J. Biochem. 129, 87–91 (2001)

Monoclonal Antibody That Binds to the Central Loop of the Tn10-Encoded Metal Tetracycline/H$^+$ Antiporter of *Escherichia coli*[1]

Shigeyuki Nada,[*,‡] Satoshi Murakami,[*,‡] Shizuka Okamoto,[*,†] Yoshiyuki Kubo,[*,†] and Akihito Yamaguchi[*,†,‡,2]

[*]Department of Cell Membrane Biology, Institute of Scientific and Industrial Research, Osaka University, Ibaraki, Osaka 567-0047; [†]Faculty of Pharmaceutical Science, Osaka University, Suita, Osaka 565-0871; and [‡]CREST, Japan Science and Technology Corporation, Japan

Received September 14, 2000; accepted October 25, 2000

Mouse monoclonal antibodies were prepared using His-tagged Tn10-encoded metal-tetracycline/H$^+$ antiporter [TetA(B)His] as an antigen. From them, those reacting equally with His-tagged and wild-type TetA(B) were selected and named TCL-1. Cysteine-scanning mutants were used to determine the TCL-1 binding site on the TetA(B) protein. First, 12 Cys mutants of TetA(B) in which one residue in a protruding loop region was replaced by cysteine were constructed. Western blot analysis revealed the binding of TCL-1 to all of these Cys-mutants except for R186C. Then, we constructed 13 cysteine-scanning mutants, F179C to T191C. Among them, eight mutants, F179C to T182C, N184C, and T189C to T191C, exhibited TCL-1 binding, whereas the other five, K183C, T185C, R186C, D187C, and N188C, exhibited no or lower TCL-1 binding. These results clearly indicate that the sequence recognized by TCL-1 is ^{183}Lys-X-Thr-Arg-Asp-Asn^{188} in the central loop region of TetA(B). TCL-1 is the first reported antibody that binds to a region other than the C-terminus of TetA(B), and the recognized amino acid sequence was identified.

Key words: drug resistance, epitope, exporter, monoclonal antibody, tetracycline.

The Tn10-encoded metal-tetracycline/H$^+$ antiporter, TetA(B), is a typical bacterial drug export protein (*1*, *2*), and its molecular mechanism and molecular structure have been studied as a paradigm of antiporters. The twelve membrane-spanning structure of TetA(B) (*3*) was determined by means of site-directed competitive chemical modification of cysteine-introduced mutants, in which a cysteine residue was introduced into one of the putative loop regions of the cysteine-free TetA(B), with membrane-permeable and membrane-impermeable maleimide derivatives (*4*). In addition, the boundaries between water-exposed loop regions and the transmembrane regions were also determined in detail on the basis of the reactivity of the cysteine-scanning mutants with maleimide derivatives (*5–9*). In these studies, a rabbit anti–TetA(B) C-terminus antibody (*10*) was used for detection of the mutant proteins. This antibody was developed using a synthetic oligopeptide corresponding to the C-terminal 14 amino acid sequence of TetA(B) as an antigen. This anti–C-terminus antibody exhibits a high reactivity against TetA(B). We have tried to develop an antibody that binds to TetA(B) at a site other than the C-terminus using the purified TetA(B) protein or a synthetic oligopeptide as an antigen, but antibodies with a high titer have not yet been obtained. In this study, we used a His-tagged TetA(B) protein as an antigen and succeeded in obtaining a mouse monoclonal antibody. In addition, cysteine-scanning mutants were revealed to be useful tools for determination of the sequence recognized by an antibody.

EXPERIMENTAL PROCEDURES

Materials—All materials used in this study were of reagent grade and obtained from commercial sources unless otherwise stated.

Construction of pSYTETHis—A plasmid encoding His-tagged TetA(B) was constructed as follows: first, a unique *Aor51HI* site (AGCGCT) was introduced at the end of the coding region of *tetA(B)* by site-directed silent mutation as 5′-AGTGCT-3′ (Ser400-Ala401)→AGCGCT using a template plasmid pCT1184 (*11*), and the resulting plasmid was named pCT1185. Then, the *Eco*RI–*Bam*HI fragment of pCT1185, which includes the whole *tetA(B)* gene, was transferred into pSYTET (*11*) by corresponding fragment exchange, the resulting plasmid being named pSYTET2. A DNA linker encoding Ala-Gly-$(His)_6$-Gly-End was obtained by annealing two synthetic oligonucleotides, BctH6-1 (5′-GCTGGCCACCATCATCATCACCATGGCTAG-3′) and BctH6-2 (5′-CGACCGGTGGTAGTAGTAGTGGTACCGATCCTAG-3′). The 5′- and 3′-termini of this linker DNA correspond to *Aor51*HI and *Bam*HI restriction fragments, respectively. This linker was inserted into the *Aor51*HI–*Bam*HI site of pSYTET2. As a result, the C-terminus of TetA(B) was changed from ^{400}Ser-Ala^{401}-End to ^{400}Ser-Ala-Gly-$(His)_6$-Gly^{409}-End. The insertion was verified by DNA sequencing of the resulting plasmid pSYTETHis.

Purification of TetA(B)His—*Escherichia coli* RB791 (W3110 *lacI^{q}L8*) (*12*) cells harboring a plasmid, pSYTETHis, were grown at 37°C on minimal medium supple-

[1] This work was supported by Grants-in-Aid from the Ministry of Education, and the Ministry of Science and Technology of Japan.

[2] To whom correspondence should be addressed at: Institute of Science and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki, Osaka 567-0047. Phone: +81-6-6879-8545, Fax: +81-6-6879-8549, E-mail: akihito@sanken.osaka-u.ac.jp

mented with 0.2% glucose and 0.1% casamino acids. Expression of the *tetA* gene was induced with 0.1 mM isopropyl β-D-thiogalactopyranoside at the late log phase for 2 h. Cells were harvested, washed once with phosphate-buffered saline, and then disrupted with a French press at 12,000 psi in 50 mM Tris-HCl (pH 7.0) containing 10 mM $MgCl_2$. The membrane fraction was collected by ultracentrifugation at 145,000 ×*g* for 1 h. After washing once with 50 mM Tris-HCl (pH 7.0) containing 0.5 mM EDTA, the inner and outer membranes were separated by sucrose density gradient centrifugation (*13*). The inner membranes were washed with 5 mM Tris-HCl (pH 7.0) containing 0.5 mM EDTA, then resuspended in 50 mM Tris-HCl (pH 7.0). The resulting inner membrane suspensions (10 mg protein/ml) were solubilized with 2% dodecylmaltoside. After removal of the insoluble materials by ultracentrifugation, the solubilized proteins were applied to a Chelating Sepharose Fast Flow column (Pharmacia Biotech) charged with Ni^{2+} ions that had been equilibrated with buffer A (20 mM Tris-HCl, pH 7.0, containing 0.3 M NaCl, 10% glycerol, and 0.2% dodecylmaltoside). After two-step washing of the column with 25 mM imidazole and 100 mM imidazole in buffer A, TetA(B)His was eluted with 300 mM imidazole in buffer A.

Preparation of Anti-TetA(B)His Monoclonal Antibodies—A mixture of 50 μg of purified TetA(B)His and Z-Max (Zonagen, The Woodlands, TX) pellet was injected into SPF mice (BALB/c male) several times at one-week intervals. The reactivity of the mouse antiserum against inner membranes containing the TetA(B) protein was checked by Western blotting. On four days after the final boost of 25 μg of purified TetA(B) into tail blood vessel, splenocytes were recovered and fused with myeloma P3-X63-Ag8.653 cells in the presence of 50% PEG6000 in PBS. ELISA screening was done by using purified TetA(B)His as an antigen. ELISA-positive hybridoma cells were grown in RPMI1640 medium supplemented with 100 U/ml IL-6 and 10% fetal calf serum.

Purification of Monoclonal Antibody TCL-1—Mouse ascites was gained by injecting TCL-1 hybridoma intraabdominally in BALB/c mice. Immunoglobulin fraction was recovered by precipitating with ammonium sulfate at 50% saturation followed by protein G affinity chromatography (MAbTrap GII Kit, Pharmacia).

Cysteine-Introduced Mutants of TetA(B)—Cysteine-introduced mutants of TetA(B), in which any one amino acid residue of the Cys-free TetA(B) was replaced by cysteine, were previously constructed by means of oligonucleotide-directed site-specific mutagenesis (*4–9*). The mutant genes were subcloned into a low-copy-number plasmid, pLGT2, by fragment exchange (*4*).

Preparation of Sonicated Membranes—Sonicated membranes were prepared from *E. coli* W3104 (*14*) cells carrying the low-copy-number mutant plasmid as described previously (*4*).

RESULTS AND DISCUSSION

Purification of His-Tagged TetA(B)—The *tetA(B)his* gene, which codes for amino acids 2–401 of the TetA(B) protein with an extra eight amino acid residues ($GlyHis_6Gly$) at the C-terminus and three amino acid residues (MetIleThr) derived from *LacZ* at the N-terminus (Fig. 1A), was placed downstream of the *lac* promoter in pUC118, and the resulting plasmid was named pSYTETHis (Fig. 1B). The insertion of these residues did not affect the tetracycline/H^+ antiport activity (data not shown). The *E. coli* strain required the $lacI^q$ mutation for transformation with a plasmid carrying the *tetA(B)* gene under the control of the *lac* promoter (*11*). Following 2 h of induction of *E. coli* RB791 cells harboring pSYTETHis with 0.1 mM isopropyl β-D-thiogalactopyranoside, the cells were harvested and frac-

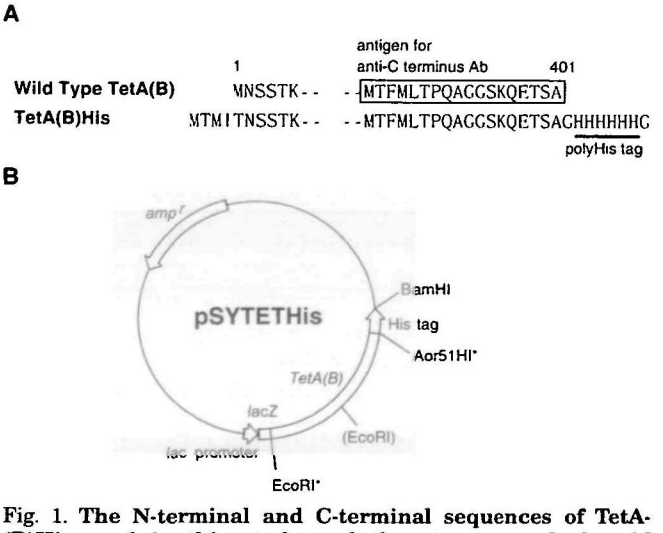

Fig. 1. **The N-terminal and C-terminal sequences of TetA-(B)His used in this study and the structure of plasmid pSYTETHis.** (A) The amino acid sequences of TetA(B)His around its N-terminal and C-terminal regions. Extra amino acid residues derived from LacZ and a polyhistidine tag are underlined. (B) Plasmid pSYTETHis was constructed by insertion of a GH_6G linker into the end of the *tetA(B)* gene of pSYTET at the *Aor51*HI–*Bam*HI site. The *Aor51*HI site had been introduced at the end of the *tetA(B)* gene by site-directed mutagenesis as described under "EXPERIMENTAL PROCEDURES." The *Eco*RI–*Aor51*HI fragment of *tetA(B)* gene derived from pCT1185 is indicated with asterisks.

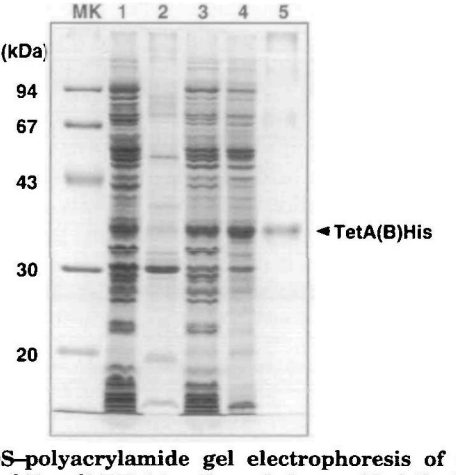

Fig. 2. **SDS–polyacrylamide gel electrophoresis of various fractions of *E. coli* RB791 cells and the purified TetA(B)His.** Protein bands were visualized by Coomassie Brilliant Blue staining. Lane 1, total membrane fraction (20 μg protein); lane 2, outer membrane (10 μg protein); lane 3, inner membrane (20 μg protein); lane 4, EDTA-washed inner membrane (20 μg protein); lane 5, TetA(B) (5 μg protein) purified by Ni-IDA Sepharose column chromatography.

tionated. A dense 37-kDa band was observed for the total membrane fraction on SDS–polyacrylamide gel electrophoresis (Fig. 2), which was identified as the His-tagged TetA(B) protein by Western blotting using anti-polyhistidine antibodies (data not shown). The membrane fraction was separated into inner and outer membrane fractions on a sucrose density gradient. Then the inner membrane was washed with a low ionic strength buffer containing 0.5 mM EDTA to remove peripheral membrane proteins. The EDTA-washed inner membrane fraction was solubilized with 2% dodecylmaltoside and TetA(B)His was purified by one-step column chromatography with chelated Ni^{2+} as described under "EXPERIMENTAL PROCEDURES." The purified TetA(B) gave one band on SDS–polyacrylamide gel electrophoresis (Fig. 2).

Anti-TetA(B)His Monoclonal Antibodies—For immunization of mice with TetA(B)His, a Z-Max adjuvant system was used to obtain high titers as to a native part of the protein. The resulting mouse antiserum was equally reactive with TetA(B)His and native TetA(B) (Fig. 3), indicating that the antibodies in the serum recognize a native part of the TetA(B) protein as an epitope. On the other hand, anti-TetA(B) C-terminus antibodies did not bind to TetA(B)His (Fig. 3). The splenocytes recovered from the mouse producing this serum were fused with mouse myeloma cells, and an ELISA-positive hybridoma cell line was established. The antibody produced by this hybridoma cell line, named TCL-1, was subjected to further study.

Purification of Monoclonal Antibody TCL-1—TCL-1 producing hybridoma cells were injected intra-abdominally in BALB/c mice and ascites was obtained. Immunoglobulin fraction was precipitated at 50% saturation of ammonium sulfate. Resuspended immunoglobulin was further purified by Protein G affinity chromatography (Fig. 4). This procedure yielded 26.8 mg of purified immunoglobulin from 7.5 ml of ascites.

Determination of the TCL-1 Epitope by Use of Cysteine-Scanning Mutants—To determine the region that binds to TCL-1, we used mutants in which one amino acid residue in any of the water-protruding loop regions of TetA(B) was replaced by cysteine as an antigen. The reactivity of TCL-1 with the mutant proteins was assessed by immunoblotting. We first selected 12 mutants in which the cysteine-replacing mutation was located in relatively large loop regions as depicted in Fig. 5. Each mutant was expressed in *E. coli,* and cells were disrupted by brief sonication in PBS. The supernatant containing membrane vesicles was subjected to SDS–polyacrylamide gel electrophoresis, followed by immunoblotting using either TCL-1 (Fig. 6A) or rabbit anti–C-terminus antiserum (Fig. 6B). With the exception of mutant R238C, these mutants were expressed in similar amounts. R238C showed lower protein expression concomitant with the lower minimum inhibitory concentration of tetracycline for cell growth (MIC value) of 25 μg/ml. Of the 12 mutants, TCL-1 failed to detect only R186C mutant. This indicated that R186 was indispensable for the epitope recognition by TCL-1. The amino acid residues around R186 were tested for their involvement as epitopes of TCL-1 (Fig. 5) using 13 cysteine-introduced mutants from F179 to T191 in central loop region. TCL-1 did not react with K183C, T185C, R186C, and D187C (Fig. 7A) and showed

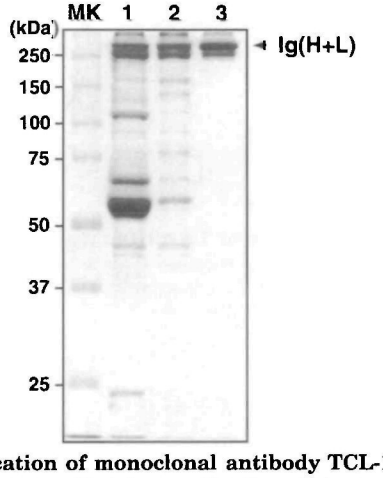

Fig. 4. **Purification of monoclonal antibody TCL-1.** TCL-1 immunoglobulin was purified from ascites (lane 1) by ammonium sulfate precipitation (lane 2) and Protein G affinity chromatography (lane 3). Samples of each step were applied to SDS–polyacrylamide gel electrophoresis (15 μg protein in lane 1, and 5 μg protein in lanes 2 and 3) and stained with CBB.

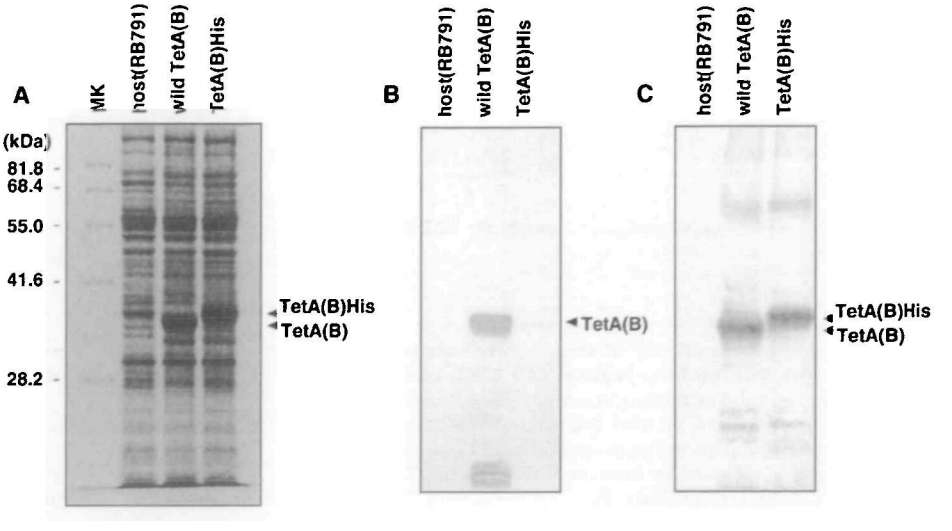

Fig. 3. **Western blot analysis of native and His-tagged TetA(B) with mouse anti TetA(B)His antiserum and rabbit anti-TetA(B) C-terminus antibodies.** Sonicated membranes prepared from *E. coli* RB791 cells carrying pSYTET or pSYTETHis were subjected to SDS–polyacrylamide gel electrophoresis (20 μg protein per lane). After electroblotting, the immunoreactive bands were visualized by staining with Coomassie Brilliant Blue (CBB) (A), rabbit anti–C-terminus antibodies (B) or mouse anti-TetA(B)His antiserum (C).

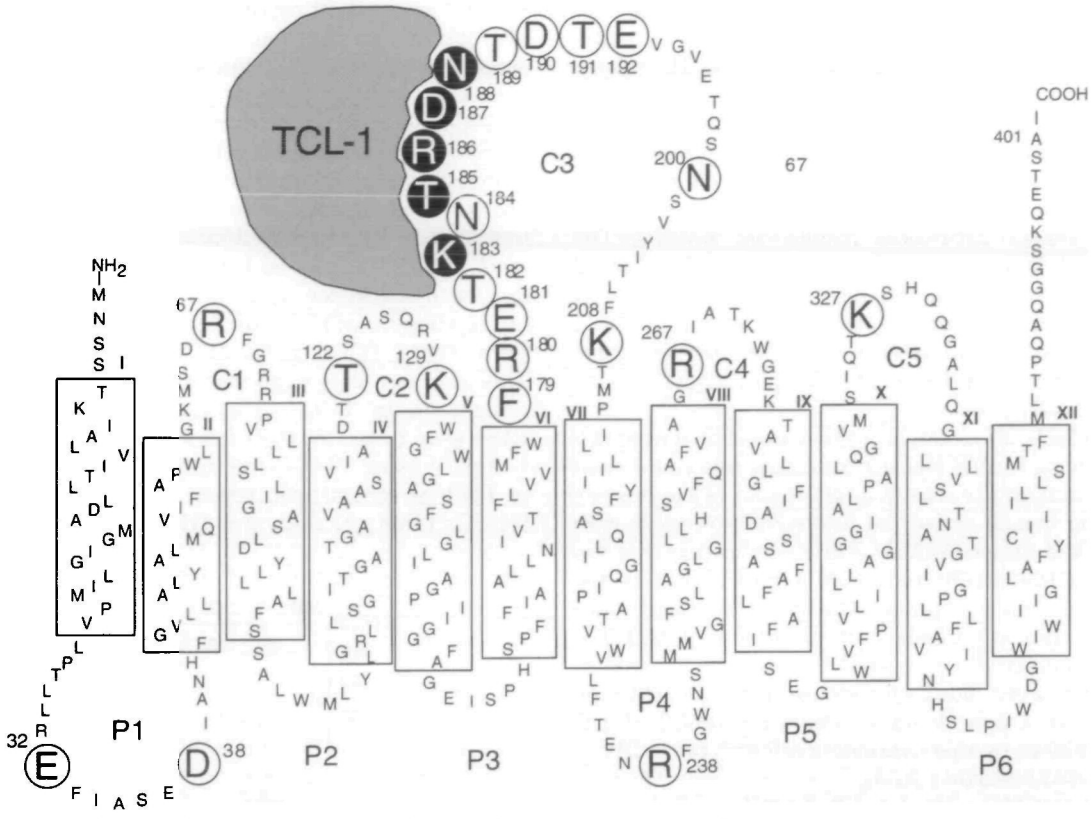

Fig. 5. **Putative topology of TetA(B) and the residues replaced with cysteine.** Putative transmembrane segments are enclosed in boxes. Bold letters indicates residues replaced with cysteine. Open circles and closed circles indicate that the cysteine mutants having high and no reactivity with TCL-1, respectively. C1 to C5 and P1 to P6 indicate the cytoplasmic and periplasmic loops, respectively. The TCL-1 antibody is depicted schematically.

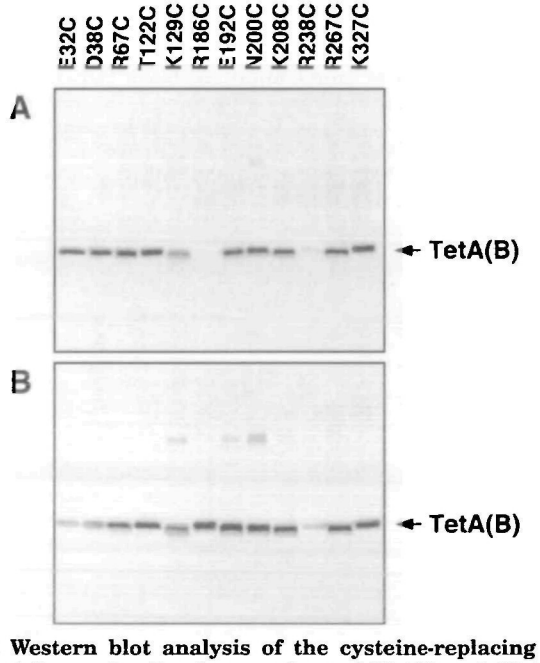

Fig. 6. **Western blot analysis of the cysteine-replacing mutants at the protruding loop regions of P1, P4, and C1 to C5 of TetA(B).** *E. coli* cells expressing one of the Cys-mutants were disrupted by sonication, and cleared supernatants containing membrane vesicles (20 μg protein) were applied to SDS–polyacrylamide gel electrophoresis followed by immunoblotting using TCL-1 (A) or anti-TetA(B) C-terminus antibody (B).

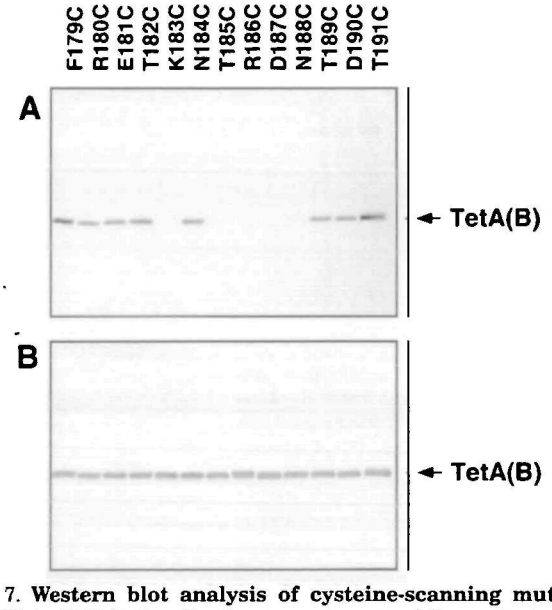

Fig. 7. **Western blot analysis of cysteine-scanning mutants F179C to T191C.** *E. coli* cells expressing one of Cys-mutants were disrupted by sonication and cleared supernatants containing membrane vesicles (20 μg protein) were applied to SDS–polyacrylamide gel electrophoresis followed by immunoblotting using TCL-1 (A) or anti–TetA(B) C-terminus antibody (B).

much weaker reactivity toward N188C than toward F179C to T182C, N184C, and T189C to T191C. The expression of these mutants was normal and anti–TetA(B) C-terminus antibodies were reactive with these mutants at the same level (Fig. 7B). These results clearly indicated that the binding site of TCL-1 was ^{183}Lys-X-Thr-Arg-Asp-Asn^{188}. TCL-1 is the first reported antibody that binds to tetracycline exporters at a site other than the C-terminal region of TetA(B), and the amino acid sequence recognized as an epitope was identified. The antibody recognizing the central loop region of TetA(B) will be useful for studying the molecular structure and function of this protein. The current study also revealed that cysteine-scanning mutants are very useful tools for epitope mapping and determination of amino acid sequences that bind to antibodies, as well as a method utilizing alanine-scanning mutants (*15*, *16*).

REFERENCES

1. McMurry, L., Petrucci, R.E., Jr., and Levy, S.E. (1980) Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **77**, 3974–3977
2. Yamaguchi, A., Udagawa, T., and Sawai, T. (1990) Transport of divalent cations with tetracycline as mediated by the transposon Tn10-encoded tetracycline resistance protein. *J. Biol. Chem.* **265**, 4809–4813
3. Eckert, B. and Beck, C.F. (1989) Topology of the transposon Tn10-encoded tetracycline resistance protein within the inner membrane of *Escherichia coli*. *J. Biol. Chem.* **264**, 11663–11670
4. Kimura, T., Ohnuma, M., Sawai, T., and Yamaguchi, A. (1997) Membrane topology of the transposon 10-encoded metal-tetracycline/H^+ antiporter as studied by site-directed chemical labeling. *J. Biol. Chem.* **272**, 580–585
5. Kimura, T., Suzuki, M., Sawai, T., and Yamaguchi, A. (1996) Determination of a transmembrane segment using cysteine-scanning mutants of transposon Tn10-encoded metal-tetracycline/H^+ antiporter. *Biochemistry* **35**, 15896–15899
6. Kimura, T., Shiina, Y., Sawai, T., and Yamaguchi, A. (1998) Cysteine-scanning mutagenesis around transmembrane segment III of Tn10-encoded metal-tetracycline/H^+ antiporter. *J. Biol. Chem.* **273**, 5243–5247
7. Kimura-Someya, T., Iwaki, S., and Yamaguchi, A. (1998) Site-directed chemical modification of cysteine-scanning mutants as to transmembrane segment II and its flanking regions of the Tn10-encoded metal-tetracycline/H^+ antiporter reveals a transmembrane water-filled channel. *J. Biol. Chem.* **273**, 32806–32811
8. Kimura-Someya, T., Iwaki, S., Konishi, S., Tamura, N., Kubo, Y., and Yamaguchi, A. (2000) Cysteine-scanning mutagenesis around transmembrane segments 1 and 11 and their flanking loop regions of Tn10-encoded metal-Tetracycline/H^+ antiporter. *J. Biol. Chem.* **275**, 18692–18697
9. Iwaki, S., Tamura, N., Kimura-Someya, T., Nada, N., and Yamaguchi, A. (2000) Cysteine-scanning mutagenesis of transmembrane segments 4 and 5 of the Tn10-encoded metal-tetracycline/H^+ antiporter reveals a permeability barrier in the middle of a transmembrane water-filled channel. *J. Biol. Chem.* **275**, 22704–22712
10. Yamaguchi, A., Adachi, K., and Sawai, T. (1990) Orientation of the carboxyl terminus of the transposon Tn10-encoded tetracycline resistance protein in *Escherichia coli*. *FEBS Lett.* **265**, 17–19
11. Someya, Y., Moriyama, Y., Futai, M., Sawai, T., and Yamaguchi, A. (1995) Reconstitution of the metal-tetracycline/H^+ antiporter of *Escherichia coli* in proteoliposomes including F0F1-ATPase. *FEBS Lett.* **374**, 72–76
12. Brent, R. and Ptashne, M. (1981) Mechanism of action of the *lexA* gene product. *Proc. Natl. Acad. Sci. USA* **78**, 4204–4208
13. Osborn, M.J., Gander, J.E., Paris, E., and Carson, J. (1972) Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterization of cytoplasmic and outer membrane. *J. Biol. Chem.* **247**, 3962–3972
14. Yamamoto, T., Tanaka, M., Nohara, C., Fukunaga, Y., and Yamagishi, S. (1981) Transposition of the oxacillin-hydrolyzing penicillinase gene. *J. Bacteriol.* **145**, 808–813
15. Jin, L., Fendly, B.M., and Wells, J.A. (1992) High resolution functional analysis of antibody-antigen interactions. *J. Mol. Biol.* **226**, 851–865
16. Lubin, I.M., Healey, J.F., Barrow, R.T., Scandella, D., and Lollar, P. (1997) Analysis of the human factor VIII A2 inhibitor epitope by alanine scanning mutagenesis. *J. Biol. Chem.* **272**, 30191–30195