We purified and characterized a 39-kDa *Bacillus subtilis* 168 nuclease that has been suggested in this laboratory to be involved in chromosomal DNA degradation induced by lethal heat and cold shock treatments *in vivo*. The nuclease activity was inhibited *in vitro* by aurintricarboxylic acid but not by Zn\(^{2+}\). By the mutant analysis, we identified the 39-kDa nuclease as a product of *yokF* gene. The *yokF* gene contained a putative lipoprotein signal peptide motif. After *in vitro* exposure to lethal heat and cold stresses, the chromosomal DNA fragmentation was reduced in the *yokF* mutant, which demonstrated about a 2–10-fold higher survival rate than the wild type. The *yokF* mutant was found to be more sensitive to mitomycin C than the wild type. The transformation efficiency of the *yokF* mutant was about 10 times higher than that of the wild type. It is suggested that when *B. subtilis* cells are exposed to a stressful thermal shock resulting in membrane perturbation, YokF nuclease consequently dislocates into the cytoplasm and then attacks DNA.

In cells damaged irreversibly by a lethal stress treatment, a variety of structural molecules are subjected to the activated cellular degradation system. This degradation is due to functions of endogenous degradative enzymes, such as autolysins (peptidoglycan hydrolases), proteases, phospholipases, RNases, and DNases.

Evidence has shown that a variety of structures and functions in bacterial cell are damaged by heat stress, but the relationship between the damage and cell death is still unclear. Among those, DNA and its functions should be of critical importance for cell survival. Earlier physiological studies have dealt with the effect of heat on the production of single and double strand DNA breaks. In *Escherichia coli*, the occurrence of single strand breaks at a lethal temperature of 52 °C was first demonstrated by Bridges et al. (1), and afterward, it was reported that the double strand break was also incurred on DNA by *in vivo* heating at the same temperature (2). Although a single strand break has been detected by using the alkaline sucrose gradient technique (3), this technique also picks up apurinic sites in DNA strands (4). In *E. coli*, endonuclease IV is a representative DNA repair enzyme. After exposure to 52 °C, an *E. coli* mutant defective in this enzyme demonstrates only 20–30% of the viability of the wild type strain (5).

Endonuclease IV is a major endonuclease acting on apurinic sites in *E. coli* DNA, it may be involved in the first stage of the DNA excision-repair pathway. The mutant had less DNA breaks after heat treatment, confirming that the production of DNA breaks in this case is part of the DNA repair process.

Cold shock has also been reported on *E. coli* to result in DNA damage as well as cell death (6–8). It has been suggested that one possible mechanism for cold shock lethality is the loss of magnesium ion from cells, leading to the inactivation of the magnesium-dependent DNA ligase, which joins phosphodeoxyribo-linkage gaps in the strand produced during the DNA replication and repair processes (8).

As for *Bacillus subtilis* cells, only few studies have been carried out on lethal cold shock and heat shock (9, 10). It has been reported that peptidoglycan-degradative autolytic enzymes are activated by cold shock to induce cell lysis and subsequent death (10–12). In our preliminary experiment, however, when a *B. subtilis* autolysins deficient mutant, FJ2 strain, was exposed to cold shock treatment, it still demonstrated about 50% reduction in viability.† We have therefore presumed that some additional factor(s), other than the autolysis induction, are involved in the cold shock-induced death and hypothesized the DNA damage as one of them.

In another study, in fact, we have reported that DNA is cleaved in *B. subtilis* 168 cells by a certain endogenous DNase after cold and heat shock treatments.‡ The resultant DNA fragmentation was only detected in the presence of Ca\(^{2+}\) or Mn\(^{2+}\) in a minimal synthetic medium and was also observed with the above *B. subtilis* FJ2 cells, suggesting that cell lysis is not a prerequisite for the intracellular DNA degradation. We have further concluded that the DNA fragmentation is caused by the 39-kDa nuclease.‡

In this study, we purified and characterized this DNase from *B. subtilis* 168 and then identified its encoding gene. Further, we constructed its knock-out mutant to investigate the relationship between the DNA cleavage level and the viability of cells exposed to thermal shock treatments and also to obtain a clue of understanding the physiological role of YokF nuclease.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—The strains and plasmids used are listed in Table I. *B. subtilis* strain 168 (trpC2) and its mutants were cultivated at 37 °C to an *A*\(_{600}\) of 0.3, unless otherwise stated, in either Lennox broth (L broth; 1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.0) or Spizizen minimal salts medium (15) supplemented with 0.5 g of glucose, 2 g of glutamate and 20 mg of L-tryptophan/liter. *E. coli* JM109 was used as a cloning vector for construction of plasmids to be supplied for this study (14). Cells of the JM109 strain were grown at 37 °C L broth or on L agar plates (L broth plus 1.5% agar) with an appropriate antibiotic.

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Purification of the 39-kDa Nuclease—B. subtilis 168 nucA cells were cultivated in L broth containing 1 mM MgCl₂ and 1 mM CaCl₂ at 37 °C for 5 h, until the A₆₀₀ of the culture reached 2.0. The cells were harvested, washed, and resuspended in TM buffer (50 mM Tris-HCl, pH 8.0) containing 1 mM PMSF. The cells were disrupted by incubation with 5 mg ml⁻¹ lysozyme for 1 h in ice-cold water. The resultant lysate was centrifuged at 8,500 × g for 5 min and washed twice with TM buffer. The pellet was suspended in TM buffer containing 1 mM PMSF and 1% Nonidet P-40, and the suspension was sonicated gently in ice-cold water for 1 h with an ultrasonic automatic washer (US-1; NSD Co.). After the homogenate was centrifuged at 10,000 cut) under conditions equilibrated with buffer B (20 mM Hepes-NaOH, 0.1% Triton X-100, 0.1 mM PMSF, pH 8.0). The sample was applied to a HiTrap-DEAE column (bed volume, 6 ml; Amersham Pharmacia Biotech) equilibrated with buffer B. The column was washed with buffer B containing 40 mM NaCl and then eluted with a linear gradient of 40–100 mM NaCl. Fractions containing activity were collected, and the activity was concentrated by ultrafiltration with a Q010 membrane (Advantec, molecular mass of 10,000 cut) under conditions equilibrated with buffer B (20 mM Hepes-NaOH, 0.1% Triton X-100, 0.1 mM PMSF, pH 8.0). The sample was applied to a Resource S column (bed volume, 6 ml; Amersham Pharmacia Biotech) equilibrated with buffer B. The column was washed with buffer B containing 80 mM NaCl and then eluted with a linear gradient of 80–160 mM NaCl.

**Endonuclease Assay**—DNase or RNAse activity was measured by evaluating the degree of fragmentation of B. subtilis 168 chromosomal DNA (2 μg; assay 1), supercoiled pUC19 plasmid DNA (5 μg; assay 2), single strand 13Mmp19 DNA (10 μg; assay 3) in 1% agarose gel, or total B. subtilis 168 RNA (10 μg; assay 4) in Tris/boric acid/EDTA/PAGE (6%) gel. The reaction mixture (total volume, 15 μl) for assay of endonuclease activity contained 50 μM Tris-HCl, pH 8.0, 3 mM CaCl₂, 3 mM MgCl₂, and 0.01% Triton X-100. The reaction was carried out at 37 °C for various periods and then stopped by addition of EDTA at a final concentration of 10 mM. DNase activity was assayed by measuring the amount of chromosomal DNA under a 9.4 kilobase trace × optical density level/1 min of reaction time/1 μg of protein (using Image Master, Amersham Pharmacia Biotech).

**Construction of Plasmids and Knock-out Mutants**—To construct knock-out plasmids and expression plasmids of endonuclease encoding genes, we first searched for DNA endonuclease homolog genes in B. subtilis 168 using the BLAST homology search system based on the B. subtilis genome project data base and DDBJ data base and found five unknown genes, ywJD, yqfS, yokF, yncB, and yosQ. These genes, which were amplified by polymerase chain reaction from the B. subtilis 168 chromosomal DNA with oligonucleotide primers, and the nucA gene derived from pSH19 were cloned into pET-19b or pET-21a to generate the overexpression plasmids pET-21a-yokF, pET-21a-yokF-His, pET-21a-yncB, pET-21a-nucA, pET-19b-ywjD, pET-19b-yqfS, and pET-19b-yosQ. The knock-out plasmids pBluescriptII KS(+)–yokF-Cmr, pBluescriptII KS(+)–yokF-Nm', pUC18-yncB-Cmr, pUC18-yncB-Tc', pUC19-nucA-Tc', pBluescriptII KS(+)–ywjD-Cmr, pBluescriptII KS(+)–yqfS-Cmr, and pUC18-S-Q-Cmr, were generated by subcloning of polymerase chain reaction products or nucA gene followed by ligation with ligation with Cmr, Nm', or Tc' cartridge from pMSG-CAT, pBES'T513, or pHY300PLK, respectively. The knock-out plasmids were digested with an appropriate restriction enzyme to be linearized for subsequent knock-out of chromosomal endonuclease genes by homologous recombination.

**Viability Assay**—Cell samples were appropriately diluted and plated on L agar plates containing 4 mg ml⁻¹ chloramphenicol, 7 μg ml⁻¹ neomycin, or 10 μg ml⁻¹ tetracycline, depending on the type of antibiotic for resistance.

**Thermal Shock Treatments**—Heat shock treatment was performed in medium by transfer of a flask containing cells from an incubator at 37 °C to another at 55 °C followed by incubation 55 °C for 30 min with shaking. For cold shock treatment, after the cells were concentrated by centrifugation (8,500 × g, 5 min, 25 °C), their suspension was diluted 10-fold with ice-cold medium at 0 °C and then kept for 30 min at this temperature. After that, the cells were further incubated at 37 °C for 30 min.

**Analyses of DNA Fragmentation and DNase Activity**—DNA fragmentation was evaluated as described elsewhere by using a modification of Ishizuwa's method (18).

**Expression of Recombinant DNA Endonucleases in E. coli**—We used E. coli JM109 (DE3) strains carrying the DNA endonuclease homolog encoding plasmid for heterogeneous expression. Transformants were grown at 37 °C in L broth containing 100 μg ml⁻¹ ampicillin to an A₆₀₀ of 0.15, were 0.99 and 1.03 h for the wild type and yokF mutant, respectively.

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Therefore, we cultivated the mutant and its parent 168 strain have similar levels of both thermal shock-induced DNA fragmentation, because the nucA mutants revealed that the yokF gene encoded a 39-kDa nuclease but also a 28-kDa nuclease on zymogram (Fig. 3). The latter enzyme was presumed to be a proteolytically processed but still active form like the 39-kDa type. Further, a 26-kDa enzyme having a weak DNase activity was identified to be the yncB gene product (Fig. 3A). Therefore, both yokF and yncB genes were not pseudo-genes. The YokF protein consisted of 296 amino acids with a calculated molecular mass of 32,000, and it was a basic protein with pI 8.9. The yokF gene was localized in the SPβ prophage region, and the sequence of 20 amino acid residues at the N terminus had a feature of a signal peptide with a −31LXC−1 lipobox cleavage site motif of lipoprotein (20), suggesting the ability of YokF to associate with the cytoplasmic membrane. The YokF nuclease was a homolog of a member of the thermonuclease family from Staphylococcus groups and highly resembled to B. subtilis YncB, which was also presumed to be a lipoprotein as a paralog (21).

In ywjD, yqfS, and yosQ deficient mutants, all DNase activity bands detected remained on the zymogram (Fig. 3A). In addition, in E. coli JM109 (DE3) carrying plasmids containing ywjD, yqfS, or yosQ gene, no increases in DNase activity were obtained in their cell-free extracts, and no additional bands were seen on zymogram (Fig. 4A). These results suggest that the products of ywjD, yqfS, and yosQ-encoding open reading frames have no activity of cleaving randomly double strand DNA.

Analysis of the DNA Fragmentation in DNase-deficient Mutants—In another study,2 we have reported that the nucA gene disruption reduces levels of neither DNase activity in the cell-free extract nor in vivo DNA cleavage. To clarify which DNase is involved in the DNA fragmentation caused by thermal stresses, we measured the DNase activities of mutants deficient in yokF and yncB genes. As a result, in the cell-free extract of the yokF mutants, the DNase activity was little detected, whereas the parent strain and yncB mutant had substantial levels of activity (Fig. 5A). Correspondingly, in cells of the yokF mutant exposed to these stresses, no substantial fragmentation of chromosomal DNA was found (Fig. 5B). These results indicate that YokF is a major DNase in vegetative cells at the exponential growth phase and also that it is a critical factor of thermal stress-induced DNA fragmentation.

Involvement of the 39-kDa Nuclease in Cell Death by Thermal Stresses—To know whether the DNA cleavage by YokF nuclease is a cause of thermal shock-induced cell death, we compared survival rates of cells exposed to thermal stresses between the parent strain and yokF mutant. After heat treatment at 55 °C for 30 min, the survival of the yokF mutant was 0.0113%, whereas that of the wild type 0.00396%. After cold shock treatment, the survival of the yokF mutant was 0.76%, whereas that of the wild type 0.31%. A similar result on cold-shocked cells was obtained by using the growth delay analysis method (17). The survivals of each strain were 0.0034 and 0.035%, respectively. These results demonstrate that the YokF nuclease is one of the death factors in thermally shocked cells of B. subtilis.
The Physiological Roles of YokF—To obtain a clue of understanding what is the primary role of YokF nuclease, several phenotypes were compared among single, double, and triple mutants of yokF, yncB, and nucA genes as well as their parent strain. We found the following characteristics of the yokF mutant different from other mutants and the parent. First, the yokF mutant was found to be sensitive to mitomycin C as an alkylating agent (22). Although the growth curves of these strains were similar until about 2 h after inoculation, hereafter, the growth of only yokF-deficient mutant was inhibited by mitomycin C (data not shown). This sensitivity might be induced by accumulation of damaged DNA. Second, the yokF mutant demonstrated an altered ability of competence (Table III). The transformation efficiency in the presence of 1 mM CaCl2 of the yokF mutant was 10 times as much as that of the wild type. This result suggests that one of the functions of YokF may possibly be the degradation of extracellular DNA. Third, the yokF mutant was not able to metabolize chromosomal DNA added externally, whereas the parent was able to do so and grew well. In a medium containing chromosomal DNA as a phosphagen and 3 mM CaCl2, the wild type grew faster than the DNase-deficient mutants tested, including yokF mutant. In the absence of CaCl2, all strains tested could not grow. At the exponential growth phase, all strains had little activity of extracellular DNase, and also no individual activities of YokF, YncB, and NucA were detected in the medium (data not shown).

**DISCUSSION**

In our other paper, we have demonstrated on B. subtilis that cold shock and heat shock treatments cause DNA fragmentation accompanied by cell death and further that the 39-kDa nuclease may be involved in the DNA cleavage. The results obtained in this study strongly substantiate the involvement of this nuclease.

From zymographic study B. subtilis 168 vegetative cells apparently have at least three major DNases, YokF (39 kDa and its possibly processed form, 28 kDa), YncB (26 kDa), and NucA (17 kDa) (23, 24). An additional 60-kDa DNase is an inactive enzyme in the cell-free extract and is encoded in an unidentified gene. Up to date, the presence of several DNases, including a Ca2+-dependent exonuclease (25), an ATP-dependent nuclease (26, 27), and a Mg2+-dependent endonuclease (28, 29), have been reported in B. subtilis. Merchante et al. (30) have found several DNases in the periplasm, membrane, and cytoplasm by using zymogram, and Coughlin et al. (31) have also analyzed B. subtilis DNases by using two-dimensional zymography analysis and detected 83 nuclease spots. However, we wonder whether many of these enzymes are proteolytic products generated during the lysozyme treatment at 37 °C. In fact, we have observed that YokF (39-kDa form) is very sensitive to serine protease in cell-free extract, and therefore it seems difficult to detect all DNases as intact forms.

Although we attempted to determine the N-terminal amino acid sequence of 39-kDa YokF by the Edman method, we did not succeed because of its blocking by a lipid. From the genome analysis project, YokF and also YncB have been found to possess a putative signal peptide of lipoprotein, called the lipobox cleavage site, LXXC (Ref. 20 and Fig. 6). The lipobox-processed YokF is suggested to consist of 277 amino acids with a calculated molecular mass of 31,000. The modification of cysteine residue in the lipobox of YokF by the diacylglycerol transferase, Lgt (32), is a prerequisite for processing of the lipoprotein precursor by signal peptidase II (33). The molecular mass of mature YokF protein was indicated to be 39,000 from SDS-PAGE but was 31,000 when we estimated by mass spectrometry. An overestimated molecular mass obtained with SDS-PAGE is probably due to the presence of a lipid in the molecule and highly basic property. The purified YokF seems to contain no cysteine residues in the active site of the molecule, or the disulfide bond may not be involved in its activity and conformation. The fact that the purified YokF nuclease is not inactivated even after heated at 55 °C for 30 min is consistent with an idea that the DNase is involved in DNA cleavage observed in B. subtilis 168 cells heated to 55 °C.

In the other study, we have suggested that the 39-kDa YokF is localized in the membrane fraction by using Triton X-114 two-phase preparation system. Although NucA has also been reported to be a membrane protein, both NucA and its inhibitor NIn have no signal peptide domain of lipoprotein (23, 24). Two examples of nuclease modified with a lipid have been known. One is a Ca2+- and Mg2+-dependent DNase purified from the membrane fraction of *Mycoplasma penetrans*, and its structural gene *mnuA* has already been identified (34, 35). Because MnuA has a signal peptide motif (TISC) of lipoprotein, this is probably the first enzyme identified as a lipoprotein DNase (35). As another example is a modified *S. aureus* nuclease constructed for protein secretion study. In this nuclease, its inherent signal peptide I is artificially converted to a signal peptide II of Nlp lipoprotein derived from *Lactococcus lactis* (36). The 28-kDa YokF may possibly be a product processed at a site different from the lipobox cleavage site of YokF molecule.

![Fig. 2. Substrate specificity of the 39-kDa nuclease. A and B, the purified 39-kDa nuclease was incubated at 37 °C for different periods (0, 10, 20, or 30 min) with 0.5 μg of native double strand (ds) DNA of M13mp19 (A) or 1 μg of single strand (ss) DNA of M13mp19 (B). C, the 39-kDa nuclease was incubated at 37 °C for different periods (0, 30, or 60 min) with 2 μg of B. subtilis 168 RNA extracted by acid-phenol method. Samples of no addition of the DNase and of cell-free extract from B. subtilis 168 cells were also treated similarly.](http://www.jbc.org/)
ity bands are indicated with arrowheads. The DNase activity bands are indicated with arrowheads. The positions of the size markers (M.W.) are indicated on the left of the stained gel. The DNase activity bands are indicated with arrowheads.
YokF Nuclease of Bacillus subtilis

nucleotide synthesis and metabolism. Considering the results obtained in this study, it is likely that YokF may function as a member of a possible cellular DNA recycling system consisting of the degradation of intracellular damaged DNA and reuse of the degraded products. YokF may also work as a cellular self-defense system to protect cell from invasion or infection by an extracellular foreign DNA, plasmid, or bacteriophage, as suggested by an increased ability of competence in yokF mutant. YokF and its homologs might have a role for prevention of horizontal transfer and recombination of genes between bacterial cells in the natural environment. When the extracellular DNA is taken up by a bacterial cell, it may be metabolized at the cytoplasmic membrane for the supply of nucleotide and inorganic phosphate as substrates for the cell itself.

Once cells are exposed to thermal shock stress, however, because of induced membrane injury, YokF may be released from the membrane and enter the cytoplasm to attack chromosomal DNA. Further, it might also be likely that, in such stressed cells, YokF plays a role for metabolizing DNA and RNA released from dead cells to supply the resulting products for residual survived cells in the bacterial population.

In E. coli, EndA has been known as a periplasmic DNase randomly cleaving double strand DNA (46), and endA mutants have been so far used for providing plasmid DNA at a high yield and of highly quality because of demonstrating little DNase activity (13, 47). It is also possible, therefore, that a YokF nuclease deficient mutant is used for efficient plasmid DNA production in B. subtilis in laboratory work as well as for industrial application.

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Purification and Characterization of a *Bacillus subtilis* 168 Nuclease, YokF, Involved in Chromosomal DNA Degradation and Cell Death Caused by Thermal Shock Treatments

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