FEBS Letters 442 (1999) 34–38 FEBS 21358

Characterization of the bleomycin resistance determinant encoded on the transposon Tn*5*

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Received 12 October 1998; received in revised form 27 November 1998

Abstract The transposon Tn*5* carries a gene, *ble*, which confers resistance to bleomycin (Bm) and gives a survival advantage to its host cell. We found that the *ble* gene product, designated BLMT, is a binding protein with a strong affinity for Bm. BLMT quenched both the antibacterial and DNA-cleaving activities of Bm, when incubated with the antibiotic. An electron spin resonance spin-trapping analysis showed that BLMT inhibits the generation of Bm-induced hydroxyl radical, by trapping Bm but not the hydroxyl radical. Western blot analysis using an anti-BLMT monoclonal antibody revealed that BLMT is immunologically distinct from Bm-binding proteins from *Streptomyces verticillus*, *Staphylococcus aureus* and *Streptoalloteichus hindustanus*. *Escherichia coli*, transformed with a mutant *ble* having leucine instead of proline at N-terminal amino acid position 7, lost resistance to Bm, although the cell maintained the survival benefit. This suggests that the Bm resistance mediated by *ble* is independent of its ability to give a survival advantage to the host bacterium.

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Key words: Transposon Tn*5*; Bleomycin resistance; Bleomycin-binding protein; Electron spin resonance spin trapping; Site-directed mutagenesis

1. Introduction

Bleomycin (Bm), produced by *Streptomyces verticillus*, is a glycopeptide antibiotic which is clinically used in the treatment of certain tumors [1]. The Bm-Fe(II) complex, in conjunction with reducing agent and oxygen, causes nucleotide sequence-specific DNA cleavage [2]. A two-step mechanism has been suggested for DNA cleavage, involving the interaction of the bithiazole moiety of Bm into DNA followed by decomposition of a labile Fe(II)-Bm-O_2 complex to generate a Fe(III)-Bm complex and either hydroxy or superoxide radicals that can then degrade the DNA [2].

Bm-producing *S. verticillus* must be protected from the lethal effect of its own product. We have cloned and sequenced two independent genes, designated *blmA* and *blmB*, encoding Bm resistance determinants from *S. verticillus* ATCC 15003 [3]. The genes *blmA* and *blmB* were shown to encode a Bm-binding protein and an *N*-acetyltransferase, respectively [4,5]. Tallysomycin-producing *Streptoalloteichus hindustanus* has a gene, designated Sh*ble*, encoding a protein which binds to Bm [6,7].

Interestingly, although Bm has never been used as an antibacterial agent, most strains of methicillin-resistant *Staphylococcus aureus* (MRSA), clinically isolated in Hiroshima University Hospital, Japan, were resistant to the drug at a high level. We have cloned and sequenced a Bm resistance gene, designated *blmS*, from the chromosomal DNA of MRSA strain B-26 [8,9]. The gene was identical to that located on a staphylococcal plasmid pUB110. We have shown that pUB110 was integrated into the chromosomal DNA with the mediation of IS*431*mec [9]. We have found that the *blmS* gene product, designated BLMS, is also a Bm-binding protein [4].

A Bm resistance gene, *ble*, encoded on the transposon Tn*5* [10,11], has been reported to confer a survival advantage to *Escherichia coli* host cells [12,13]. Although it has been suggested that this advantage to the bacterium might be mediated via an induction of the DNA repair system, the contributory mechanism to this repair system and physico-chemical properties of the *ble* gene product have not yet been determined.

The aim of the present study is to overproduce and characterize the *ble* gene product, designated BLMT. This is the first purification of the Bm-binding protein. Moreover, this paper reports on the successful generation of the anti-BLMT monoclonal antibody, for the immunological characterization among BLMT and other Bm-binding proteins. We defined the function of *ble* gene product by creating a mutant *ble* protein lacking resistance to Bm.

2. Materials and methods

2.1. Bacterial strains and plasmid constructions

E. coli HB101 and plasmid pKK*trp* [4] having the *trp* promoter were used to overexpress *ble*. *E. coli* BMH71-18 *mut*S and plasmid pUC18 were used for site-directed mutagenesis.

The plasmid pUC4KIXX [14], purchased from Pharmacia (Sweden), was used as a source of *ble*. The *ble* structural gene having *Eco*RI and *Hin*dIII sites at the 5′- and 3′-adjacent regions, respectively, was amplified by PCR using a sense 5′-GGGGGAATTCATGACCGACCAAGCGACGCC-3′ primer and an antisense 5′-GAAGAAGCTTTCATGAGATGCCTGCAAGCA-3′ primer, and pUC4KIXX as the DNA template. The amplified *ble* was subcloned into the *Eco*RI- and *Hin*dIII-digested pKK*trp* to generate pKKblmT.

To create a mutant *ble* having leucine instead of proline at N-ter-

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Abbreviations: *ble*, a gene encoding Bm-resistant determinant carried by the transposon Tn*5*; Bm, bleomycin; ELISA, enzyme-linked immunosorbent assay; ESR, electron spin resonance; MIC, minimum inhibitory concentration; PAGE, polyacrylamide gel electrophoresis; PBN, α-phenyl-*N*-*tert*-butylnitron; SDS, sodium dodecyl sulfate; Tn, transposon

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PII: S0014-5793(98)01613-5

minal position 7 by site-directed mutagenesis, the amplified *ble* was subcloned into pUC18 digested with *Eco*RI and *Hind*III to generate pUCblmT. The two oligonucleotides, 5′-CTTCCTTTTTCGATAT-CATTGAAGCATTT-3′ as a selection primer and 5′-GTGATGG-CAGGTTCAGCGTCGCTTGGTCG-3′ as a mutagenic primer, were chemically synthesized and annealed to the template pUCblmT which was denatured at 100°C. The mutant DNA strand, synthesized with T4 DNA polymerase, was introduced into *E. coli* BMH71-18 *mut*S. This mutagenic experiment was performed using Transformer Site-Directed Mutagenesis Kit (Clontech, USA), according to the supplier's protocol based on the method described in [15]. The mutant *ble* was cloned into pKK*trp* to generate pKKblmTM.

2.2. Isolation of BLMT

E. coli HB101 harboring pKKblmT was grown in M9-casamino acid medium [16] containing ampicillin 100 μg/ml. At the exponential phase of growth, 3-indoleacrylic acid (0.13 mM) was added to the culture to induce the *trp* promoter and incubated for 24 h. The harvested cells, washed with 20 mM Tris-HCl (pH 7.6), were ground with quartz sand and extracted with buffer A (20 mM Tris-HCl, pH 7.6, 30 mM NH_4Cl, 10 mM Mg-acetate, 6 mM 2-mercaptoethanol, 5 mM Mg-titriplex and 3.45 mM phenylmethylsulfonyl fluoride). The resulting supernatant fluid was collected by centrifugation at $18\,000\times g$ for 20 min. Solid ammonium sulfate was added to the supernatant at 20% saturation and the resulting precipitate was removed by centrifugation. BLMT was precipitated from the supernatant by addition of ammonium sulfate at 40% saturation, dissolved in small volumes of 20 mM Tris-HCl (pH 7.6) and dialyzed against the same buffer. The dialysate was subjected to a DEAE-Sepharose CL-6B column (1.8×20 cm) equilibrated with the same buffer and eluted with 20 mM Tris-HCl (pH 7.6) buffer containing 0–1 M NaCl in a linear concentration gradient. The fractions containing BLMT, concentrated using ULTRACENT (Tosho, Japan), were finally applied on a Sephadex G-75 superfine column (1.8×120 cm) to purify to homogeneity.

2.3. Measurement of molecular weight of BLMT

The molecular weight of BLMT was estimated by the gel filtration method using a Sephadex G-75 superfine column equilibrated with 25 mM Tris-HCl (pH 7.6) and 10 mM NaCl. Albumin (66.2 kDa), ovalbumin (43 kDa), chymotrypsinogen (25.7 kDa) and RNase A (13.7 kDa) were used as the size marker proteins.

2.4. N-terminal amino acid sequence

The N-terminal amino acid sequence of BLMT was determined by Edman degradation method using the auto-sequencer (Shimadzu, Japan).

2.5. Spin-trapping and ESR measurements

To make the 1:1 Fe(III)-Bm complex, 0.1 mM $FeCl_3$ and Bm A_2 sulfate were mixed in 50 mM Tris-HCl (pH 7.8). After the complex was incubated together with 80 mM α-phenyl-*N*-*tert*-butylnitron (PBN) as a spin trap reagent, the spin-trapping experiment was started under atmospheric conditions by adding 2-mercaptoethanol as a reductant. An aliquot of the reaction mixture was rapidly transferred into a quartz cell and analyzed for electron spin resonance (ESR) at 298 K. Generation of radicals by the xanthine-xanthine oxidase system was performed according to the method described previously [17].

X-Band ESR spectra were recorded with a JES-FE-1X spectrometer (JEOL, Japan) operating with 100 kHz magnetic field modulation. The ESR conditions were as follows: frequency, 9.43 GHz; power, 8 mW; modulated amplitude, 0.8 Gauss; sweep time, 2 min; time constant, 0.1 s.

2.6. Generation of anti-BLMT monoclonal antibody

The purified BLMT was used as an antigen and injected intraperitoneally to BALB/c mice. An anti-BLMT monoclonal antibody was generated by the same method as described previously [4].

2.7. SDS-PAGE and Western blotting

The tricine-SDS-PAGE system [18] was employed for the resolution of small proteins such as BLMT at 10% acrylamide concentration. The proteins, separated by the system, were electrophoretically transferred onto a nitrocellulose membrane (Hybond-C super, Amersham, UK) and detected by the anti-BLMT monoclonal antibody using *Elite* ABC kit (Vectastain, USA).

2.8. Cell survival analysis

The cell survival assay was performed as described in [12]. *E. coli* harboring a plasmid was grown in Erlenmeyer flasks with 40 ml of Luria-Bertani (LB) medium [16] supplemented with ampicillin 200 μg/ml and gently shaken at 37°C. Portions of the culture were collected at given periods and diluted appropriately. The cells, grown on LB agar containing 200 μg/ml ampicillin, were counted to determine the colony-forming units.

3. Results and discussion

3.1. Physico-chemical properties of the ble gene product

To characterize BLMT physico-chemically, we overexpressed *ble* under the control of the *trp* promoter and purified the gene product to homogeneity. Its N-terminal five amino acid sequence was MTDQA, identical with that deduced from the nucleotide sequence of *ble*. This result indicates that the N-terminal methionine is not processed in the same way as BLMA and BLMS described in [4]. The molecular weight of BLMT, deduced from its nucleotide sequence described in [11], is 14 058 Da. However, the molecular weight of BLMT estimated by a Sephadex G-75 column chromatography, was about 34 kDa, suggesting that this protein is dimeric. The X-ray crystallographic analysis of BLMA from *S. verticillus* showed that the protein takes a dimer structure (submitted). The isoelectric point of BLMT determined with PAGE containing ampholytes (pH 3–5, Bio-Lyte, Bio-Rad, USA), was 4.5. This value is almost the same as the theoretical isoelectric point (4.34) estimated from the deduced amino acid sequence, clearly indicating that BLMT is also an acidic protein like BLMA, BLMS and Sh*ble* protein. Even when incubated with BLMT in the absence of co-factors, such as acetyl coenzyme A or ATP, Bm lost the ability to degrade DNA. In addition, heat treatment at 100°C for 20 min or digestion with protease K (200 μg/ml) of the BLMT/Bm complex restored the DNA-cleaving activity of the antibiotic. These results suggest that BLMT protects against Bm-induced DNA

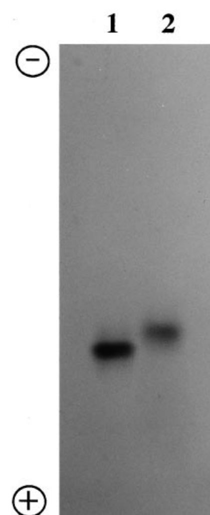

Fig. 1. The electrophoretic migration profile of the BLMT protein pre-incubated with Bm. BLMT (8 μg) was incubated with Bm (8 μg) for 3 h at 16°C and subjected to native PAGE containing 15% acrylamide. Lanes 1 and 2, BLMT without and with Bm, respectively.

breakage, which occurs without covalent modification of the drug, like BLMA [4]. Fig. 1 shows that BLMT migrated more slowly on native PAGE when pre-incubated with Bm, than without Bm.

The Fe(III)-Bm-oxygen system, in the presence of a reductant, or the Fe(II)-Bm-oxygen system without the reductant produces potentially reactive free-radical species [19–22]. We examined, using an ESR spin-trapping technique, whether BLMT interferes with the production of hydroxyl radicals by the Fe(III)-Bm system in the presence of 2-mercaptoethanol under atmospheric conditions [19,20]. Reduction of the Fe(III)-Bm (1:1) complex by adding 2-mercaptoethanol in the presence of PBN gave the ESR spectrum shown in Fig. 2A. This signal is essentially identical to the PBN-OH adduct, which is the same as that identified previously [19]. However, ESR signals of the Fe(III)-Bm complex, pre-incubated with an equimolar amount of BLMT for 10 min at room temperature, were not observed on addition of 2-mercaptoethanol (Fig. 2B).

Hydroxyl radicals are also generated by the xanthine-xanthine oxidase reaction system [17]. The ESR spectrum, given by this system using PBN, is shown in Fig. 2C. This PBN-OH signal was observed when the system was pre-incubated with BLMT (data not shown), indicating that BLMT traps neither hydroxyl radicals nor PBN. These results clearly show that BLMT inhibits the DNA cleavage which is caused by the production of hydroxyl radical.

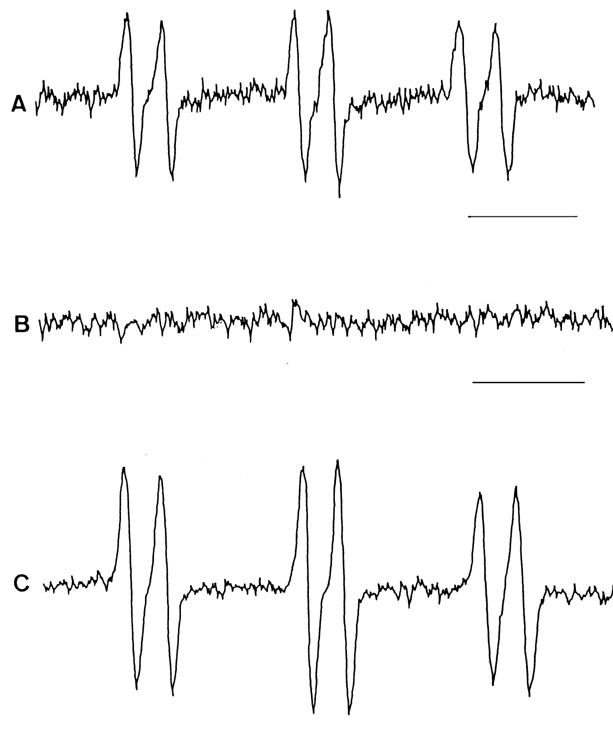

Fig. 2. ESR spectra obtained from chemical reduction of Fe(III)-Bm (A and B) and xanthine-xanthine oxidase reaction (C) using PBN as a spin trap. A: 0.1 mM Bm-Fe(III) complex and 80 mM PBN. B: 0.1 mM Fe(III)-Bm complex pre-incubated with 0.1 mM BLMT and 80 mM PBN. C: 0.5 mM xanthine, xanthine oxdase (25 mU), 0.014 mM EDTA and 80 mM PBN. The scale bars indicate 10 Gauss.

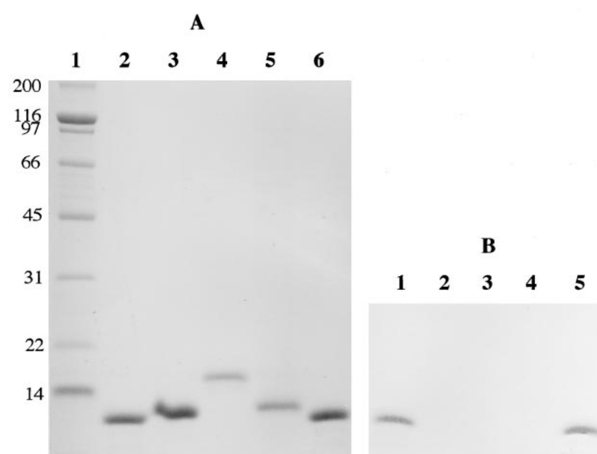

Fig. 3. Tricine-SDS-PAGE (A) and Western blotting (B) profiles of Bm-binding proteins. A: The proteins were stained with Coomassie brilliant blue R-250. Lanes: 1, molecular size marker; 2 and 6, BLMT (2 μg); 3, Sh*ble* protein (2 μg); 4, BLMA (2 μg); 5, BLMS (2 μg). B: The proteins, Western-blotted using anti-BLMT monoclonal antibody, were stained with Elite ABC Kit. Lanes: 1 and 5, BLMT (10 ng); 2, Sh*ble* protein (1 μg); 3, BLMA (1 μg); 4, BLMS (1 μg).

3.2. Immunological properties of BLMT

To generate an anti-BLMT monoclonal antibody, we used the purified BLMT (Fig. 1) as an antigen. Eight hybridomas, which secrete antibodies showing an anti-BLMT activity, were screened by enzyme-linked immunosorbent assay (ELISA). The antibody designated No. 925-1 showed the highest activity among the eight candidates. Western blot analysis reveals that No. 925-1 strongly reacts with BLMT (Fig. 3B, lanes 1 and 5), but not with Sh*ble* protein, BLMA and BLMS, even when used at 100-fold the concentration used for BLMT (Fig. 3B). BLMT always migrated on Tricine-SDS-PAGE [18] at about 12 kDa, which is smaller than the molecular size (14 kDa) deduced from the nucleotide sequence. On the other hand, BLMA always migrated on the same PAGE at 15.5 kDa, which is larger than expected [4]. In addition, an anti-BLMA monoclonal antibody [4] did not cross-react with

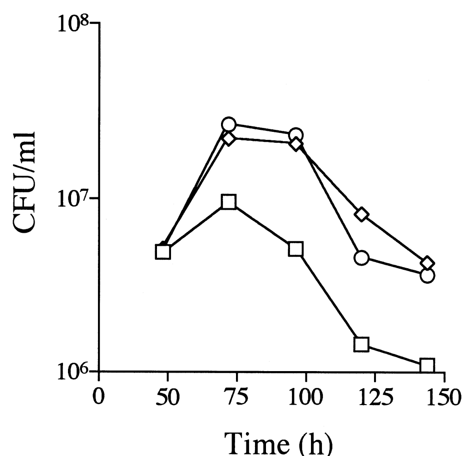

Fig. 4. Survival curves of *E. coli* HB101 transformed with the following plasmids. □, pKK*trp*; ◇, pKKblmTM; ○, pKKblmT. The colony-forming units (CFU) were determined as described in Section 2.

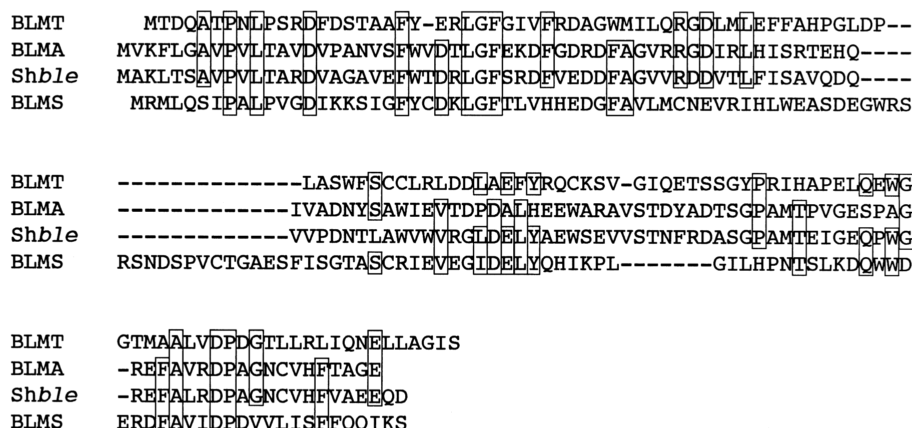

Fig. 5. Alignment of the amino acid sequences of BLMA, Sh*ble* protein, BLMS and BLMT. The boxes indicate conserved amino acids.

BLMT (data not shown). We have also shown that a polyclonal antibody, raised against Sh*ble* protein in rabbit, did not cross-react with BLMA [4]. These results suggest that BLMT is immunologically distinct from the other Bm-binding proteins, although they share the same function.

3.3. The ble-mediated Bm resistance is independent of its survival advantage

In addition to the function of the *ble* gene product as a Bm resistance determinant, the protein has been suggested to confer a survival advantage to the host cells, as a result of the enhancement of the DNA repair system [12,13]. To analyze the relationship between the *ble*-mediated Bm resistance and the survival advantage on the host cell, we created a mutant *ble* protein which lacked Bm-resistant activity. The minimum inhibitory concentration (MIC) of Bm against *E. coli* carrying *ble* was over 250 μg/ml, whereas the same host transformed with the plasmid carrying a mutant *ble*, replacing proline with leucine at N-terminal amino acid position 7, lost resistance to Bm (MIC $<$ 4 μg/ml). Fig. 4 shows that *E. coli* harboring the mutant *ble* exhibited the same survival curve as that harboring the wild-type *ble* (Fig. 4). These results suggest that even if the *ble* gene product lost Bm resistance activity, it retained the ability to provide a survival advantage to the host cell.

Fig. 5 shows conservative regions among the alignment of amino acid sequences of the four proteins. These Bm-binding proteins share similar physico-chemical properties of a low isoelectric point and a low molecular weight (14 kDa). Judging from these characteristics, their encoding genes might be derived from the same ancestor.

We discovered that BLMT inhibits the generation of hydroxyl radical by the Fe(III)-Bm complex in the presence of a reductant, as a result of its binding to Bm. This result suggests that the inhibition results in the disappearance of the Bm-induced DNA breakage and antibacterial activities [23].

Our established anti-BLMT monoclonal antibody showed specific binding to BLMT. Although BLMT inhibits Bm-induced DNA breakage, restoration of the Bm-induced DNA breakage was observed when BLMT was pre-incubated with the anti-BLMT monoclonal antibody (data not shown), suggesting that the antibody-bound BLMT no longer binds the drug. This result not only exhibits specificity of the antibody to BLMT, but also definitely confirms that BLMT is a Bm-binding protein.

The X-ray crystal structure analysis of the Sh*ble* protein revealed that the N-terminal proline 9 residue may be necessary for arm exchange to form the dimer structure [24,25]. In fact, the proline residue is conserved in all four Bm-binding proteins, including BLMT (Fig. 5). Replacement of proline 9 by leucine in BLMA from *S. verticillus* abolished Bm resistance in *E. coli*. We confirmed that this mutant BLMA lost the Bm-binding ability due to its forming no dimer structure (our unpublished data). In the present study we created a mutant protein in which proline 7 in BLMT was replaced by leucine. The *E. coli* cells transformed with the mutant *ble* lost Bm resistance. This may be due to the loss of Bm-binding ability of BLMT like the mutant BLMA. The *ble*-mediated Bm resistance in *E. coli* has been demonstrated to require the expression of host genes, such as *aidC* and *polA*, which induce the DNA repair system [26]. Several advantages to *E. coli* carrying *ble* are proposed to be associated with the *ble*-induced enhancement of the DNA repair system, which increases the repair of spontaneous DNA lesions. The present study showed that the survival of *E. coli* carrying the mutant *ble* lacking Bm resistance was the same as that of *E. coli* carrying the wild-type *ble*, indicating that even the mutant *ble* can induce the DNA repair system of the host cells.

The present study suggests that BLMT is a bifunctional protein, in which the two functions are independent. Judging from the result of the mutant BLMA, the mutant BLMT, created by replacing proline with leucine, might also not form a dimer structure. This suggests that the mutant protein is likely to abolish the function as the Bm resistance determinant. It is of interest that the monomeric BLMT, which does not confer Bm resistance, can improve the survival advantage to the host cell as a result of a putative enhancement of the DNA repair system. To elucidate the unique biological function of BLMT, crystallization and X-ray diffraction studies of the BLMT protein are in process. These results will be submitted elsewhere.

Acknowledgements: We thank Nippon Kayaku Co., Ltd. for the gift of bleomycin A_2 sulfate. This investigation received support from the Ministry of Education, Science, Sports and Culture, Japan.

References

[1] Kross, J., Henner, W.D., Hecht, S. and Haseltine, W.A. (1982) Biochemistry 21, 4310–4318.
[2] Stubbe, J. and Kozarich, J.W. (1987) Chem. Rev. 87, 1107–1136.
[3] Sugiyama, M., Thompson, C.J., Kumagai, T., Suzuki, K., Deblaere, R., Villaroel, R. and Davies, J. (1994) Gene 151, 11–16.
[4] Sugiyama, M., Kumagai, T., Matsuo, M., Bhuiyan, M.Z.A., Ueda, K., Mochizuki, H., Nakamura, N. and Davies, J.E. (1995) FEBS Lett. 362, 80–84.
[5] Sugiyama, M., Kumagai, T., Shionoya, M., Kimura, E. and Davies, J.E. (1994) FEMS Microbiol. Lett. 121, 81–86.
[6] Drocourt, D., Calmels, T., Reynes, J.-P., Baron, M. and Tiraby, G. (1990) Nucleic Acids Res. 18, 4009.
[7] Gatignol, A., Durand, H. and Tiraby, G. (1988) FEBS Lett. 230, 171–175.
[8] Bhuiyan, M.Z.A., Ueda, K., Inouye, Y. and Sugiyama, M. (1995) Appl. Microbiol. Biotechnol. 43, 65–69.
[9] Sugiyama, M., Yuasa, K., Bhuiyan, M.Z.A., Iwai, Y., Masumi, N. and Ueda, K. (1996) Appl. Microbiol. Biotechnol. 46, 61–66.
[10] Genilloud, O., Garrido, M.C. and Moreno, F. (1984) Gene 32, 225–233.
[11] Mazodier, P., Cossart, P., Giraud, E. and Gasser, F. (1985) Nucleic Acids Res. 13, 195–205.
[12] Blot, M., Meyer, J. and Arber, W. (1991) Proc. Natl. Acad. Sci. USA 88, 9112–9116.
[13] Blot, M., Hauer, B. and Monnet, G. (1994) Mol. Gen. Genet. 242, 595–601.
[14] Barany, F. (1985) Gene 37, 111–123.
[15] Deng, W.P. and Nickoloff, J.A. (1992) Anal. Biochem. 200, 81.
[16] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[17] Beauchamp, C. and Fridovich, I. (1970) J. Biol. Chem. 245, 4641–4646.
[18] Schägger, H. and von Jagow, G. (1987) Anal. Biochem. 166, 368–379.
[19] Tero-Kubota, S., Ikegami, Y., Sugioka, K. and Nakano, M. (1987) Biochem. Int. 14, 879–887.
[20] Sugiura, Y., Suzuki, T., Kuwahara, J. and Tanaka, H. (1982) Biochem. Biophys. Res. Commun. 105, 1511–1518.
[21] Sugiura, Y. and Kikuchi, T. (1978) J. Antibiot. 31, 1310–1312.
[22] Oberley, L.W. and Buettner, G.R. (1979) FEBS Lett. 97, 47–49.
[23] Lown, J.W. and Sim, S.K. (1977) Biochem. Biophys. Res. Commun. 77, 1150–1157.
[24] Dumas, P., Bergdoll, M., Cagnon, C. and Masson, J.-M. (1994) EMBO J. 13, 2483–2492.
[25] Bergdoll, M., Remy, M.-H., Cagnon, C., Masson, J.-M. and Dumas, P. (1997) Structure 5, 391–401.
[26] Blot, M., Heitman, J. and Arber, W. (1993) Mol. Microbiol. 8, 1017–1024.