Characterization of *Bacillus subtilis* ExoA Protein: a Multifunctional DNA-repair Enzyme Similar to the *Escherichia coli* Exonuclease III

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To discover the physiological role of the *Bacillus subtilis* ExoA protein, which is similar in amino acid sequence to *Escherichia coli* exonuclease III, an *exoA::Cm* disruption was constructed in the chromosomal DNA of *B. subtilis*. There was no clear difference in tolerance to hydrogen peroxide and alkylating agents between the disruptant and the wild type strain. An expression plasmid of the ExoA in *E. coli* was constructed by inserting the *exoA* gene into the expression vector pkP1500. The purified ExoA was used to clarify enzymatic characterizations using synthetic DNA oligomers as substrates. A DNA oligomer containing a 1', 2'-dideoxyribose residue as an AP site, a DNA-RNA chimera oligomer, and a 3' end 32P-labeled oligomer were synthesized. It has been shown that the ExoA has AP exonuclease, 3'-5' exonuclease, ribonuclease H, and 3'-phosphomonoesterase activities. Thus, it has been confirmed that ExoA is a multifunctional DNA-repair enzyme in *B. subtilis* that is very similar to *E. coli* exonuclease III except that ExoA has lower 3'-5' exonuclease activity than that of *E. coli* exonuclease III.

**Key words:** *Bacillus subtilis*; ExoA; AP endonuclease; synthetic DNA; multifunctional DNA-repair enzyme

DNA is the target of numerous physical and chemical agents such as ionizing radiation, ultraviolet irradiation, and mutagenic compounds. Appearance of an apurinic/apyrimidinic (AP) site is one of the most commonly occurring types of DNA damage. AP sites arise spontaneously under physiological conditions as a result of depurination, and can also arise as the product of DNA N-glycosylases that catalyze the removal of damaged or misincorporated bases on DNA. The AP site inhibits normal DNA replication and then causes cell death, mutation, and so on.

Because the AP site is a common lesion that arises incessantly in cellular DNA, AP endonucleases are an important in the repair of damaged DNA containing AP sites. The major AP exonuclease in *E. coli* is exonuclease III, which belongs to the family of class II AP endonucleases. Class II AP endonucleases repair DNA by cleaving 5' to an AP site, leaving a 3'-hydroxyl group. Exonuclease III is a multifunctional DNA-repair enzyme that has AP endonuclease, 3'-5' exonuclease, ribonuclease H, 3'-phosphomonoesterase, and 3'-repair phosphomonoesterase activities. Cloning and base sequencing of the class II AP endonuclease genes from various organisms, such as human, bovine, mouse, Drosophila melanogaster, and Streptococcus pneumoniae enzymes, have been done. The amino acid sequences of these enzymes show a remarkably high homology to those of *E. coli* exonuclease III. With respect to the exonuclease III and the human AP endonuclease HAP1, the crystal structures have been reported. Based on comparison of the crystal data of these AP endonucleases and DNase I, which has significant structural similarity to the AP endonucleases, an AP site recognition mechanism of the type II AP endonuclease has been speculated on. Recently, we have shown that exonuclease III cleaves not only the 5' side of the AP site in the DNA duplex but also the 5' side of the AP site of single-stranded DNA.

The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis* has been published. Its genome of 4,214,810 base pairs (bp) comprises about 4,100 protein-coding genes. The amino acid sequence of the ORF at the location from 4,197,746 to 4,196,988 bp in the 358.5° region of the *B. subtilis* genome is very similar to that of exonuclease III (Fig. 1). Consequently, it is expected that the substrate specificity of this gene product (ExoA) is the same as that of exonuclease III. However, in the case of the bovine AP endonuclease (BAP1), this endonuclease is devoid of 3'-5' exonuclease activity, in spite of the high similarity of the amino acid sequence to that of *E. coli* exonuclease III. In addition, the HAP1 AP endonuclease has reducing activity, which activates the human transcription factors Fos and Jun.

In this study, we constructed the insertion mutant, *B. subtilis* (exoA), to compare the tolerances to an oxidizing and an alkylating reagent of the disruptant with those of the wild strain. Moreover, to obtain the ExoA protein, we constructed the plasmid for overexpression of the ExoA protein in *E. coli*. The ExoA protein produced in exonuclease III-deficient *E. coli* was

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**Abbreviations:** AP site, apurinic/apyrimidinic site; Ap, ampicillin; Cm, chloramphenicol; Dig, digoxigenin; EthBr, ethidium bromide; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine
purified by various column chromatographies. The sub-
strate specificity of the purified ExoA protein was exam-
ined, using synthetic oligonucleotides as substrates.

Materials and Methods

Bacterial strains and plasmids. The commercial strain
E. coli JM109 and plasmid pMW119 were purchased from
Takara Shuzo (Kyoto, Japan) and Nippon Gene
(Toyama, Japan), respectively. Chromosomal DNA was
isolated from B. subtilis AC327 (purB, his-1, smo-1). 20
E. coli JM109 and BW2001 (nfo-2, xth-11) 21 were used
as host cells to construct plasmids and to overproduce
the ExoA protein, respectively. The expression vector
pKPI500 22 was provided by Dr. T. Miki (Kyushu Univ.).
E. coli and B. subtilis were grown in modified Luria-Ber-
tani medium (5 g of yeast extract, 10 g of polypeptone,
10 g of NaCl per liter [pH 7.2]). When necessary, am-
icillin (Ap: 50 μg/ml) and/or chloramphenicol (Cm: 20 μg/ml) were added to the medium. Schaeffer medium
(8 g of nutrient broth, 0.25 g of MgCl2, 1 g of KCl per
liter, 1 mM Ca(NO3)2, 10 mM MgSO4, and 1 mM FeCl3)
was used to obtain the spores.

Gene amplification by PCR and DNA labeling. The
exO A gene fragment was amplified from the chromo-
somal DNA of B. subtilis AC327 by PCR using Taq
DNA polymerase (Perkin Elmer) with 5'-CCGCG-
GAATTCATGAGTATTTCTGAAATGTA- 3' (the exO A
sequence is italicized, the initiation codon is boldfaced,
and the EcoRI site is underlined) and 5'-
GCTGGACCTGATCCTGCTAAGAAG-
CAGG-3' (the complimentary sequence of the exO A
sequence is italicized, the complimentary sequence of
the TGA termination codon is boldfaced, and the HindIII
site is underlined) as primers. The Cm' gene was
amplified by PCR with plasmid pUD1 (Cm') 20 as a tem-
plate and with 5'-GGCTGCTATGCTCCAATACAAAG-
CACCATTAGTT-3' and 5'-CCGAGCATGCAAC-
TAACCGGCGAGTTAGTACG-3' as primers.

Northern blot analysis. A culture suspension was cen-
trifuged at various times. Each pellet (cell density, 0.01
OD) was used for RNA preparation with Isogen
(Nippon Gene). RNAs were fractionated by electropho-
resis in agarose-formaldehyde gels. The labeled exO A
DNA fragment was obtained using the Dig labeling Kit
(Boehringer Mannheim), followed by Southern hybrid-
ization. Hybridization and detection were done with a
Dig Luminescent Detection Kit (Boehringer Mannheim).

Observation of phenotypic change of an exoA-
deficient mutant. Growth and spore formation of
ExoA- mutant in LB and Schaeffer mediums were com-
pared with those of the wild-type strain. Heat resistance
of the purified spores was measured by germination of
heated spores in boiling water. Heating times were va-
ried from 5 min to 30 min. Tolerances to chemical rea-
gents were judged by the size of the growth inhibitory
circle. Concentrations of H2O2, N-methyl-N'-nitro-N-
nitrosoaniline (MNNG), and ethidium bromide (EthBr)
at the center of the disks were 0.5%, 2.5 mg/ml,
and 0.025 mg/ml, respectively.

Overproduction and purification of ExoA. Compet-
ten E. coli BW2001 was transformed with plasmid
pKPE1. E. coli BW2001 harboring the plasmid was grown in
LB-medium containing ampicillin (50 mg/ml)
at 28°C overnight. The culture (5 ml) was added to 1.5 l
of LB-medium and incubated at 28°C. Cells were har-
ested by centrifugation (6,000 rpm, 4°C, 5 min), and
then suspended in 50 ml of the ultrasonication buffer
(50 mM KCl, 1 mM EDTA, 0.002% phenylmethanesul-
fonyl fluoride, 50 mM Tris-HCl, pH 8.0) followed by
the addition of 4 g of glass beads, on ice. Cells were
ultrasonicated with a Tomy UD200 (15 cycles of 1 min
treatment and 2 min rest) on ice. The supernatant was
collected by centrifugation (15,000 rpm, 5 min, 4°C).
The ExoA protein was precipitated in 70%-90% ammio-
nium sulfate concentration. The precipitated proteins
were dissolved in 100 ml of 20 mM Tris-HCl (pH 8.0),
0.1 mM DTT, and 0.002% sodium azide and then dia-
lized with 2 l of 20 mM Tris-HCl (pH 8.0) and 0.1 mM
DTT. The resulting solution was put on a DEAE cellul-
lose column (Whatman DE52) and proteins were eluted
with a linear gradient from 0 to 1 M NaCl (total 240 ml,
20 mM Tris-HCl (pH 8.0)). The fraction containing
ExoA was collected and dialyzed against the dialysis
buffer (20 mM potassium phosphate (pH 6.5), 0.1 mM
DTT). The dialyzed solution was put on a Whatman
P11 phosphocellulose column (Whatman), and proteins
were eluted with a linear gradient from 0.02 to 1 M
potassium phosphate buffer (pH 6.5) containing 0.1 mM
DTT. The fraction containing ExoA was dialyzed
against the dialysis buffer (20 mM Tris-HCl (pH 8.0),
0.1 mM DTT) and the dialyzed solution was put on a
Resource Q column (Pharmacia Biotech.). The ExoA
protein was eluted with a linear gradient from 0 to 0.5 M
NaCl.

Oligonucleotide synthesis and labeling. The oligo-
nucleotides used in this study were synthesized on an
Applied Biosystems 391 DNA synthesizer. Protected DNA,
RNA, and 1,2-dideoxyribose phosphoroamidite
monomers were purchased from Applied Biosystems
and Glen Research (Sterling, VA). The oligomers were
purified by HPLC on a reverse-phase C18 column. The
5' end of the oligomer was labeled with [γ-32P]ATP
(Amersham) by T4 polynucleotide kinase (Takara).
To detect the 3'-phosphomonoesterase activity of the
ExoA protein, the 3' end labeled oligomer was pre-
pared. The oligomer (d-5'CCCTCTCTTTCTCTCT-
CTCTCT') was elongated with [α-32P]d-ATP and the other cold d-NTP (N: G, C, and T) by the Klenow frag-
ment (Takara) using d-5'ACACGATAAGAGGAG-
AGAGAGAAGGGGAAGG3' as a template. The elongated oligomer (d-CCCTCTCTTTCTCTCTCT-
CTCTCT')pATTGTCTGT, asterisk: [32P]labeling site) was heated
in 0.1 M formic acid at 57°C for 16 h. After evapora-
tion of formic acid solution, the oligomer was dissolved
in 0.1 M piperidine and heated at 90°C for 1 h. The
resulting oligomer (d-CCCTCTCTTTCTCTCTCT-
CTCTCT')p was purified in a MicroSpin S-200 HR column
Fig. 1. Alignment of Amino Acid Sequences of the ExoA Protein of *B. subtilis* and Bacterial AP Endonucleases.

The consensus amino acids are indicated by bold type, and the amino acids in the catalytic site of exonuclease III of *E. coli* are indicated by closed triangles.

(Pharmacia). Purity of the sample was confirmed by 7 m urea polyacrylamide gel electrophoresis. DNA bands were made visible by autoradiography. The duplex (d-CCTCTCCTTCTTCTCTCTCCTCp/d-ACACGAA-TAGAGGAAGGAGAAAGAGGG) was used as a substrate to detect the 3’-phosphomonoesterase activity of the ExoA protein.

**Assay for enzymatic activities of the ExoA protein.** The AP endonucleolytic activity of ExoA was assayed as previously described by use of the 5’-end labeled oligonucleotide containing a 1',2'-dideoxyribose residue as an AP site. The 3’-5’ exonuclease activity of ExoA was assayed by use of the duplex with a 5’-end overhanging duplex. The RNase H activity of ExoA was assayed by use of a duplex consisting of the DNA-RNA chimera and DNA oligonucleotides. After addition of the enzyme, the samples were incubated for 10 min at 37°C and chilled on ice. In order to detect the released phosphate in the reaction mixture, the Norit adsorption procedure was done.

**Results and Discussion**

**Construction of an insertional mutant, *B. subtilis* AC327 (exoA::Cm)**

To construct the *exoA* insertional mutant of *B. subtilis* AC327, the plasmids shown in Fig. 2A were prepared. The *exoA* gene fragment was amplified from the chromosomal DNA of *B. subtilis* AC327 by PCR. This *exoA* gene fragment was cloned into the multicloning site of pMW119, and the resulting plasmid was named pMW1. To disrupt the gene by the Cm' gene, the Cm' gene fragment was amplified by PCR with pUD1 (Cm') as a template, and then the fragment was cloned at the *SpI* site on the *exoA* gene of pMW1. The resulting plasmid pMWEC1 was linearized by Scal digestion, and the resulting DNA fragment was used to transform competent *B. subtilis* subtilis AC327 cells to construct the insertional mutant, *B. subtilis* EC327 (exoA), by double cross-over integration. To confirm the gene structure of the insertional inactivated mutants, chromosomal DNA was extracted from the transformants, digested with *PstI* and *SmaI*, and electrophoresed on an agarose gel, followed by Southern hybridization analysis with a Dig-labeled *exoA* gene fragment (data not shown). The *SmaI-PstI* fragment from pMW1 containing the *exoA* gene as a probe hybridized to a *SmaI-PstI* chromosomal DNA fragment of *B. subtilis* BC327 at 1.3 kbp and hybridized to a *SmaI-PstI* fragment of *B. subtilis* 168S (wild type) chromosomal DNA at 0.4 kbp. Since this 0.9 kbp difference between the hybridization bands corresponded to the 0.9 kbp Cm' gene insert in pMW1, the desired double cross-over integration, leading to inactivation of the *exoA* gene, was indicated.

**Observations of phenotype of the *exoA*-deficient mutant**

To confirm the expression of the *exoA* gene in vivo, Northern blot analysis with the *exoA*-specific probe was done. The result of Northern blot analysis indicated that the *exoA* gene was transcribed during the exponential growth phase in the wild type strain *B. subtilis* AC327 (unpublished results). With respect to growth and spore formation of the mutant in LB and Schaeffer media, there were no significant differences between the *exoA*-deficient mutant and wild type strain (data not shown).

Heat resistances of the purified spores were measured by survival of boiled spores. The germination ratios of the *exoA*-deficient mutant and wild type strain after boiling for 10 min were 53% and 44%, respectively. After boiling for 30 min, the ratios were further reduced to 14% and 11%, respectively. Thus heat resistances of both spores were almost identical. Tolerances to chemical reagents (MNN, H₂O₂, and EthBr) were judged by the sizes of the growth inhibitory circles. No differences in the sizes of the growth inhibitory circles were observed among any of the reagents (data not shown). In *E. coli*, exonuclease III accounts for approximately 80%
Fig. 2. Construction of Plasmid DNAs and an exoA-Deficient Mutant of B. subtilis.

A. Insertional mutation of the chromosomal exoA gene by transformation with linearized pMEC1. B. Construction of pKPE1 used in expression of the exoA gene. The arrows indicate the directions of transcription of the genes. Restriction endonuclease sites related to the construction of the plasmids and the disruptant are presented.

of the cellular AP endonuclease activity. En- donuclease IV, which is the minor AP endonuclease in E. coli, accounts for no more than 10% of the AP endonuclease activity. In B. subtilis, however, the extent of the contribution of the ExoA protein is not known in the repair of the AP sites. xthA- and/or nfo- E. coli, which are missing AP endonucleases (endonuclease III and/or endonuclease IV, respectively), are more sensitive to the alkylating agent MNNG and the oxidizing agent H2O2 than wild type strain. The results in this study suggested that AP endonucleases, other than the ExoA protein, might contribute mainly the repair of AP sites or chemical agent degradation enzymes such as catalase might act effectively to suppress the formation of DNA damages.

Expression of the ExoA protein in E. coli

The exoA gene fragment was amplified from the chromosomal DNA of B. subtilis AC327 by PCR. The amplified exoA gene was cloned between EcoRI and HindIII sites within multicloning sites of the expression vector pKP1500, which preserves the lac promoter, the lac SD sequence, and the rnbA terminator. Thus, we constructed an expression plasmid, pKPE1, as shown in Fig. 2B. This plasmid was introduced into E. coli BW2001 (xth, nfo). The ExoA protein produced in E. coli was purified as described in Materials and Methods. Figure 3 shows SDS-PAGE of the total cellular proteins of E. coli BW2001 (lane 1) and of the harvested cells harboring plasmid pKPE1 (lane 2). Proteins in each

Fig. 3. SDS-Polyacrylamide Gel Electrophoresis of Fractions from the ExoA Purification and Zymography of the Purified ExoA Protein.

SDS-PAGE was stained with Coomassie brilliant blue R-250 (lanes from 1 to 7) and zymography was stained with ethidium bromide (lane 8). Lane 1, cell-free extract from E. coli BW2001; lane 2, cell-free extract from E. coli BW2001 harboring pKPE1; lane 3, supernatant fraction after ultrasonication; lane 4, ammonium sulfate precipitate; lane 5, DEAE cellulose fraction; lane 6, phosphatecellulose fraction; lane 7, Resource Q fraction. For activity staining (lane 8), calf thymus DNA containing AP sites was obtained by heating in 100 mM NaCl, 10 mM sodium citrate (pH 5.0) at 70°C for 20 min. After SDS-PAGE was done on a gel containing the calf thymus DNA (25 mg/ml), the gel was shaken in renaturation buffer (50 mM Tris-HCl (pH 8.0), 5 mM MgCl2, 0.02% sodium azide) for 1 h at room temperature followed by ethidium bromide staining.
puriﬁcation step are shown in lanes 3 to 7. When BW2001 cells harboring pKPE1 were grown at 28°C, overproduction of the ExoA protein (29.3 kDa) was observed as a band at the 33-kDa position. The amount of the puriﬁed ExoA protein ﬁnally reached 1.32 mg from 1.5 l cultivation. Lane 8 in Fig. 3 represents activity staining (zymography) of the puriﬁed enzyme in AP site-containing DNA-cast SDS-polyacrylamide gel. As can be seen, the ExoA protein shows some nucleolytic activity. At the same time, the results for this lane indicate that the enzyme preparation does not include nucleolytic enzymes, with the exception of the ExoA protein.

Characterization of the ExoA protein

The puriﬁed ExoA protein was experimented on to clarify its enzymatic characterizations using synthetic DNA oligomers as substrates. Nucleolytic activities of the ExoA protein were compared with those of the multifunctional exonuclease III of E. coli. Since it is known that the 1′,2′-dideoxyriboburanose residue (Fig. 4, H) is essentially isosteric with the predominant form of a 2′-dideoxyribose residue (Fig. 4, dR) and that the H residue is not alkaline-labile, compared with the 2′-dideoxyribose residue, we prepared the DNA oligomer containing an H residue as an AP site and used as an enzyme substrate.

To detect the AP endonuclease activities of the enzymes on the duplex, the duplex containing an AP site was used (Fig. 5B). Both 3′-ends of the duplex were overhanged to prevent the digestion from the 3′-end of DNA by the 3′-5′ exonuclease activities of the enzymes. To a reaction mixture containing 5 pmol of the duplex, either the ExoA protein or exonuclease III was added 1 pmol, 0.1 pmol, or 0.01 pmol, respectively. After 5 minutes of incubation at 23°C, the reaction mixtures were electrophoresed in a 7 M urea polyacrylamide gel (Fig. 5A). When each enzyme was added to the reaction mixture, the digest (13-mer) at the 3′ side of the phosphodiester bond adjacent to the AP site was produced. In the case of the ExoA protein, the 13-mer product was the main product. Products smaller than the 13-mer were produced from the 13-mer substrate by the exonuclease activity. The ExoA protein of B. subtilis shows strong AP endonuclease activity and weak 3′-5′ exonuclease activity, when compared with exonuclease III of E. coli. This tendency was reproduced when single-strand DNA containing an AP site was used as a substrate (Fig. 6). In the case of exonuclease III, only a slight amount of 13-mer product was produced as a result of AP endonuclease ac-

![Fig. 4. The Structures of an Artificial (H) and a Natural (dR) AP Site Residue.](image)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Exonuclease III</th>
<th>ExoA</th>
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<tr>
<td>pmol</td>
<td>M</td>
<td>0.01</td>
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![Fig. 5. Detection of AP Endonuclease Activities of Exonuclease III and ExoA Using Double-stranded DNA Containing an Abasic Residue.](image)

A. Detection of cleavage products by exonuclease III and ExoA. B. Schematic representation of double-stranded DNA used in this study as a substrate. The arrows indicate the marker oligomers.

![Fig. 6. Detection of AP Endonuclease Activities of Exonuclease III and ExoA Using Single-stranded DNA Containing an Abasic Residue.](image)

A. Detection of cleavage products by exonuclease III and ExoA. B. Schematic representation of single-stranded DNA used as a substrate. The arrows indicate the marker oligomers.
Fig. 7. Detection of 3'-5' Exonuclease Activities of Exonuclease III and ExoA Using Double-stranded DNA.
A, Detection of cleavage products by exonuclease III and ExoA. B, Schematic representation of double-stranded DNA used as a substrate. The arrow indicates the marker oligomer.

Fig. 8. Detection of RNaseH Activities of Exonuclease III and ExoA using a Duplex Constructed of DNA-RNA Chimera Oligomer and DNA Oligomer.
A, Detection of cleavage products by exonuclease III and ExoA. B, Schematic representation of the duplex used in this study as a substrate. The arrow indicates the oligomer used as a marker.

Activity, while the 12-mer produced from the 13-mer by the exonuclease activity was the main product. On the other hand, the main product produced by the ExoA protein was 13-mer. The 12-mer band appeared clearly, when 1 pmol of the ExoA protein was used (Fig. 6A). The band of DNA product smaller than the 11-mer was not entirely absent in the case of both enzymes (Fig. 6). This phenomenon seems to be attributable to low stability of the complex between the AP endonuclease and a single-stranded oligonucleotide containing an AP site.

To confirm the 3'-5' exonuclease activity of ExoA, we used the duplex shown in Fig. 7B as a substrate. Only the 32P-labeled upper strand of the duplex was digested by the exonuclease activities of both ExoA and exonuclease III. Figure 7A shows faint ladder bands by both enzymatic digestions.

To detect RNaseH activity, we prepared a chimera oligonucleotide (Fig. 8B). The 3'-side half of the upper strand of the duplex shown in Fig. 7B was replaced by RNA. The series of ladder bands formed from the starting oligomer (25-mer) appeared in both enzymes. Since there was no apparent difference in the band patterns (Fig. 8A), the RNaseH activity of ExoA seems to be equal to that of exonuclease III.

To detect the 3'-phosphomonoesterase activity of ExoA, we prepared an oligonucleotide containing 3' terminal phosphate labeled with 32P. The desired substrate oligomer (22-mer) represented 53% of the recovered oligonucleotides. The ratios of the 25-mer and the 28-mer byproducts and 30-mer undecomposed oligomer were 17%, 16%, and 14%, respectively. Release of the labeled phosphoric acid was detected by the Norit adsorption method. The amounts of released phosphoric acid by exonuclease III and ExoA for 10 minutes reaction time were 16.2% and 15.4%, respectively (data not shown). It was proved that the strength of the 3'-phosphomonoesterase activities of the two enzymes were nearly equal.

This study demonstrated that the ExoA protein shows many of the same enzymatic activities as members of the exonuclease III family, such as AP endonuclease, 3'-5' exonuclease, ribonuclease H, and 3'-phosphomonoesterase, except that the ExoA has lower 3'-5' exonuclease activity than that of E. coli exonuclease III. Consequently, it is presumed that this ExoA protein in B. subtilis plays the role of a repair enzyme, like exonuclease III in E. coli.

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