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Construction of the combinatorial library of *Rhizopus oryzae* lipase mutated in the lid domain by displaying on yeast cell surface

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Abstract

The lid domain of lipase is an interesting portion which has a large effect on the substrate specificity of the enzyme. To investigate the relationship between the amino acid sequence of the lid domain of *Rhizopus oryzae* lipase (ROL) and its substrate specificity, six amino acids (Phe88–Arg89–Ser90–Ala91–Ile92–Thr93) consisting the lid domain were combinatorially changed and mutated ROLs were displayed on the yeast cell surface by cell surface engineering. Clones exhibiting halos around colonies on the plates containing tributyrin or soybean oil were screened. As the preliminary results, seven clones among 20,000 clones showed clear halos on tributyrin-containing plates, while no halos were detected on soybean oil-containing plates. Assays using fluorescent substrates (fluorescein dibutyrate and fluorescein dilaurate) indicated that these cells displaying mutated enzymes had a lower activity than the cells displaying the wild-type enzymes, but there were several cells which exhibited a unique substrate specificity. The results obtained from the determination of the DNA sequences of the lid domain of combinatorially mutated enzymes indicated that the sequential alignment of the (basic amino acid)–(polar amino acid)–(non-polar amino acid) might be important for the function of the lid domain. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Display systems of libraries of mutated proteins or peptides on phage or some biological resources [1,2] seem useful for assaying and analyzing a large number of mutated proteins, being used to improve proteins and enzymes. Phage display is the most popular method at present because of its high transformation efficiency ($<10^{11}$), but post-translational glycosylation and proteolytic modification cannot be expected in the phage system. Furthermore, the sizes of molecules to be displayed on the phage surface are limited to small size, and clones can be screened only when they have an improved affinity for substrates or ligands [3,4]. We have developed a technique, termed as cell surface engineering [5,6], using the yeast *Saccharomyces cerevisiae*. This method allows the display of libraries of mutated proteins on the surface of yeast cells as the fusion protein to the C-terminal half of α-agglutinin. As the yeast can express many functional proteins necessary for post-translational modifications, yeast-display system seems unique and useful among various display systems reported hitherto. In this study, we have screened a library of combinatorially mutated enzymes to obtain clones exhibiting a modified catalytic activity, *Rhizopus oryzae* lipase (ROL), displayed on the yeast cell surface. ROL, which has been used to produce diesel fuels from

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vegetable oil [7], needs some modification to form a mature type [8] and has the substrate specificity to long-chain substrates. Its three-dimensional structure can be expected from that of *R. niveus* lipase [9] which has 99% amino acid identical to ROL. Although there are many reports about the structures of microbial lipases [10,11], almost all lipases, including ROL, have a lid domain on its active site and the movement of a lid, which occurs above the critical micellar concentration of the substrate, is necessary for the activation of lipases [12,13]. The lid domain directly contacts with substrates and is an interesting domain which has big effects on the substrate specificity of the lipases.

This paper deals with the construction of a combinatorial library of ROL mutated in the lid domain on yeast cell surface and investigation of the effect of the amino acid sequence of the lid domain on its substrate specificity.

2. Experimental

2.1. Strains and media

Escherichia coli DH5α (*F*$^-$, *endAl*, *hsdR17(rK*$^-$, *mK*$^+$*)*, *supE44*, *thi-1*, λ$^-$, *recAl*, *gyrA96*, *ΔlacU196*, *φ80dlacZΔM1*) was used as a host for recombinant DNA manipulation. *S. cerevisiae* strain MT8-1 (*MATa*, *ade*, *his3*, *leu2*, *trp1*, *ura3*) [14] was used as the host for protein display. *E. coli* was grown in LB medium (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride) containing 50 μg/ml ampicillin. Yeast was cultivated in SD-W medium (2% glucose and 0.7% yeast nitrogen base without amino acid (Difco, MI, USA) with appropriate supplements) containing 2% casamino acids.

2.2. Construction of plasmid

The plasmid for the expression of ROL on the cell surface of *S. cerevisiae* was constructed as follows: the genes encoding the *GAPDH* promoter and the terminator from pYE22m [15], and C-terminal 320 amino acids of α-agglutinin from pGA11 [16] into pMW1 [17], the resulting plasmid being named pWGSD1. The gene encoding pre-α-factor sequence fused to the gene encoding ProROL was amplified by polymerase chain reaction (PCR) with pWRL2 [18] as the template. The primers used are 5′-CTATAGATCTGTCGACATGAGATTTCCTTC-3′ (*Sal*I site, underlined) and 5′-CCTCCTCGAGCCCAAACAGCTTCCTTCGTTGATATC-3′ (*Xho*I site, underlined). The amplified fragment was digested by *Sal*I and *Xho*I, and introduced into the *Xho*I site of pWGSD1, resulting pWRSL2. To insert the linker sequence, 20 μl of two oligonucleotides (5′-TCGAGTGGTGGTTCTGGTGGTTCTGGTGGTTCTGGC-3′ and 5′-TCGAGCCAGACCACCAGAACCACCAGAACCACCAGAACCACCAC-3′) were resolved in Tris–EDTA buffer (pH 8.0), incubated at 95 °C for 10 min, and gradually cooled to room temperature for annealing. After phosphorylation on their ends, the fragment having the *Xho*I adapter site on their ends was digested by *Xho*I and introduced into the *Xho*I site of pWRSL2. The resulting expression plasmid for ROL on the cell surface of *S. cerevisiae* was named pWRSL17. Insertion of the *Sac*II site near the lid-coding region of pWRSL17 was accomplished as follows: a 438-bp fragment containing the lid-coding region and *Nco*I and *Ehe*I sites were amplified by PCR with pWRSL17 as a template. The primers used are 5′-ATGGTATGAGTCCCATGGTGGCAACC TGACATCCAT-3′ (*Nco*I site, underlined) and (5′-TGAACCTTGGCGCCCTTGACAGGCT TG TAGT CGGAAAAGTTGAAGACAATATCAGTGATGGCACT TCTGAAGGAGTTGGTACCGCGGAAA-3′ (*Sac*II and *Ehe*I sites, underlined). The amplified fragment was digested by *Nco*I and *Ehe*I, and then introduced to the isolated larger product of pWRSL17 after digestion with *Nco*I and *Ehe*I. The resulting plasmid was named pWRSL17S. The plasmid for the expression of ROL mutated in the lid domain was constructed as follows: an oligonucleotide named m-Lid (5′-TGTTTTCCGCGGTACCAAC TCCNNKNNKNNKNNKNNKNNKGATATCGTCTTC AACTT TTCTGACTACAAGCCTGTCAAGGGCGCCAAAGT TCATG-3′; N, mixture of A, T, G, and C; K, mixture of G and T; *Sac*II and *Ehe*I sites, underlined.) was first amplified by PCR using primers, 5′-TGTTTTCCGCGGTACCAACTCC-3′ and 5′-CATGAACTTTGGCGCCCTTGACAGGCTTGTAG-3′. The amplified fragment was digested by *Sac*II and *Ehe*I, and introduced to the isolated larger product of pWRSL17S after digestion with *Sac*II and *Ehe*I. The resulting expression plasmid for mutated ROLs (the lid domain library) was used to transform the yeast.

2.3. Screening by halo assay

Transformants having mutated ROL displayed on the yeast cell surface were spread on SD-W + 2% casamino acids medium agar plates containing 0.2% (v/v) tributyrin or 0.2% (v/v) soybean oil and 1% (w/v) gall powder as an emulsifier. Colonies which can hydrolyze tributyrin or soybean oil were identified as clear halos.

2.4. Assay of lipase activity using fluorescent substrates

Fluorescein dibutyrate ($C = 4$) or fluorescein dilaurate ($C = 12$) (5.0×10^{-5} M) was dissolved in methyl cellosolve/0.1 M Tris-buffer (w/w, 1/19), pH 8.0 [19]. Yeast cells were cultivated in 10 ml of SD-W medium and collected by centrifuging at $3000 \times g$ for 10 min. After cells ($A_{600} = 2.0$) were washed, the reaction was started by adding 1 ml of the substrate solution. After 3 h incubation at 37 °C, the mixtures were centrifuged at $3000 \times g$ for 10 min, and the fluorescence intensity of the supernatants was measured by a Fluoroscan Ascent Fluorometer (Labsystems OY, Helsinki, Finland) on the tissue culture plates (353047 Multiwell 24-well, Becton Dickinson Labware, NJ, USA). Filters with an excitation at 485 nm and with an emission at 527 nm were employed.

2.5. DNA sequencing

About 200-bp DNA fragments, encoding the mutated portion of the lid domain, were amplified from yeast colonies by PCR [20]. Amplified fragments were purified by quantum prep PCR kleen spin columns (Bio-Rad, CA, USA) and were sequenced by using a Dye Terminator Cycle Sequencing Ready Reaction Kit and an ABI Prism 373A DNA Sequencer (Perkin-Elmer/Applied Biosystems, CA, USA).

3. Results

3.1. Halo assay of yeast cells having combinatorially mutated ROL library displayed on the cell surface

For the display of ROLs mutated in the lid domain on yeast cell surface, the ROL-coding portion involving the fragment m-Lid, described in Section 2 (Fig. 1) was expressed in the yeast cells. Transformed cells were spread on two kinds of plates containing tributyrin or soybean oil as substrate to detect 200∼900 colonies per plate and incubated for 72 h at 30 °C. Totally, about 2×10^4 and 1×10^4 colonies were obtained on the soybean oil plates and on the tributyrin plates, respectively. First of all, we selected seven colonies, which formed larger halos than the negative-control colony on the tributyrin plate, but no halo on the soybean oil plates (Fig. 2).

3.2. Evaluation of lipase activity of mutants by fluorescence substrates

Relative lipase activities of seven colonies formed clear halos on the plate were assayed with fluorescence substrates (Fig. 3). Four colonies among them had higher activity than the negative-control cells, and exhibited 42–77% of the activity toward fluorescein dibutyrate ($C = 4$) and 10–35% of the activity toward fluorescein dilaurate ($C = 12$) (Fig. 3A and B), comparing with the cells displaying wild-type ROL. The negative-control cells showed about 22% of the activity toward fluorescein dibutyrate and 3% of the activity toward fluorescein dilaurate. The remaining three colonies had the same activity as the negative-control cells toward respective substrates. Although there were no mutant enzymes having higher activity than the wild-type enzyme, these four mutants showed different chain length specificity from that of the wild-type (Fig. 3C). These results demonstrated that combinatorial mutagenesis of the lid domain could alter the chain length specificity of ROL and that our surface-display system would be applicable to screen some potential ROL mutants having desired chain length specificity.

3.3. Analysis of DNA sequence of the lid-coding region

DNA sequences of the lid-coding region for the seven mutant enzymes that formed clear halos were determined as described in Section 2 (Table 1). In *Rhizomucor miehei* lipase, ‘Arg’ in the lid domain is important to stabilize the open conformation of the lid [21]. In the mutated ROL library examined, three mutants (clone no. 1–3), except for clone no. 4, that formed clear halos had a conserved sequence of

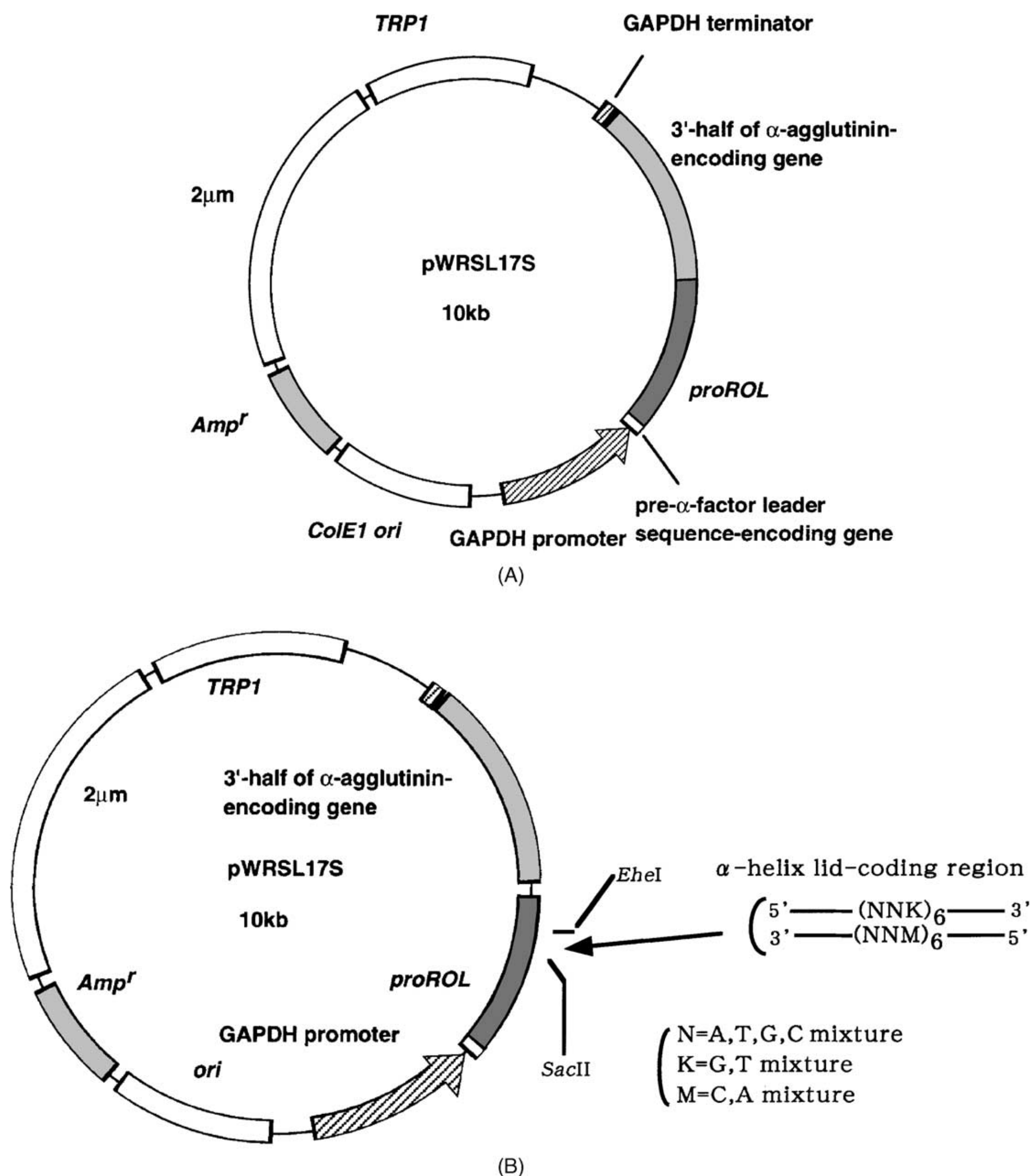

Fig. 1. The plasmid constructed to display ROL on the cell surface of *S. cerevisiae* (A) and the construction of the plasmid for display of mutated ROL library (B).

(basic amino acid)–(polar amino acid)–(non-polar amino acid). These results indicated that this sequential alignment would be important for the lid to activate ROL. In clone no.1, the position of the sequence of (basic amino acid)–(polar amino acid)–(non-polar amino acid) was shifted to left by one amino acid, but this enzyme exhibited 57% of the activity toward fluorescein dibutyrate ($C = 4$) and 11% of the activity toward fluorescein dilaurate($C = 12$) when compared with that of wild-type ROL. The ratio (activity for

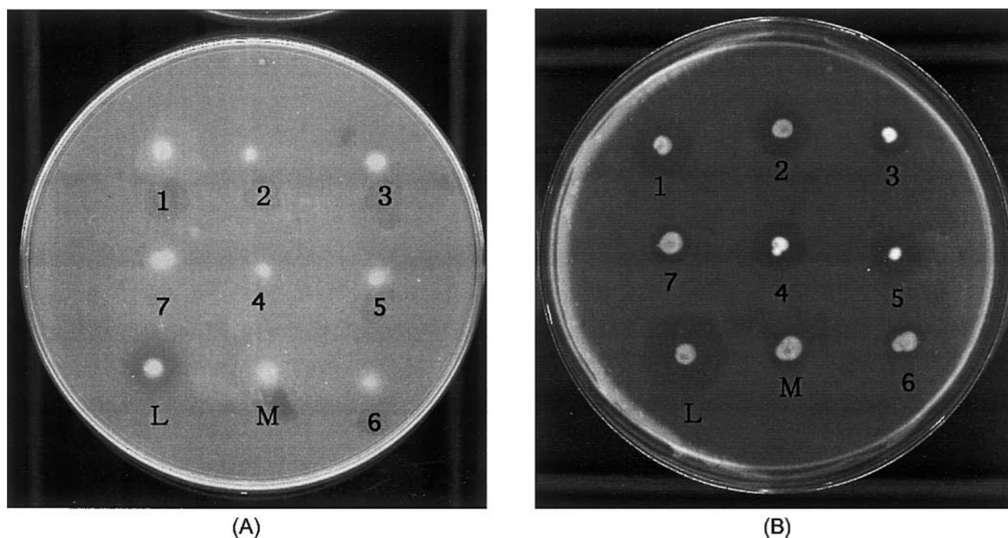

Fig. 2. Halo formation of *S. cerevisiae* colonies displaying combinatorial mutated ROLs on SD-W + 2% casamino acids agar plates with 0.2% (v/v) soybean oil and 1% (w/v) gall powder (A) or 0.2% (v/v) tributyrin (B). 1–7, Cells displaying combinatorial mutant enzymes; M, negative-control cell harboring pMW1; L, positive-control cell harboring pWRSL17S.

dibutyrate/dilaurate) was largely changed in comparison with that of the wild-type. These results indicated that, not only the position of basic amino acid, but also the sequence of (basic amino acid)–(polar amino acid)–(non-polar amino acid) should be essential for the determination of the substrate specificity by the lid domain. In clone no. 4, studies from the structure of the lid domain will be further necessary.

4. Discussion

Here we reported a novel protein library-displaying method using eukaryotic yeast cells. Alteration of the chain length specificity of ROL was generated by combinatorial mutagenesis of the lid domain. Library of ROL with the combinatorial mutation of the lid domain was functionally displayed on the yeast cell surface and screened by halo assay. Yeast-display method would be one of effective techniques to obtain an interesting clone from functional protein libraries by the introduction of the high-throughput screening. There are many reports on the study of microbial lipases by the site-directed mutation [22]. The site-directed mutagenesis requires information of conformation and electrostatic properties. However, all studies using the site-directed mutations have not lead to the desired results. Recently, Gaskin et al. [23] carried out the combinatorial mutation of Phe94 in the groove of *R. miehei* lipase to alter the chain length specificity. They purified 14 mutants of the possible 19 mutants from the culture supernatants of *Pichia pastoris* and obtained several mutants with high activity to short-chain substrates. Yeast-display method allows us to carry out the combinatorial mutagenesis of multi-point amino acids because of its feasibility for the assay of mutants. Here, we tried the combinatorial mutagenesis of the six amino acid residues of the lid domain of ROL. As the number screened (20,000 clones) was too small compared with the theoretical value (6.4×10^7), we have not yet got mutants with a higher activity to short- or long-chain substrates than the wild-type enzyme, but could find several mutant enzymes shifting the chain length specificity with a conserved amino acid sequence. Further studies, including combinatorial mutagenesis of the alignment of two or three amino acids in the lid domain, will lead to get interesting mutant enzymes.

These results obtained from the combinatorial mutagenesis by yeast surface display may give an instructive idea for the construction of the strategy of protein engineering.

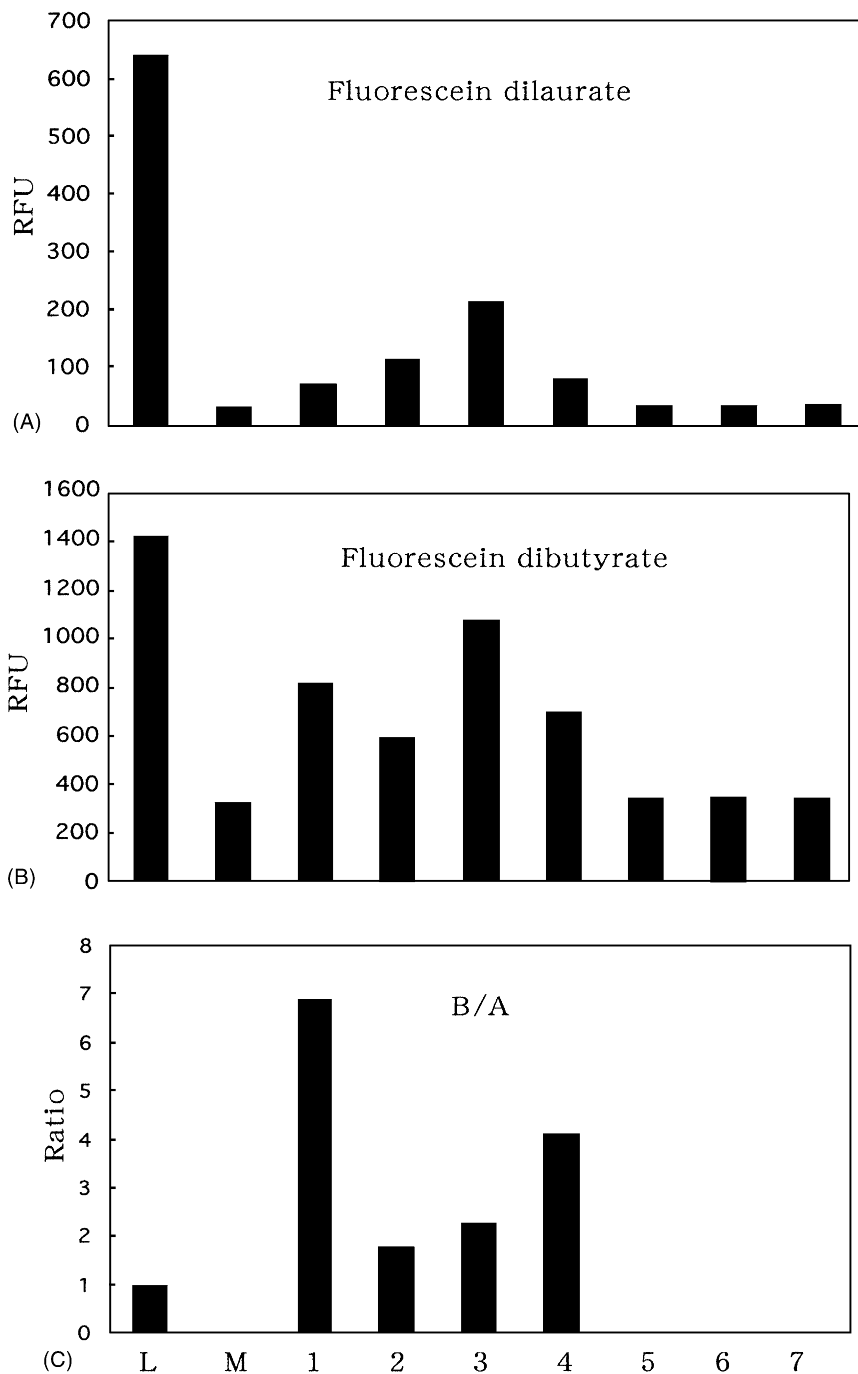

Fig. 3. Comparison of lipase activities to fluorescein dilaurate (A) or fluorescein dibutyrate (B) and the ratio, B/A (C). For 1–7, M, and L, see Fig. 2. In (C), each calculated ratio, when B/A in L is represented as 1, is illustrated. In each calculation, the activity of the negative-control cells is subtracted, respectively. RFU, relative fluorescence unit.

Table 1
Amino acid sequences of the lid domain of combinatorial mutant enzymes

Wild-type sequence of the lid domain	tcc- ttc aga agt gcc atc act - gat S - F R S A I T - D
Clone no.	Determined sequence
1	tcc- cgt agg gct gcg ctt gct - gat S - R S A V L A - D
2	tcc- ggt cgt aat gtg ctg act - gat S - G R N V L T - D
3	tcc- cgg cgt tct tgg att agt - gat S - R R S W I S - D
4	tcc- tgg gct gcg gtt tgt agt - gat S - W A A V C S - D
5	tcc- cag tgg ccg tgt cat ttt - gat S - Q W P C H F - D
6	tcc- tct acg gct tgg cag ttt - gat S - S T A W Q F - D
7	tcc- atg gat cgg cct gga agg - gat S - M D R P G R - D

Clones are the same as those in Fig. 2. Underlines: amino acid sequences similar to that of the wild-type.

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