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Cloning and Characterization of the *yjeA* Gene, Encoding a Novel Deoxyribonuclease, from *Bacillus subtilis*

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The *yje*A gene, encoding a secreted protein, YjeA, of *Bacillus subtilis*, was cloned and characterized. A derivative of YjeA, the recombinant YjeA-H, which contained a C-terminal His_6-tag, was purified from *Escherichia coli* for functional studies. YjeA-H was shown to be an endonuclease, which hydrolyses both single-stranded and double-stranded DNA, but not RNA. Covalently closed circular pBR322 DNA incubated with YjeA-H was shown by gel electrophoresis to be first nicked to an open circular form, and then to a linearized structure on a background of DNA smear, and finally to small species of linear molecules that accumulated gradually. When ^{32}P-labelled pBR322 DNA was used as substrate, YjeA-H was shown to progressively nick both DNA strands in a random fashion, creating intermediates of various structures, as well as DNA smears comprising linear molecules of different sizes. The final products were found to consist essentially of degraded species of DNA. The detection of a putative signal peptide at the N-terminus of YjeA, together with the purification of YjeA-H from the culture supernatants of *E. coli yjeA-H* clones, and the identification of YjeA in the culture medium of *Bacillus subtilis*, supports the conclusion that YjeA is a secretory protein of *Bacillus subtilis*.

Key words: DNA-specific, *Escherichia coli*, extracellular, gram positive bacterium, nicking endonuclease.

Abbreviations: Ap, ampicillin; CCC, covalently closed circular; dsDNA, double-stranded DNA; OC, open circular; ORF, open reading frame; ssDNA, single-stranded DNA; Tc, tetracycline; YjeA-H, YjeA with a tag of six histidine residues at the C-terminus; [], denotes plasmid-carrier state.

INTRODUCTION

Deoxyribonucleases (DNases), which are ubiquitous, play important roles in various DNA processes including repair, recombination, restriction and replication. In addition to these biological roles, some DNases may enable host survival under unfavourable conditions, such as when food is depleted, or when intrusion of foreign DNA that may be harmful to host cells is threatened (*1*). To interact more effectively with their substrates, some DNases may be released into the cell milieu (*2*). Many of the better known DNases are endonucleases of the Type II restriction enzyme form, which recognize and cleave specific double-stranded (ds) DNA sequences. A variety of other endonucleases such as NucM of *Erwinia chrysanthemi* (*3*), DNase of *Vibrio cholerae* (*4*), Dns and Dns H of *Aeromonas hydrophila* (*5*), that have been shown to defend host cells against intrusion of foreign DNA, cause non-specific cleavage, however, of their dsDNA substrates (*6*).

Restriction and modification (R/M) systems in *Bacillus subtilis* were first identified in strains infected with bacteriophages (*7*, *8*). Further work (*9*) led to the identification of six different R/M systems in *B. subtilis*, among which the majority were Type II endonucleases (*10*). In addition, non-specific endonucleases, including an ssDNA-specific DNase secreted in large quantities into the medium when the cells are converted to protoplasts (*11*), have been localized to the cell membrane in *B. subtilis*. Another example is NucA, which is also membrane-bound, and which cleaves dsDNA non-specifically. Interestingly, it is speculated that this enzyme facilitates DNA transformation of *B. subtilis* (*12*).

Our laboratory has started to engineer recombinant constructs to facilitate secretory production of heterologous proteins in *B. subtilis* (*13*). We have noticed, as have other research groups (*14*, *15*), that foreign DNA components constituting part of an *E. coli*/*B. subtilis* shuttle construct are often subjected to deletion mutations in *B. subtilis*, suggesting the possibility that such constructs might be susceptible to hydrolysis in this bacterium. Because pBR322 or its derivatives are commonly employed for the construction of *B. subtilis* shuttle vectors, we began to search for novel nucleases that might hydrolyse pBR322 DNA in *B. subtilis*. In the present study, we report that a recombinant DNA construct harboring a 6.2 kb insert cloned from *B. subtilis* is capable of degrading pBR322 DNA.

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We further provide evidence to support the idea that *yjeA*, one of the genes identified on the insert, is responsible for encoding a novel endonuclease, YjeA. Using a recombinant YjeA derivative, we have also studied the mode of action of YjeA, which exhibits a non-specific DNase activity similar to that of DNase I (*16*). Moreover, YjeA was shown to be a secretory endonuclease in *B. subtilis*. To our knowledge, this represents the first report on the functional properties of YjeA, although the protein has been previously identified and localized (*17*, *18*).

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, DNA Manipulation and Chemical Reagents—*Bacillus subtilis* 1A751 [*eglS*△102, *bglT*/*bglS*△EV, *npr*, *apr*, *his*] (*19*), used as the source of genomic DNA, was obtained from the *Bacillus* Genetics Stock Centre at Ohio State University, Ohio. *Escherichia coli* JM101 [△(*lac-proAB*), *supE*, *thi1*; F', *proAB*$^+$, *lacI*^{q}, *lacZ*△M15, *traD36*] (*20*) was employed as the host for expression and propagation of plasmids. *Bacillus subtilis* was grown at 37°C in LB medium (*21*), and transformants thereof were grown in the same medium supplemented with 20 μg/ml of kanamycin (Km). *Escherichia coli* transformants were grown at 30°C in 2 × YT medium (*21*) supplemented with 70 μg/ml of ampicillin (Ap) and/or 12.5 μg/ml of tetracycline (Tc). For solid media, Bacto agar was added to a final concentration of 1.5% (w/v). Polymerase chain reactions (PCRs) were performed as previously described (*13*) with the KOD HiFi DNA polymerase (Novagen, Madison, WI). Other DNA techniques were performed according to standard protocols (*21*). Restriction and modifying enzymes were purchased from Pharmacia (Uppsala, Sweden), Promega (Madison, WI) and New England Biolabs (Beverly, MA). Chemical reagents were obtained from Sigma Chemical Co. (St Louis, MO) unless otherwise specified.

Bacillus subtilis Genomic DNA Library Construction and Screening—A *B. subtilis* genomic DNA library was constructed as previously described (*22*) with *Eco*RI partial digestion, and cloned into the p184M vector. This vector was constructed by inserting a PCR product of the LacUV5 cassette (*23*) containing the *lac*UV5 promoter, the *lac* operator, the consensus ribosome binding site and the ATG start codon, into pACYC184 digested with *Pvu*II and *Eco*RI, to provide more effective transcription of an inserted gene. To screen for recombinant constructs harbouring a candidate nuclease gene, individual transformants randomly picked from the library were cotransformed with a derivative of the pRB373 shuttle vector (*24*), named pM2 (*13*), and cotransformants were selected on agar plates supplemented with both Tc and Ap. A clone containing and expressing the target nuclease gene would result in the restriction of pM2, and subsequently the digestion of the rest of the plasmid including the *bla* gene. As a consequence, such cotransformants would be Ap-sensitive. The number of cotransformants was expected to be small.

Comparison of sequence similarities between the nuclease gene and available *B. subtilis* sequence was facilitated using Blast search programs obtainable from the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/BLAST/). The SignalP 3.0 Server of the Center for Biological Sequence Analysis at the Technical University of Denmark (http://www.cbs.dtu.dk/services/) was used to predict the presence of signal peptide cleavage sites in the nuclease.

DNA Manipulation—As we felt that a fusion between YjeA and a sequence of His residues might facilitate purification of the recombinant product, a sequence encoding 6 His residues was fused to the 3′ end of the *yjeA* gene. To enhance the expression of the YjeA-His$_6$ fusion product, YjeA-H, which was employed for DNA restriction analysis, the *yjeA-H* gene was inserted into a pACYC184 derivative, in which transcription of the P_R promoter was controlled by the temperature sensitive cI857 repressor, to form a temperature-inducible expression plasmid, P_R-*yjeA*-H.

Expression and Purification of Recombinant YjeA-H—JM101 [P_R-*yjeA*-H] cultures were grown in 2 × YT medium supplemented with Tc at 28°C, shaking at 200 rpm. After growth had reached an A_{550} value of 1.0, YjeA-H expression was induced by switching the growth temperature to 39°C for 16 h before harvesting. The supernatant from 100 ml of an induced culture was loaded onto a Co^{2+}-resin column (BD Talon^{TM} Metal Affinity Resins, BD Biosciences Clontech, Palo Alto, CA) equilibrated with buffer A (1 × PBS). The flowthrough collected was applied again to the Co^{2+}-resin column, and this procedure was repeated twice. After washing with 200 ml of buffer B (50 mM NaH_2PO_4, 35 mM Na_2HPO_4, 0.3 M NaCl; pH 7.0), the proteins were eluted with buffer C (50 mM NaH_2PO_4, 0.3 M NaCl, 150 mM imidazole; pH 8.0). The fractions containing YjeA-H protein (detected by Western-blot analysis with an anti-YjeA serum) were concentrated by ultrafiltration (Amicon, Billerica, MA; 10 kDa mol wt cutoff), and the concentrates were used for detailed restriction activity analysis.

Analyses of YjeA-H and Preparation of Anti-YjeA Serum—The identity of the purified YjeA-H was confirmed by mass spectrometry using a quadrupole time-of-flight tandem mass spectrometer equipped with an orthogonal matrix-assisted laser desorption/ion source, by the Mass Spectrometry Facility of our University. The protein was subjected to sequencing by automated Edman degradation (Department of Physiology, Tufts University Core Facility, Boston, MA).

Purified recombinant YjeA was used to raise anti-YjeA antibodies in rabbits. The *yjeA* gene was fused to the glutathione S-transferase (GST) gene carried on a commercially available vector, pGEX2T (Amersham Biosciences, UK). Expression, purification and cleavage (using thrombin) of the GST-YjeA fusion protein were performed according to standard protocols (Amersham Biosciences). The authenticity of the YjeA product purified from an SDS–polyacrylamide gel was confirmed by amino acid sequencing, and the purified protein was used to raise antibodies against YjeA using a method described previously (*25*). The resolution of proteins on tricine–SDS–polyacrylamide gels and other protocols for

Western-blot analysis of proteins have also been described (*25*).

Characterization of the Nuclease Activity—The single-stranded M13mp18 bacteriophage (*20*) and the covalently closed circular (CCC) pBR322 plasmid (*26*) were purified using low melting-point agarose gels and employed as substrates for the analysis of the YjeA-H endonuclease activity. In brief, 0.5 μg of DNA was incubated with 2 μg of purified YjeA-H in a commercial reaction buffer (50 mM Tris–Cl, 100 mM NaCl, 10 mM $MgCl_2$; pH 7.5) at 37°C. Small aliquots of the reaction mixture were collected after various incubation times and analysed by agarose gel electrophoresis.

To investigate whether YjeA-H would hydrolyse RNA, pBR322 DNA prepared by the alkaline lysis procedure (*21*), in which the host RNA was either removed (with RNase A treatment) or retained (without RNase treatment), was treated with purified YjeA-H, followed by agarose gel analysis.

The mode of action of YjeA-H was studied using radioactively labelled pBR322 DNA. The protruding ends of *Eco*RI-linearized pBR322 DNA were labelled with α^{32}P-dATP (10 mCi/ml, 5 μl) using Klenow DNA polymerase. The labelled DNA was self-ligated to an open circular (OC) form, which was then purified by passage through an S-200 micro-spin column (Pharmacia Biotech; Piscataway, NJ). The cleavage assay was performed as described earlier for unlabelled DNA.

RESULTS

Cloning of the yjeA Gene from B. subtilis—When recombinant constructs formed between *B. subtilis* DNA and the p184M vector were used to cotransform *E. coli* with pM2, a cotransformant expressing a Tc^+ and Ap^- phenotype was selected on agar plates. Restriction analysis of the plasmid isolated from this cotransformant revealed the presence of a 6.2 kb insert cloned in p184M, designated p184M-6.2 (Fig. 1). It was interesting to note that pM2 transformed *E. coli* harbouring p184M-6.2 at a frequency *ca*. 10^3 to 10^4-fold less efficient than that harbouring p184M. This supported the idea that the 6.2 kb insert in p184M-6.2 encoded a nuclease responsible for the reduced cotransformation efficiency observed.

Deletions of the insert of p184M-6.2 were effected to help localize the candidate nuclease gene. When a 2.3 kb fragment located within the insert was deleted (Fig. 1), the deletion mutant was found to cotransform *E. coli* with pM2 with normal efficiency, suggesting that the 2.3 kb might encode part of the target nuclease. Comparison of the DNA sequence of the 2.3 kb fragment with the genomic sequence of *B. subtilis* available from the GeneBank SubtiList database (*27*) showed that the 2.3 kb fragment contained the *cotT* gene coding for a spore-coat protein and the *yjeA* gene coding for a membrane protein, YjeA. The *yjeA* gene appeared to be an independent gene because the open reading frames

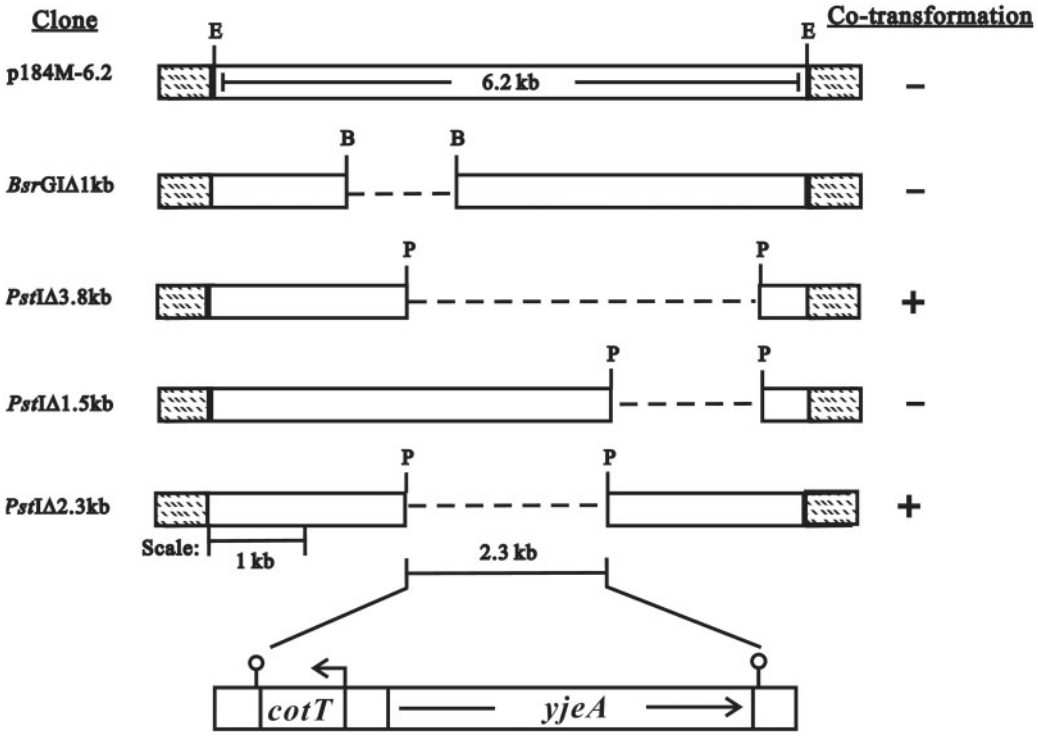

Fig. 1. **Localization of the gene encoding the target nuclease on the 6.2 kb insert.** The parental clone, p184M-6.2, and its deletion derivatives, which were constructed using appropriate restriction digestions of p184M-6.2, are shown. Cotransformation with plasmid pM2 was conducted to determine which deletions would interrupt expression of the target nuclease gene. Some clones (denoted by the '–' sign) did not tolerate the coexistence of pM2 in cotransformants, while other clones (denoted by the '+' sign), did. Other symbols are: open bars, the 6.2 kb insert and its derivatives; dotted bars, vector DNA; B, *Bsr*GI; E, *Eco*RI; P, *Pst*I; ⚲ transcription terminator. The *cotT* gene and the *yjeA* gene in the 2.3 kb deleted segment are shown, and their directions of transcription are indicated by arrows.

(ORFs) mapped upstream and downstream of *yjeA* are transcribed in the orientation opposite to that of *yjeA*. The large difference in transformation efficiencies between constructs with or without the 2.3 kb insert supported the idea that YjeA was the target nuclease. Moreover, the ability of p184M-6.2 to affect the stability of pM2 suggested that YjeA was an endonuclease.

Analysis of YjeA—The *yjeA* gene was predicted to code for a 467 amino acid YjeA protein possessing a molecular mass of 53.7 kDa and an isoelectric point of 9.1 (*27*). Although homology searches showed that YjeA contained a polysaccharide deacetylase domain, and was predicted to be a xylanase or a chitooligosaccharide deacetylase (*27*, *28*, *29*), published data (*30*, *31*) do not support this idea. Moreover, our observation that *E. coli* JM101 [p184M-6.2] transformants could not hydrolyse xylan in an agar-plate assay (*19*) indicated that YjeA is not a xylanase (data not shown). An open reading frame (ORF) analysis of *yjeA* using the ORF Finder available from NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) suggested that an ATG codon 14 codons upstream from the formerly assigned start codon, a TTG codon (*27*), is the real start codon (data not shown). The molecular size of the YjeA protein would thus be 2 kDa larger than the previously predicted size (*27*). The adjusted size is in line with a recent publication (*17*) in which YjeA was shown by gel analysis to possess a larger size of 59.8 kDa. Based on these results, we accepted the aforementioned ATG as the putative start codon in subsequent manipulations of the *yjeA* gene, where the revised ORF has 481 codons (Fig. 2).

It has been shown that YjeA is a membrane protein in *B. subtilis* (*17*). Using the TMHMM v2.0 server system available from the Center for Biological Sequence Analysis at the Technical University of Denmark (http://www.cbs.dtu.dk/services/), it was predicted that a region of the revised YjeA protein, covering amino acids 20–42 from the ATG start codon, was a transmembrane region. Signal peptide search predicted that the N-terminus of YjeA was a signal peptide and that a peptide cleavage site lay between amino acids 45 and 46 of the sequence (Fig. 2). The detection of recombinant YjeA in the culture medium of its *E. coli* host (data not shown), and the identification of the first amino acid of purified YjeA-H (a YjeA derivative, see Section 'Expression and purification of recombinant YjeA' later) as a Met residue at position 50 of the sequence, very close to the predicted cleavage site, strongly suggest that YjeA is in fact secreted by *B. subtilis*.

Expression and Purification of Recombinant YjeA—To facilitate the detection and purification of YjeA, a tag sequence encoding six His residues (His_6) was fused to the 3′ end of the *yjeA* gene to form a derivative, *yjeA-H*. Using a temperature-inducible expression plasmid, P_R-*yjeA*-H (Section 'Expression and Purification of Recombinant YjeA-H', Materials and Methods), over-expression of the YjeA-His_6 fusion product, YjeA-H, was achieved by incubating an *E. coli* recombinant at 39°C for 16 h. YjeA-H was found to be excreted to the culture supernatant and the protein was purified to homogeneity using a Co^{2+}-resin column (Fig. 3). The identity of the purified YjeA-H was confirmed by Western-blot analysis (Fig. 3) and mass spectrometry (data not shown).

Fig. 2. **The 481 amino acid residues of the YjeA protein.** The predicted signal peptide cleavage site (between residues 45 and 46) and the first amino acid of the mature protein, a Met residue at position 50 (determined by sequencing of YjeA-H purified from culture supernatant), are indicated by arrows. The predicted transmembrane region from residues 20 to 42 of YjeA is underlined.

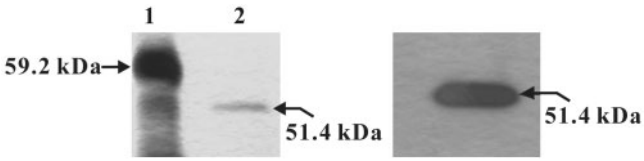

Fig. 3. **Purification of YjeA-H from the culture medium of *E. coli* JM101 [P_R-*yjeA*-H].** Left panel: silver staining of purified YjeA-H analysed by SDS–PAGE. Lane 1, protein markers. Lane 2, purified YjeA-H, which was shown by sequencing to start from Met 50, is 51.4 kDa in size. Right panel: Western-blot analysis of purified YjeA-H, which reacted strongly with a polyclonal anti-YjeA antibody.

The first residue of the purified YjeA-H was shown by sequencing to be Met 50, which is close to the peptidase cleavage site between amino acids 45 and 46. It was concluded that the excreted YjeA-H was subjected to slight proteolysis.

Nuclease Activity of YjeA-H—To investigate whether purified YjeA-H protein possessed any nuclease activity, the CCC form of pBR322 DNA (CCC DNA) was incubated with different quantities of purified YjeA-H and the digests analysed by agarose gel electrophoresis. The CCC DNA was processed to yield an OC form, a linearized intermediate, and a smear of DNA bands. It was found that the extents and rates of formation and

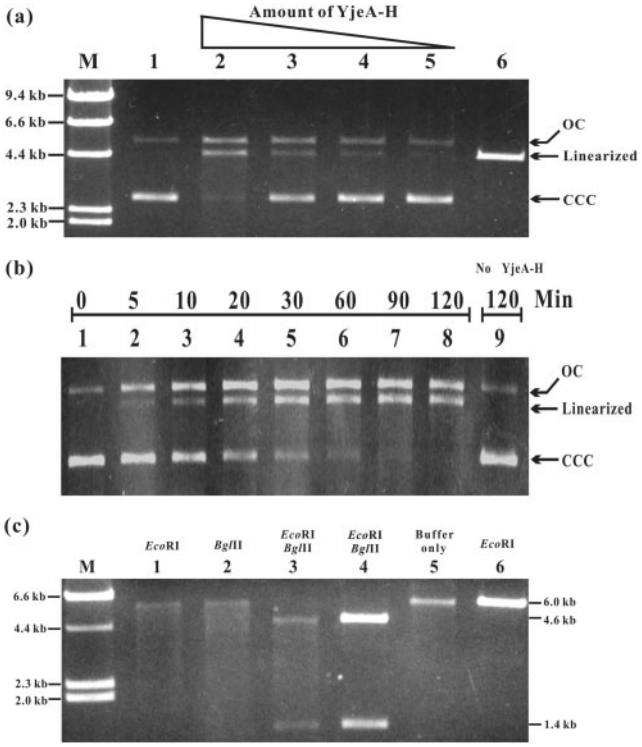

Fig. 4. **Demonstration of DNase activity exhibited by YjeA-H.** (a) Dose-dependent hydrolysis of DNA. Different quantities of purified YjeA-H were incubated with 0.03 μg of CCC pBR322 DNA in a commercial reaction buffer at 37°C for 1 h. The digests were then resolved on a 0.7% (w/v) agarose gel. Lanes 2 to 5, digests containing 2, 1, 0.5 and 0.25 μg of YjeA-H, respectively. Lane 1, DNA incubated with buffer alone. Lane 6, linearized pBR322 DNA. Lane M, *Hin*dIII-restricted λ DNA markers. Positions of OC, linear and CCC forms of pBR322 DNA are indicated. (b) Time-course study of pBR322 DNA hydrolysed by YjeA-H. 0.5 μg of CCC pBR322 DNA was incubated with 2 μg of YjeA-H in 100 μl of a commercial buffer. Lanes 1 to 8, aliquots withdrawn from the reaction mixture at 0, 5, 10, 20, 30, 60, 90 and 120 min after incubation commenced, and analysed on a 0.7% (w/v) agarose gel. Lane 9, DNA incubated with buffer alone for 120 min. (c) Mode of action of YjeA-H as studied by restriction analysis. Lane 5, DNA substrate, which was composed of linearized pM2Veg plasmid gel-purified after YjeA-H hydrolysis, incubated with buffer alone. Lanes 1 to 3, the same DNA substrate as in lane 5, incubated with *Eco*RI, *Bgl*II and *Bgl*II plus *Eco*RI, respectively. Lane 4, pM2Veg plasmid restricted by *Bgl*II and *Eco*RI to yield size markers. Lane 6, pM2Veg plasmid linearized by *Eco*RI as a linear DNA marker. The sizes of linear pM2Veg (6 kb) and the two product fragments (4.6 and 1.4 kb), resulting from *Bgl*II and *Eco*RI restriction, are indicated.

disappearance of these various forms of DNA were dependent on the concentration of YjeA-H in the digestion mixtures, in line with the idea that YjeA-H is a DNase, and, apparently, an endonuclease (Fig. 4a).

Studies on the Mechanism of YjeA-H Hydrolysis—In time-course studies on the hydrolysis of CCC DNA by YjeA-H, the appearance of OC DNA as an early intermediate product (Fig. 4b) supported the idea that YjeA-H attacked substrates by nicking. To study the distribution of nicks created by YjeA-H, an *E. coli*/*B. subtilis* shuttle vector, pM2Veg (*13*), which could be restricted by *Eco*RI and *Bgl*II to yield two distinct fragments with sizes of 4.6 and 1.4 kb (Fig. 4c), was employed as substrate. The 6 kb linearized intermediate pM2Veg product resulting from YjeA-H hydrolysis was purified and subjected to *Eco*RI and *Bgl*II restriction. The observation of DNA smears instead of discrete smaller bands beneath the *Eco*RI and *Bgl*II restriction fragments (Fig. 4c) supported the interpretation that the nicks formed by YjeA-H occurred randomly on the DNA substrate. The results revealed that the linearized pM2Veg product was composed of a mixture of molecules of similar lengths, but with different open ends, which were formed when two nicks were effected at diametrically opposed locations. In addition, the DNA smears beneath the restriction fragments (Fig. 4c) suggested the existence of other nicks, which might vary greatly in number, within linearized molecules.

These interpretations gained further support from an independent experiment in which the progress of YjeA-H hydrolysis was monitored using ^{32}P-labelled DNA (Section 'Characterization of the Nuclease Activity', Materials and Methods). The concomitant appearance of large and small digested products, which were shown to be fragments of different sizes containing labelled ends, even shortly after onset of hydrolysis (lane 3, Fig. 5) confirmed that YjeA-H formed random nicks on the DNA substrate. The hydrolysis appeared to result in accumulation of products that varied widely in size and composition, and hence in the formation of DNA smears. Furthermore, the accumulation of increasingly smaller DNA products at later time points indicated that YjeA-H thoroughly degraded the DNA substrate (Fig. 5).

Substrate Specificity of YjeA-H—YjeA-H was found to be active on both dsDNA and ssDNA (Fig. 6a). The nuclease could not, however, hydrolyse *E. coli* RNA that was available in mini-preparations of pBR322 DNA (Fig. 6b). Indeed, RNA seemed to inhibit YjeA-H activity (Fig. 6b). Although YjeA-H hydrolyses only DNA but not RNA, it is yet to be proven whether the enzyme recognizes the sugar-moiety or some other structural features of the nucleic acid substrates.

DISCUSSION

The *yjeA* gene sequence is one of 4100 possible ORFs of *B. subtilis* cataloged in 1997 (*32*). Although the predicted YjeA protein was first thought to be a xylanase (*27*), and then a peptidoglycan deacetylase (*27–29*), by homology comparisons, both predictions are incorrect (*30*, *31*, *this study*). It was then shown by proteomic studies that YjeA was a membrane protein in *B. subtilis* (*17*). The primary structure and location of YjeA did not greatly assist in understanding the biological function of YjeA. A major difficulty in studying the biological role of YjeA was likely due to the lack of information regarding the identity of the substrate recognized by YjeA (*28*). With the cloning of the *yjeA* gene and the characterization of the recombinant derivative, YjeA-H, reported here, we are able to provide the first evidence that YjeA is a novel secretory endonuclease of *B. subtilis*.

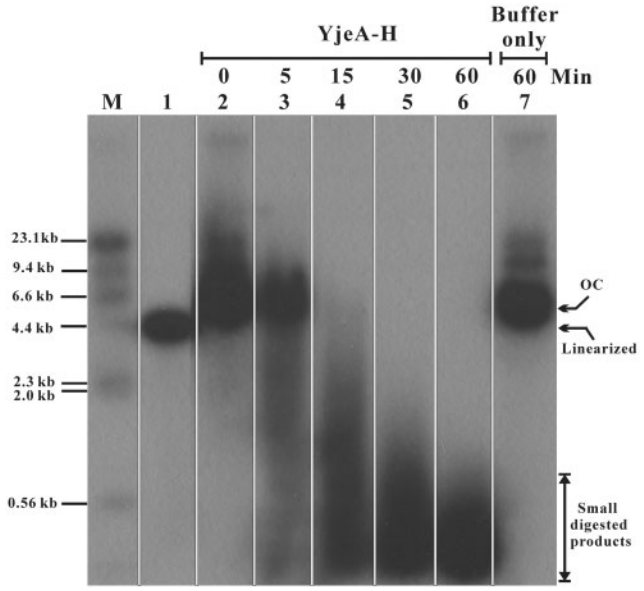

Fig. 5. **Study of YjeA-H hydrolysis using ^{32}P-labelled DNA substrate.** Lane M, ^{32}P-labelled λ DNA markers restricted by *Hin*dIII. Lane 1, labelled pBR322 linearized by *Eco*RI. Lane 2, self-ligated DNA (in OC form) from lane 1, which was used as substrate in the timecourse hydrolysis by YjeA-H. Lanes 2 to 6, aliquots of the digest withdrawn at 0, 5, 15, 30 and 60 min, respectively. Lane 7, the substrate incubated with buffer alone for 60 min. The DNA digests were resolved on a 0.7% (w/v) agarose gel. OC stands for open circular.

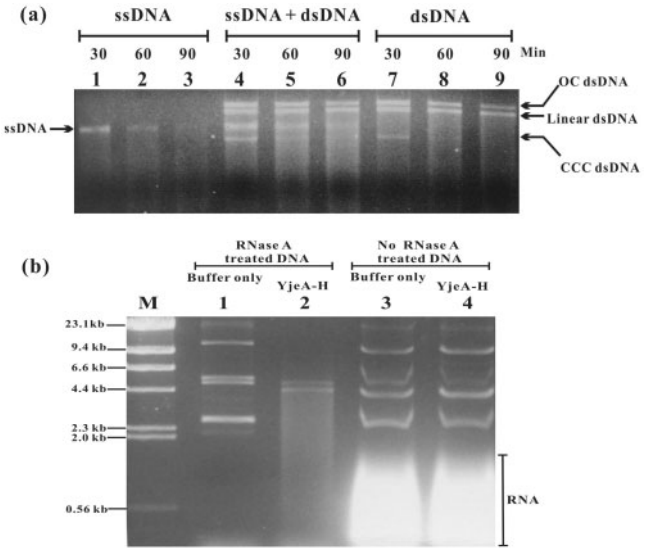

Fig. 6. **Substrate specificity of YjeA-H.** (a) Hydrolysis of single-stranded and double-stranded DNA. YjeA-H was used to digest different DNA substrates for different lengths of time (30, 60 and 90 min). Lanes 1 to 3, ss M13mp18 DNA as substrate. Lanes 7 to 9, CCC pBR322 as DNA substrate. Lanes 4 to 6, equal amounts of ss M13mp18 DNA and ds pBR322 DNA as substrate. The positions of the various forms of DNA are indicated. (b) Hydrolysis of *E. coli* RNA available in mini-preparations of pBR322 DNA. Lanes 1 and 2 contained DNA that had been treated by RNaseA, whereas lanes 3 and 4 contained untreated DNA. Lanes 1 and 2 treated DNA incubated with buffer alone and with YjeA-H, respectively. Lanes 3 and 4 untreated DNA incubated with buffer alone and with YjeA-H, respectively. All reactions were carried out at 37°C for 12 h. Lane M, *Hin*dIII-restricted λ DNA markers.

The His_6-tag at the C-terminus of YjeA was useful in the purification of YjeA-H. YjeA-H retained function despite the presence of the His_6-tag, enabling the use of YjeA-H for the development of functional assays. YjeA was unequivocally demonstrated to be an endonuclease by, first, the adverse effect of YjeA-H expression on *E. coli* cotransformation, which resulted presumably from attack by YjeA-H on the newly introduced plasmid, pM2, prior to its protection by modifications, and, second, the hydrolysis of DNA substrates by YjeA-H (Figs. 4 and 5).

The DNA smears, which appeared at all times in enzymatic assays (Fig. 4), were initially thought to be derived from contamination of YjeA-H by other DNases. Our use of homogeneously pure YjeA-H, and our care in undertaking the assays, argues against this idea. Most of all, the fact that the major events and patterns of intermediates/products seen in several assays were virtually identical, suggests that the DNA smears arise from YjeA-H action. In all assays, the CCC substrate was first hydrolysed to an OC intermediate, accompanied by the appearance of a weak background of linearized and smeared DNA. Then the CCC band was lost, with retention of the OC intermediate, and the appearance of larger amounts of linearized and smeared DNA. Finally, the OC intermediate was lost, the linearized band was reduced in intensity, and more intense DNA smears were formed, with increased amounts of smaller molecules. This development supports the notion that the DNA smears observed at earlier time points were genuine intermediates or products of YjeA-H enzymatic action. The DNA smears are genuine reaction products and are intermediates in the continuation and completion of hydrolysis. This proposed mode of action was further substantiated using labelled DNA, which revealed not only the development of various intermediates/products, but also illustrated the chronological scenario (Fig. 5). The results unequivocally show that YjeA is a non-specific endonuclease, which creates random nicks progressively on the two strands of dsDNA, resulting in highly distinguishable intermediates/products very different in chemical and physical compositions over time.

YjeA and the well-understood DNase I endonuclease share several enzymatic properties. Both enzymes are active on both ssDNA and dsDNA, both generate random nicks, and both require Mg^{2+} or Mn^{2+} for hydrolytic activity (unpublished data). The ability of YjeA-H to function in the absence of glycosylation, and its inability to function in the presence of Ca^{2+} (unpublished data), are differences between the two endonucleases. Further work on the mode of action of YjeA is needed.

A two-dimensional zymography analysis performed previously suggested that *B. subtilis* might produce more than 80 DNase species (*33*). Only a few of these DNases, however, including YokF, YncB (*34*), NucA (*12*), and an ssDNA-specific DNase associated with the cell membrane fraction (*11*), have been identified and characterized. In this paper, we show that YjeA is a

novel endonuclease of *B. subtilis*. Although YjeA was previously thought to be membrane-bound, our success in preparing excreted YjeA-H from the culture media of recombinant *E. coli*, the identification of a putative signal peptide (with a peptidase cleavage site between amino acids 45 and 46), the fact that the first residue of the excreted YjeA-H is Met 50 (a few residues from the predicted cleavage site; the N-terminus of mature YjeA-H might be subjected to slight proteolysis after cleavage), and, finally, the detection of a candidate YjeA product in concentrated culture supernatant of *B. subtilis* using anti-YjeA-H antibodies (unpublished data), all support the conclusion that YjeA is secreted from *B. subtilis*.

The discovery of YjeA function, and the clarification of YjeA subcellular localization in *B. subtilis*, will assist in the design of specific assays for better understanding of YjeA function, regulation and biological role, in *B. subtilis*.

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REFERENCES

1. Rangarajan, E.S. and Shankar, V. (2001) Sugar non-specific endonucleases. *FEMS Microbiol. Rev.* **25**, 583–613
2. Focareta, T. and Manning, P.A. (1987) Extracellular proteins of *Vibrio cholerae*: molecular cloning, nucleotide sequence and characterization of the deoxyribonuclease (DNase) together with its periplasmic localization in *Escherichia coli* K-12. *Gene* **53**, 31–40
3. Moulard, M., Condemine, G., and Robert-Baudouy, J. (1993) Characterization of the *nucM* gene coding for a nuclease of the phytopathogenic bacteria *Erwinia chrysanthemi*. *Mol. Microbiol.* **8**, 685–695
4. Marcus, H., Ketley, J.M., Kaper, J.B., and Holmes, R.K. (1990) Effects of DNase production, plasmid size, and restriction barriers on transformation of *Vibrio cholerae* by electroporation and osmotic shock. *FEMS Microbiol. Lett.* **56**, 149–154
5. Dodd, H.N. and Pemberton, J.M. (1999) The gene encoding a periplasmic deoxyribonuclease from *Aeromonas hydrophila*. *FEMS Microbiol. Lett.* **173**, 41–46
6. Li, C.L., Hor, L.I., Chang, Z.F., Tsai, L.C., Yang, W.Z., and Yuan, H.S. (2003) DNA binding and cleavage by the periplasmic nuclease Vvn: a novel structure with a known active site. *EMBO J.***22**, 4014–4025
7. Shibata, T. and Ando, T. (1974) Host controlled modification and restriction in *B. subtilis*. *Mol. Gen. Genet.* **131**, 275–280
8. Trautner, T.A., Pawlek, B., Bron, S., and Anagnostopoulos, C. (1974) Restriction and modification in *B. subtilis*: biological aspects. *Mol. Gen. Genet.* **131**, 181–191
9. Uozumi, T., Hoshino, T., Miwa, K., Horinouchi, S., Beppu, T., and Arima, K. (1977) Restriction and modification in *Bacillus* species: genetic transformation of bacteria with DNA from different species, part I. *Mol. Gen. Genet.* **152**, 65–69
10. Jentsch, S. (1983) Restriction and modification in *Bacillus subtilis*: sequence specificities of restriction/modification systems *Bsu*M, *Bsu*E, and *Bsu*F. *J. Bacteriol.* **156**, 800–808
11. Birnboim, H.C. (1966) Cellular site in *Bacillus subtilis* of a nuclease which preferentially degrades single-stranded nucleic acids. *J. Bacteriol.* **91**, 1004–1011
12. Provvedi, R., Chen, I., and Dubnau, D. (2001) NucA is required for DNA cleavage during transformation of *Bacillus subtilis*. *Mol. Microbiol.* **40**, 634–644
13. Lam, K.H.E., Chow, K.C., and Wong, W.K.R. (1998) Construction of an efficient *Bacillus subtilis* system for extracellular production of heterologous proteins. *J. Biotechnol.* **63**, 167–177
14. Ostroff, G.R. and Pene, J.J. (1984) Molecular cloning with bifunctional plasmid vectors in *Bacillus subtilis*. II. Transfer of sequences propagated in *Escherichia coli* to *B. subtilis*. *Mol. Gen. Genet.* **193**, 306–311
15. Schäffer, C., Wugeditsch, T., Messner, P., and Whitfield, C. (2002) Functional expression of enterobacterial O-polysaccharide biosynthesis enzymes in *Bacillus subtilis*. *Appl. Environ. Microbiol.* **68**, 4722–4730
16. Mishra, N.C. (2002) Deoxyribonuclease in *Nuclease: Molecular Biology and Applications* pp. 45–61, John Wiley & Sons, Inc., Hoboken, New Jersey
17. Eymann, C., Dreisbach, A., Albrecht, D., Bernhardt, J., Becher, D., Gentner, S., Tam, le T., Buttner, K., Buurman, G., Scharf, C., Venz, S., Volker, U., and Hecker, M. (2004) A comprehensive proteome map of growing *Bacillus subtilis* cells. *Proteomics* **4**, 2849–2876
18. Rivolta, C., Soldo, B., Lazarevic, V., Joris, B., Mauel, C., and Karamata, D. (1998) A 35.7 kb DNA fragment from the *Bacillus subtilis* chromosome containing a putative 12.3 kb operon involved in hexuronate catabolism and a perfectly symmetrical hypothetical catabolite-responsive element. *Microbiology* **144**, 877–884
19. Wolf, M., Geczi, A., Simon, O., and Borriss, R. (1995) Genes encoding xylan and beta-glucan hydrolysing enzymes in *Bacillus subtilis*: characterization, mapping and construction of strains deficient in lichenase, cellulase and xylanase. *Microbiology* **141**, 281–290
20. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mpl8 and pUC19 vectors. *Gene* **33**, 103–119
21. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual,* 2nd edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
22. Wong, W.K.R., Ali, A., Chan, W.K., Ho, V., and Lee, N.T.K. (1998) The cloning, expression and characterization of a cellobiase gene encoding a secretory enzyme from *Cellulomonas biazotea*. *Gene* **207**, 79–86
23. Lam, T.L., Wong, R.S.C., and Wong, W.K.R. (1997) Enhancement of extracellular production of a *Cellulomonas fimi* exoglucanase in *Escherichia coli* by the reduction of promoter strength. *Enzyme Microb. Technol.* **20**, 482–488
24. Brückner, R. (1992) A series of shuttle vectors for *Bacillus subtilis* and *Escherichia coli*. *Gene* **122**, 187–192
25. Fu, Z.B., Ng, K.L., Lam, T.L., and Wong, W.K.R. (2005) Cell death caused by hyper-expression of a secretory exoglucanase in *Escherichia coli*. *Protein Expr. Purif.* **42**, 67–77
26. Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L., and Boyer, H.W. (1977) Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**, 95–113
27. Moszer, I., Jones, L.M., Moreira, S., Fabry, C., and Danchin, A. (2002) SubtiList: the reference database for the *Bacillus subtilis* genome. *Nucleic Acids Res.* **30**, 62–65
28. Bisicchia, P., Noone, D., Lioliou, E., Howell, A., Quigley, S., Jensen, T., Jarmer, H., and Devine, K.M. (2007) The essential YycFG two-component system controls cell wall metabolism in *Bacillus subtilis*. *Mol. Microbiol.* **65**, 180–200
29. Fukushima, T., Yamamoto, H., Atrih, A., Foster, S.J., and Sekiguchi, J. (2002) A polysaccharide deacetylase gene (*pdaA*) is required for germination and for production of muramic delta-lactam residues in the spore cortex of *Bacillus subtilis*. *J. Bacteriol.* **184**, 6007–6015
30. Fukushima, T., Tanabe, T., Yamamoto, H., Hosoya, S., Sato, T., Yoshikawa, H., and Sekiguchi, J. (2004) Characterization of a polysaccharide deacetylase gene

homologue (*pdaB*) on sporulation of *Bacillus subtilis*. *J. Biochem. (Tokyo)* **136**, 283–291

31. Gilmore, M.E., Bandyopadhyay, D., Dean, A.M., Linnstaedt, S.D., and Popham, D.L. (2004) Production of muramic delta-lactam in *Bacillus subtilis* spore peptidoglycan. *J. Bacteriol.* **186**, 80–89
32. Kunst, F., Ogasawara, N., Moszer, I., Albertini, A.M., Alloni, G., Azevedo, V., Bertero, M.G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S.C., Bron, S., Brouillet, S., Bruschi, C.V., Caldwell, B., Capuano, V., Carter, N.M., Choi, S.K., Codani, J.J., Connerton, I.F., Curnmings, N.J., Daniel, R.A., Denizot, F., Devine, K.M., Dusterhoft, A., Ehrlich, S.D., Emmerson, P.T., Entian, K.D., Errington, J., Fabret, C., Ferrarl, E., Foulger, D., Fritz, C., Fujita, M., Fujita, Y., Furna, S., Galizzi, A., Galleron, N., Ghim, S.Y., Glaser, P., Goffeau, A., Golightly, E.J., Grandi, G., Guiseppi, G., Guy, B.J., Haga, K., Haiech, J., Harwood, C.R., Henaut, A., Hilbert, H., Holsappel, S., Hosono, S., Hullo, M.F., Itaya, M., Jones, L., Joris, B., Karamata, D., Kasahara, Y., K.-Blanchard, M., Klein, C., Kobayashi, Y., Koetter, P., Koningstein, G., Krogh, S., Kumano, M., Kurita, K., Lapidus, A., Lardinois, S., Lauber, J., Lazarevic, V., Lee, S.M., Levine, A., Liu, H., Masuda, S., Mauel, C., Medligue, C., Medina, N., Mellado, R.P., Mizuno, M., Moestl, D., Nakai, S., Noback, M., Noone, D., O'Reilly, M., Ogawa, K., Ogiwara, A., Oudega, B., Park, S.H., Parro, V., Pohl, T.M., Portelle, D., Porwolik, S., Prescott, A.M., Presecan, E., Pujic, P., Purnelle, B., Rapoport, G., Rey, M., Reynolds, S., Rieger, M., Rivolta, C., Rocha, E., Roche, B., Rose, M., Sadaie, Y., Sato, T., Scanlan, E., Schleich, S., Schroeter, R., Scoffone, F., Sekiguchi, J., Sekowska, A., Seror, S.J., Serror, P., Shin, B.S., Soldo, B., Sorokin, A., Tacconi, E., Takagi, T., Takahashi, H., Takemaru, K., Takeuchi, M., Tamakoshi, A., Tanaka, T., Terpstra, P., Tognoni, A., Tosato, V., Uchiyama, S., Vandenbol, M., Vannier, F., Vassarotti, A., Viari, A., Wambutt, R., Wedler, E., Wedler, H., Weitzenegger, T., Winters, P., Wipat, A., Yamamoto, H., Yamane, K., Yasamoto, K., Yata, K., Yoshida, K., Yoshikawa, H.F., Zumstein, E., Yoshikawa, H., and Danchin, A. (1997) The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature* **390**, 249–256
33. Coughlin, S.A. and Green, D.M. (1983) Two-dimensional zymogram analysis of nucleases in *Bacillus subtilis*. *Anal. Biochem.* **133**, 322–329
34. Sakamoto, J.J., Sasaki, M., and Tsuchido, T. (2001) Purification and characterization of a *Bacillus subtilis* 168 nuclease, YokF, involved in chromosomal DNA degradation and cell death caused by thermal shock treatments. *J. Biol. Chem.* **276**, 47046–47051