAGCTA-3′ |
| W24A | 5′-CACAAGTATTAGCGTTCTGCTTC-3′ |
| W36A | 5′-CTGTAACCATAGCGTGGACATTA-3′ |
| W53A | 5′-CTGAGCCTTCAGCGATTGTAGCA-3′ |
| Y4A | 5′-ACATAATCAGAGCTCCATCACCA-3′ |
| Y80A | 5′-AAGAGCGAATAGCGAACCCTGTG-3′ |
| Y84A | 5′-CCACACGTCTAGCAGAGCGAATG-3′ |
| D2A | 5′-TCAGGTATCCAGCACCACGCTTC-3′ |
| K9A | 5′-GGTCACAGTTAGCGCACATAATC-3′ |
| D12A | 5′-CCGTGTTTGGAGCACAGTTTTTG-3′ |
| D19A | 5′-TCTGCTTCCAAGCACAGCTACCC-3′ |
| K21A | 5′-TCCAGTTCTGAGCCCAATCACAG-3′ |
| H35A | 5′-TAACCATCCAAGCGACATTAGCT-3′ |
| D44A | 5′-CTTGCTTCCCAGCAGTGCTGCCG-3′ |
| K46A | 5′-CACACCCTTGAGCCCCATCAGTG-3′ |
| E54A | 5′-ATCCTGAGCCAGCCCAGATTGTA-3′ |
| R61A | 5′-TTGTGGTTGAAGCACCCACACAT-3′ |
| R82A | 5′-GTCTGTAAGAAGCAATGTAGAAC-3′ |
| R85A | 5′-ATTCCACACGTGCGTAAGAGCGA-3′ |
| R86A | 5′-CCTATTCCACAGCTCTGTAAGAG-3′ |
| E88A | 5′-TAATCTCCTAAGCCACACGTCTG-3′ |

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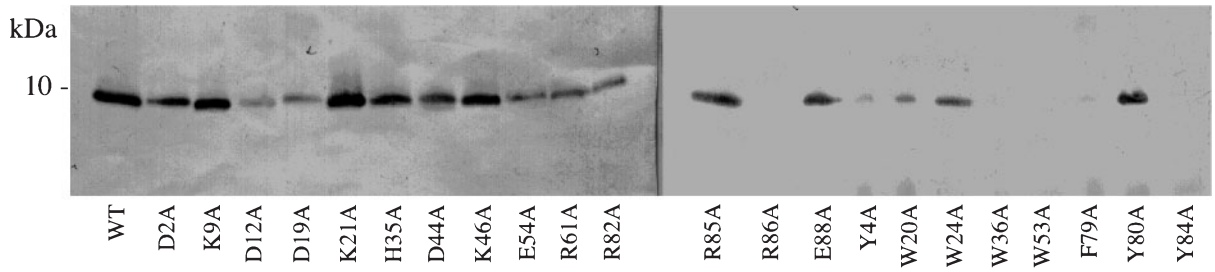


Fig. 1. **Western blotting of HM-1 analogues.** Five microliter aliquots of 20 times concentrated samples were subjected to 15% SDS-PAGE, followed by immunoblotting. WT, wild type.

*General Methods*—The recombinant DNA methods used were those of Sambrook *et al.* (*16*). The DNA sequence was determined by the dideoxy method using fluorescence labeled dNTPs with a Long Read Tower DNA sequencer (Amersham Pharmacia Biotech, Tokyo). The three-dimensional structure of HM-1 was analyzed using the NMR data (*17*). Quantification of HM-1 and its analogues was carried out by the ELISA method using the purified HM-1, and rabbit polyclonal and mouse monoclonal antibodies (*18*).

*Construction of Mutant HM-1 Genes and Their Expression*—Construction of mutant HM-1 genes and their expression were carried out as reported previously (*14*). The mutant genes were transformed into HM-1 resistant *S. cerevisiae* BJ1824 *rhk1*Δ::*URA3*. The HM-1 toxin or analogue gene-bearing yeasts were grown for 48 h at 30°C at 120 rpm in liquid medium consisting of 1% yeast extract, 2% peptone, 2% galactose and 0.5% sucrose (YPGal+suc). After the cultivation, a supernatant was obtained by centrifugation and passed through a cellulose acetate filter (pore size, 0.45 μm), and then dialyzed against deionized distilled water overnight. The dialyzate was concentrated with a centrifugal concentrator and then kept at 4°C.

*SDS-PAGE and Western Blotting*—The SDS-PAGE and immunoblotting methods used were as reported previously (*19*, *20*). HM-1 and its analogues were detected as the intensity of oxidized 3-amino-9-ethylcarbazole using the anti–HM-1 rabbit polyclonal antibody as the first antibody and horseradish peroxidase conjugated anti–rabbit goat IgG as the secondary antibody.

*Measurement of Killing Activity of HM-1*—The killer eclipse assay was performed by the method described by Kishida *et al.* using *Hansenula anomala* IFO 0569 and *Candida albicans* ATCC 10231 as test strains (*21*). Three microliters of a test strain at the concentration of $10^6$ cells/ml was plated on YPGal+suc agar plates, and then mutant HM-1 gene–bearing yeasts were planted at the edge of the test strain spot. The plates were incubated for 24 h at 30°C, and then photographed. To determine $IC_{50}$, the concentration required for 50% inhibition of growth of sensitive yeast *S. cerevisiae* A451, cells at the logarithmic phase were incubated with various concentrations of HM-1 or its analogues at 30°C with reciprocal shaking at 120 rpm. After 12 h incubation, the optical densities of the culture broth were measured with a spectrophotometer at 600 nm, and then $IC_{50}$ values were determined using semi-logarithmic graphs.

## RESULTS

*SDS-PAGE of HM-1 Analogues Secreted by Alanine Mutant Gene–Bearing Yeasts*—The concentrated culture supernatants of HM-1 gene or mutant gene–bearing yeasts were subjected to SDS-PAGE and Western blotting (Fig. 1). Almost all the supernatants gave a 9.5 kDa band, which corresponded to the molecular size of the wild type HM-1, but the band intensities varied among

Table 2. **The amounts and $IC_{50}$ values of HM-1 analogues secreted by HM-1 analogue gene–bearing yeasts.**

| Analogue | Concentration (ng/ml) | $IC_{50}$ (ng/ml) | Analogue | Concentration (ng/ml) | $IC_{50}$ (ng/ml) |
|---|---|---|---|---|---|
| wild type | 2,160 | 84.9 | D2A | 875 | 94.5 |
| F79A | 15.1 | >34 | D12A | 489 | 86.0 |
| W20A | 26.1 | >79 | D19A | 253 | 66.7 |
| W24A | 69.7 | >119 | D44A | 3,590 | 718 |
| W36A | 22.7 | >64 | E54A | 101 | 27.5 |
| W53A | ND | ND | E88A | 52.5 | 9.66 |
| Y4A | 14.7 | >41 | H35A | 870 | 2,580 |
| Y80A | 1,590 | 72.2 | K9A | 765 | 125 |
| Y84A | ND | ND | K21A | 2,000 | 519 |
| | | | K46A | 10,200 | 1,060 |
| | | | R61A | 109 | 41.4 |
| | | | R82A | 119 | 414 |
| | | | R85A | 3,040 | 821 |
| | | | R86A | 36.9 | 443 |

ND, not detected.

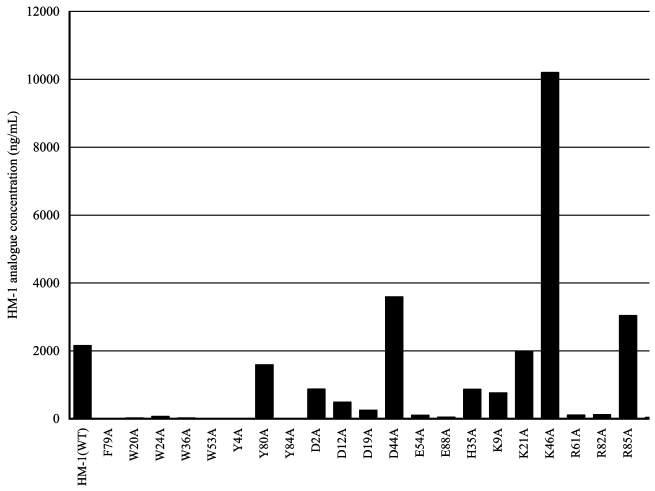


Fig. 2. **The amounts of HM-1 analogues secreted by HM-1 analogue gene–bearing yeasts.** The amounts of HM-1 analogues produced by mutant gene–bearing yeasts were determined by the ELISA method. WT, wild type.

the supernatants. W36A, W53A, Y84A and R86A HM-1 did not give this band, probably due to their low production. This finding indicates that the expression these mutant HM-1 genes was successful and that the amounts of HM-1 analogues produced varied among the mutant genes.

*Quantification of HM-1 Analogues Secreted into the Medium*—Using concentrated culture supernatants, the amounts of HM-1 analogues secreted by mutant gene–bearing yeasts were determined by the ELISA method. The amounts of HM-1 secreted varied among the analogues (Table 2 and Fig. 2). As shown in Fig. 1, two HM-1 analogues, Y80A and K21A, are produced in the culture broth in nearly equal amounts to that of the amounts of the wild type HM-1. The amounts of the D2A, D12A, D19A, H35A and K9A HM-1 analogues were lower than that of the wild type HM-1, and those of D44A and R85A HM-1 were relatively higher. The K46A HM-1 production was the highest among the analogues, being by five-fold that of the wild type HM-1. The aromatic amino acid residue-substituted analogues except for Y80A HM-1, E54A, E88A, R61A, R82A and R86A HM-1 were produced in very minute amounts.

*Determination of Killing Activity by Killer Eclipse Assaying*—To determine the killing activity of HM-1 analogues secreted by mutant gene-bearing yeasts toward sensitive yeasts, we carried out the killer eclipse assay (Fig. 3). The two yeast strains, *H. anomala* and *C. albicans,* used in this study exhibited different degrees of sensitivity to wild type HM-1. The D12A, D44A and K46A gene-bearing yeasts showed strong killing activity toward *H. anomala*, the potency being almost the same as that of wild type HM-1, whereas the D2A, D19A, E54A, E88A, K9A, K21A, R61A and R85A gene–bearing yeasts showed weaker killing activity. On the other hand, the H35A, R82A and R86A gene bearing yeasts exhibited no such activity. *C. albicans* was not killed by wild type HM-1 or any polar amino acid substituted HM-1 gene–bearing yeast (Fig. 3B). For aromatic amino acid residue substituted analogues except Y80A HM-1, activity could not be detected toward either yeast (data not shown).

*Killing Activity Determination as $IC_{50}$ Values*—The killing activities of HM-1 analogues measured as $IC_{50}$ are shown in Table 2 and Fig. 4. Y80A, D2A, D12A, D19A, E54A, K9A and R61A HM-1 showed similar activity to that of the wild type HM-1. D44A, H35A, K21A, K46A, R82A, R85A and R86A HM-1 showed high $IC_{50}$ values, that of H35A HM-1 being 30 times higher than that of the wild type. The lowest $IC_{50}$ value was found for E88A HM-1. In the case of aromatic amino acid residue–substituted analogues except Y80A, we could not determine the $IC_{50}$ values because of the very low quantities of analogues in the culture supernatants.

## DISCUSSION

To determine the important residues for the killing activity of HM-1 toxin, we substituted specific residues with alanine, and then determined the secreted amounts of analogues and their killing activities. Figure 1 shows that almost all the HM-1 mutants produced are of the same size, but their quantities differ in the culture medium. When the aromatic amino acid residues were substituted with alanine, the production of HM-1 analogues decreased strikingly except in the case of Y80A HM-1 (Table 2 and Fig. 2). These aromatic residues may contribute to the formation of the core structure of HM-1 through hydrophobic interaction through a folding process and 3-dimensional structure formation, and

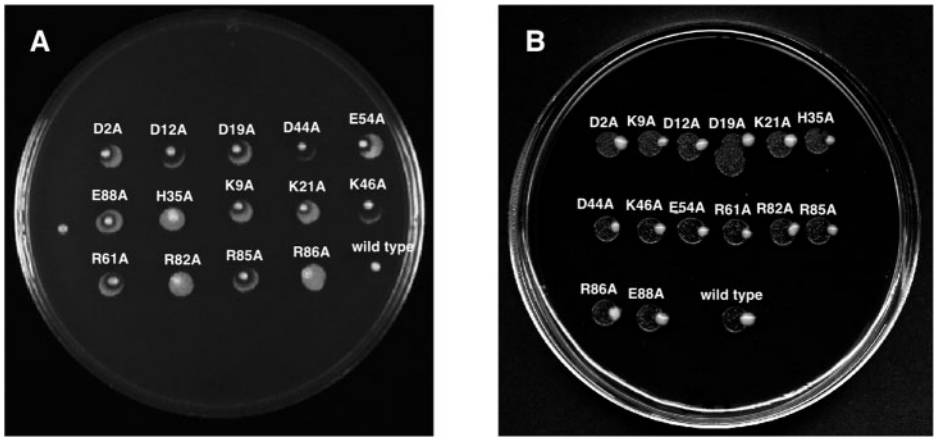


Fig. 3. **Killer eclipse assay for HM-1 analogues.** (A) *H. anomala* IFO 0569 and (B) *C. albicans* ATCC 10231 were used as test strains.

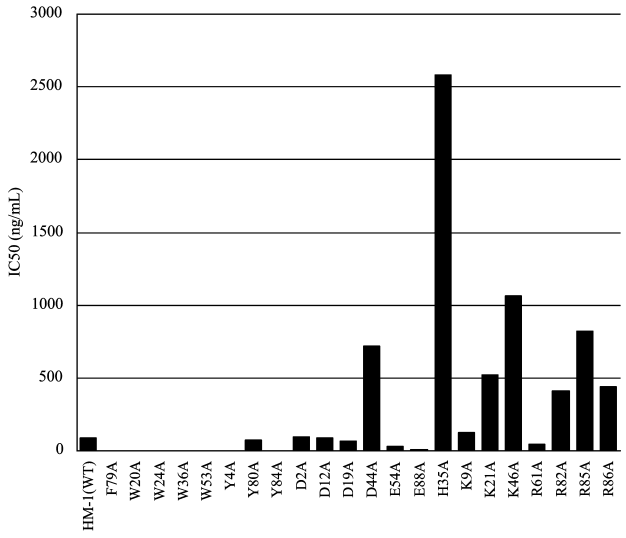


Fig. 4. **$IC_{50}$ values of HM-1 analogue toxins against *S. cerevisiae* A451.** $IC_{50}$ values were determined as described under "MATERIALS AND METHODS." WT, wild type.

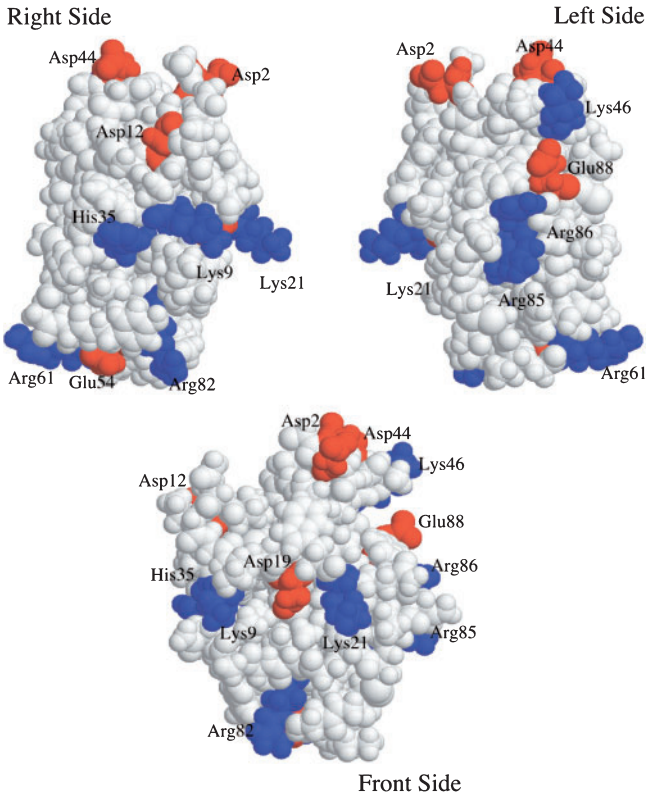


Fig. 5. **The three-dimensional structure of HM-1.** An image of the three-dimensional structure of HM-1 was obtained using RasMol software. All the basic amino acid residues in HM-1, *i.e.*, Lys-9, Lys-21, His-35, Lys-46, Arg-61, Arg-82, Arg-85 and Arg-86, are colored blue. All the acidic amino acid residues in HM-1, *i.e.*, Asp-2, Asp-12, Asp-19, Asp-44, Glu-54 and Glu-88, are colored red.

without these interactions, HM-1 analogues fold into an unstable structure and must be degraded through proteolysis (*22*, *23*).

Substitution of charged residues yielded varying amounts of HM-1 analogues. In the case of acidic amino acid residues, the amounts of HM-1 analogues secreted were low except for D44A, the amount of E88A HM-1 being 1/41 that of the wild type. Probably, glutamic acid-88 contributes to the C-terminal folding of HM-1 due to its negatively charged side chain. The K46A HM-1 production was five times higher than that of the wild type HM-1. But, some basic amino acid substitutions, R61A, R82A and R86A, decreased the secreted amount. In some proteins, charged amino acid residues form salt bridges to stabilize the biologically active structure (*24–28*). In recombinant *Torpedo californica* acetylcholinesterase, arginine-44 and glutamic acid-92 interact to form a salt bridge, which plays an important role in correct folding and transport to the cell surface (*29*). In the case of HM-1, two arginine residues, arginine-85 and -86, and the glutamic acid-88 residue may interact with each other, and may play important roles in the folding process and maintenance of the biologically active structure of HM-1. This hypothesis is supported by the results of NMR structure analysis (*17*), which showed that arginine-85, -86, and glutamic acid-88 are folded closely on the wild type HM-1 protein surface (Fig. 5).

It was reported earlier that substitution of arginine-82 and -86 with alanine decreased the killing activity, and substitution of arginine-82 with lysine restored the killing activity to some extent (*14*), these results indicating the importance of the positive charge for the killing activity. Other basic amino acid substitutions also had drastic effects on the killing activity (Table 2 and Fig. 4). In particular, H35A HM-1 exhibited strikingly decreased killing activity, possibly because histidine-35 plays an important role in the interaction with the receptor on the cell wall or the target enzyme. The K21A, R82A, R85A, R86A substitutions decreased its killing activity to similar levels. In the case of SKLP, *Streptomyces* killer toxin-like protein, the negatively charged region interacts with unidentified targets (*30*), which is contrary to the case of HM-1. But it is undeniable that replacement of these arginine residues destabilizes the biologically active structure and decreases the killing activity of HM-1.

The obtained results showed that the residues involved in the killing activity of HM-1 were scattered. Arginine-85 and -86 form a positively charged region that is located on the opposite side to histidine-35 and also far from arginine-82 in the HM-1 molecule (Fig. 5). It is assumed that the killing action of *S. cerevisiae* killer toxin, K1, is a multi-step procedure. The first step is binding of the toxin to the cell wall receptors containing β-1,6-glucan, and the next step is interaction with the plasma membrane receptors (*31–33*). Probably, in HM-1, histidine-35, arginine-82, arginine-85 and arginine-86 each play a different role in a different step of the killing action of HM-1.

The K46A substitution also decreased the killing activity greatly, but not completely ($IC_{50}$ = 1,060 ng/ml). HYI produced by *Williopsis saturnus* var. *saturnus* has a primary structure exhibiting 87% similarity with HM-1 (*6*, *34*). But, the $IC_{50}$ value of HYI against *S. cerevisiae* A451 is 2.8 times higher than that of HM-1, and HYI does not have a lysine at position 46. Lysine-46 is located near arginine-85 and -86, and may participate in the posi-

tively charged region in HM-1 (Fig. 5). Therefore, different from HYI, the positive charge of the C-terminal domain of HM-1 is reinforced by lysine-46. This evidence indicates that lysine-46 may amplify the killing activity of HM-1.

The E88A substitution decreased the $IC_{50}$ value to 1/8.8 of that of the wild type HM-1. The substitution of glutamic acid-88 with alanine causes a net positive charge gain by the C-terminal domain and, therefore, it may cause increased HM-1 killing activity.

Most of the other acidic amino acid substitutions had small effects on the killing activity. But, the D44A substitution decreased the killing activity, the $IC_{50}$ value being 8.5 times higher than that of the wild type HM-1, and the aspartic acid-44 substitution with arginine also decreased the killing activity (data not shown). Aspartic acid-44 is located near lysine-46 (Fig. 5). Moreover, HYI does not have an acidic amino acid residue at position 44. Aspartic acid-44 is not essential, but may increase HM-1 killing activity by interacting with lysine-46 of HM-1 or with the HM-1 receptor through its negative charged side chain.

In conclusion, using the site-directed mutagenesis method, we could identify the important amino acid residues for the killing action of HM-1. It is important to know the tertiary structures of mutated HM-1 molecules for elucidation of the roles of the replaced amino acid residues. But, at present, we can not analyze the tertiary structure of HM-1 because of the low HM-1 analogue production and the lack of established procedures for the purification of mutated HM-1. We are now trying to produce larger amounts of HM-1 mutants in the culture broth using recombinant DNA methods.

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