Group I Self-Splicing Intron in the *recA* Gene of *Bacillus anthracis*

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Self-splicing introns are rarely found in bacteria and bacteriophages. They are classified into group I and II according to their structural features and splicing mechanisms. While the group I introns are occasionally found in protein-coding regions of phage genomes and in several tRNA genes of cyanobacteria and proteobacteria, they had not been found in protein-coding regions of bacterial genomes. Here we report a group I intron in the *recA* gene of *Bacillus anthracis* which was initially found by DNA sequencing as an intervening sequence (IVS). By using reverse transcriptase PCR, the IVS was shown to be removable from the *recA* precursor mRNA for RecA that was being translated in *E. coli*. The splicing was visualized in vitro with labeled free GTP, indicating that it is a group I intron, which is also implied by its predicted secondary structure. The RecA protein of *B. anthracis* expressed in *E. coli* was functional in its ability to complement a *recA* defect. When *recA*-negative *E. coli* cells were irradiated with UV, the *Bacillus* RecA reduced the UV susceptibility of the *recA* mutant, regardless of the presence of intron.

While most of the introns found in eukaryotes and archaeobacteria require functions of several proteins for their processing, introns found in eubacterial species are removed by self-splicing. There are two types of self-splicing introns, classified according to their structures and splicing mechanisms. The processing of group I introns is initiated by a transesterification reaction mediated by an exogenous guanosine cofactor, generating ligated exon with a free intron bearing the guanosine at its 5' end (2, 32). The splicing of group II intron is similar to that of eukaryotic pre-mRNA, in which the 5' splice site is attacked by a 2' OH group of a bulged nucleotide, resulting in a lariat structure joined with a 2'-5' bond. Subsequently, the cleaved 5' exon attacks the 3' splice site, resulting in exon ligation and release of the intron lariat (14, 32).

Despite a lack of overall primary sequence conservation, the group I introns contain blocks of conserved nucleotide sequence elements (P, Q, R, and S), forming an active center with a secondary structure consisting of characteristic stem-loops P1 through P10 (2, 22, 23). There are also extra sequences, often including open reading frames (ORFs), which are located in loops peripheral to the catalytic intron core. The group II introns contain a different secondary structure, consisting of six conserved pairings emanating from a central wheel (24). In both introns, sequence conservations are confined to small regions of nucleotide residues. The products of internal ORFs of these introns usually play a role in splicing (in which case they are called maturases) or mobility of the intron (also referred to as "homing") (17, 32).

The bacterial group I introns are found in several tRNA genes of cyanobacteria and purple proteobacteria (16, 28, 38).

Some protein-coding genes, associated with DNA metabolism, of phages from *Escherichia coli*, *Bacillus subtilis*, *Lactococcus lactis*, *Lactococcus delbrueckii*, and *Staphylococcus aureus* contain group I introns (1, 3, 12, 18, 37). However, no group I intron has been reported so far from a protein-coding gene of a eubacterial genome. The group II introns of bacteria have been identified in cyanobacteria (*Calothrix* spp.), proteobacteria (*Azotobacter vinelandii* and *E. coli* [9]), and gram-positive bacteria (*L. lactis* and *Clostridium difficile* [27, 35]). Most of the eubacterial group II introns are found in genes on plasmids or associated with conjugal transfer (7).

The bacterial RecA protein is ubiquitous and highly conserved (15). It is involved in homologous recombination, in DNA repair, and in promoting proteolysis (25, 30). In recombination, RecA binds to DNA, promotes homologous pairing, and catalyzes strand exchange (31). When there is DNA damage, this process is used to fill the gap generated by the DNA damage during replication. RecA also serves as a regulator with an activation upon SOS signal to facilitate proteolysis of LexA, λ repressor, and SulA involved in the control of cell division (21, 29, 34). In *E. coli*, the transcription of the *recA* gene is negatively regulated by the LexA protein, which binds to the upstream regions of the SOS genes including *recA* (20). *Bacillus* species also contain LexA, called DinR in *B. subtilis*, and the SOS repair system. However, the binding consensus sequence CGAACRNRYGTTYC (36), also known as the Cheo box for LexA, is different from that of *E. coli* (26).

Several *recA* genes of mycobacteria are interrupted by an intein that is removed from its precursor by a protein splicing mechanism to generate a functional RecA protein (5, 6). The inteins in six species, including *Mycobacterium leprae* and *Mycobacterium chitae*, were found in the same insertion sites (RecA-b) immediately downstream of glycine 205 in *M. leprae*, while the one (RecA-a) for *Mycobacterium tuberculosis* is after the lysine 251 (33). Inteins are also found in other eubacteria, archaea, and eukaryotes, frequently interrupting genes involved in replication and repair.

During the study of the *recA* genes from several gram-pos-

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FIG. 1. Schematic diagram of *B. anthracis* *recA* gene. The gene includes a 327-bp IVS, located after residue 203. The thick arrows indicate locations of oligonucleotides used. The predicted transcriptional start site and the termination loop are also indicated.

itive bacteria, we discovered that a putative *recA* gene of *Bacillus anthracis* is interrupted by an intervening sequence (IVS). Here we show that the IVS is a group I intron and that the *recA* gene codes for a functional RecA protein in *E. coli*. This is the first demonstration of the presence of a group I intron in a protein-coding gene of a bacterial genome.

MATERIALS AND METHODS

Strains and media. The *E. coli* K-12 strains used are DH10 β , MG1655, and BL21(DE3). The Δ *recA*::Cm allele was transferred from CP857 (A. Wolfe) to MG1655 by P1 transduction to generate a *recA* deletion. Cells were grown in Luria broth or Luria-Bertani (LB) agar plates at 37°C. When required, ampicillin (100 μ g/ml) or chloramphenicol (30 μ g/ml) was added to the medium. The nonpathogenic derivative of *B. anthracis* (Δ Stern lacking pXO1 and pXO2) was used for RNA preparation. The *Bacillus* strain was cultured in LBG broth (LB with 1% glucose) or LBG agar plates at 37°C.

Plasmid construction. The chromosomal DNA of *B. anthracis*, obtained from Y. Chae, was used as a template for PCR with a pair of primers (RECA-A, 5'-TGT TGG CAA ATT GAA TTG AAA ATA GG-3', and RECA-B, 5'-CTC CAA CGA TAT AGA ACG CTT TC-3') to obtain the whole *recA* ORF, from 65 bp upstream of the start codon to 67 bp downstream of the stop codon. The PCR product was cloned into the *Sma*I site of pUC19 or pBluescript SK(+) to yield pUC*recA*[int⁺] and pT7*recA*[int⁺], respectively. The intronless version of the *recA* gene was constructed by making a fusion of the two exons directly, using an overlapping extension technique of PCR (13). The *recA*[int⁻] gene was cloned into pUC19 or pBluescript SK(+) to generate pUC*recA*[int⁻] and pT7*recA*[int⁻], respectively. The cloned region of pUC*recA*[int⁻] or pT7*recA*[int⁻] is the same as that of pUC*recA*[int⁺] or pT7*recA*[int⁺], except for the intron region. The sequences and orientations of the cloned *recA* genes were confirmed by DNA sequencing. The *recA* genes of pT7*recA*[int⁺] and pT7*recA*[int⁻] are under the control of the T7 promoter.

Inverse PCR and sequencing of *recA* from *B. anthracis*. Purified chromosomal DNA of *B. anthracis* was completely digested with *Sau*3AI, *Eco*RI, *Bam*HI, or *Pst*I/*Nsi*I. Each digested DNA was ligated at low concentration to facilitate formation of circles. PCR amplification was performed with the ligated template and a pair of oppositely directed primers (RECA-1, 5'-GAT AAT AGT AAT TCA TCG ATG TTA ACA C-3', and RECA-2, 5'-ACA ATC GCA ATC TTT ATT AAC CAA ATT C-3') recognizing the internal regions of *recA*. The sizes of PCR products were about 0.85 (*Sau*3AI), 1.7 (*Eco*RI), 3.8 (*Bam*HI), and 4 (*Pst*I/*Nsi*I) kb. After sequencing of the products with primers RECA-1, RECA-2, and RECA-3 (newly designed based on the initial sequencing result; 5'-GGC CTC TGA ACT TGA TAT CG-3'), the partial sequences were assembled to generate the complete sequence of the putative *recA* gene. The assembled sequence was confirmed by direct sequencing of the PCR product (about 1.5 kb) amplified with RECA-A and RECA-B from the *B. anthracis* chromosome. The locations and directions of the primers are shown in Fig. 1.

RNA isolation. RNA from *B. anthracis* Δ Stern or DH10 β containing plasmids was obtained from cells grown in appropriate media to an optical density of 1.0 at 600 nm. For preparation of RNA from BL21(DE3) containing pBluescript SK(+), pT7*recA*[int⁺], or pT7*recA*[int⁻], cells were grown to an optical density of 0.3 at 600 nm and treated with isopropyl- β -D-thiogalactoside (IPTG) at a final concentration of 0.5 mM. The IPTG-induced cells were grown further for 1 h.

Cells were harvested from 3-ml cultures and resuspended in 400 μ l of ice-cold diethyl pyrocarbonate-treated water. An equal volume of acid phenol (Amresco) was added to the cell suspension and heated to 60°C for 15 min with occasional vortexing. The mixture was then centrifuged and the upper phase was transferred to a clean tube. The total RNA was precipitated with ethanol (Merck). After the RNA pellet had been washed with 80% ethanol, RNA was resuspended in 20 μ l of diethyl pyrocarbonate-treated water. When required, RNA was further purified with the RNeasy kit (Qiagen) following the manufacturer's instructions.

In vitro transcription. In vitro synthesis of RNA from a linearized (with *Xba*I) pT7*recA*[int⁺] plasmid was performed with T7 RNA polymerase (Takara). The reaction was carried out in a total volume of 100 μ l with 1 $\times$ reaction buffer, 5 mM dithiothreitol, a 0.5 mM concentration of each ribonucleoside triphosphate, 80 U of RNase inhibitor, and 100 U of T7 polymerase. The mixtures were incubated at 37°C for 2 h, and the RNA synthesized was extracted with acid-phenol and precipitated with ethanol.

Reverse transcription-PCR (RT-PCR). Synthesis of cDNA was performed with a Moloney murine leukemia virus reverse transcriptase (Stratagene). One to five micrograms of total RNA was used as a template for cDNA synthesis initiated with the oligonucleotide RECA-5 (5'-CAC GGA ATG GTG GTG CCA C-3'), specific for the region downstream of the IVS. A portion of the cDNA was used for a PCR with oligonucleotides RECA-4 (specific for the region upstream of IVS; 5'-ATC GCA GAA GCA CTT GTA CG-3') and RECA-5. The cycling profile was as follows: 1 cycle at 94°C for 1 min; 30 cycles at 94°C for 20 s, 54°C for 30 s, and 72°C for 30 s; 1 cycle at 72°C for 2 min.

Intron labeling. Total cellular RNA (5 μ g) was incubated with [α -³²P]GTP (3,000 Ci/mmol, 1 μ Ci per μ g of RNA) and 40 U of RNase inhibitor at 37°C for 0, 10, 30, and 60 min in a splicing buffer (25 mM Na-HEPES [pH 7.5] and 15 mM MgCl₂) (11). The reaction was stopped by addition of EDTA (final concentration of 10 mM) and loading buffer (1 mg of xylene cyanol FF per ml, 1 mg of bromophenol blue per ml, and 10 mM EDTA in formamide), and the RNAs were then loaded onto a 5% acrylamide-8 M urea gel. After electrophoresis, a sheet of X-ray film (Kodak) was laid onto the gel and exposed for 12 h at -70°C.

Testing UV sensitivity. The *E. coli* strains containing pUC19, pUC*recA*[int⁺], and pUC*recA*[int⁻] plasmids were grown at 37°C in LB medium to a density of 2×10^8 to 3×10^8 cells per ml. Cells were resuspended in 0.1 M MgSO₄, and aliquots were dispensed onto petri plates. After UV irradiation for various lengths of time, cells were diluted and plated onto LB agar plates. After 20 h of incubation at 37°C, colonies were counted to measure rates of survival.

Nucleotide sequence accession number. The nucleotide and deduced amino acid sequences of the *B. anthracis* *recA* gene have been submitted to GenBank under accession number AF229167.

RESULTS

A putative *recA* gene from *B. anthracis*. A partial sequence (292 bp) of a putative *recA* gene of *B. anthracis* was obtained by PCR amplification using a set of degenerate primers recognizing conserved regions of the *recA* gene in gram-positive bacteria (Y. Chae, unpublished data). This sequence is homologous to the region encoding amino acid residues 104 to 202 of *B. subtilis* RecA with 96% identity. Using inverse PCR with a pair of outwardly directed primers, we were able to determine the full sequence of the *B. anthracis* *recA* gene (see Materials and Methods for details). As shown in Fig. 1, the putative *recA* gene is interrupted by a 327-bp fragment after amino acid residue 203. This IVS contains 11 stop codons when translated continuously. There is no inverted repeat at the ends of the IVS, indicating that the IVS is neither an insertion element nor a transposon.

In the promoter region of the *recA*-like coding sequence are putative -10 and -35 promoter sequences between 32 and 63 bp upstream of the predicted start codon. The putative promoter sequence (TTGGCA-18 bp-TATAAT) is very similar to the *E. coli* consensus (TTGACA-16 to 18 bp-TATAAT).

FIG. 2. In vivo and in vitro splicing of *recA* transcript. (A) In vivo splicing of *recA* transcript. Lane 1 shows a PCR product obtained from *B. anthracis* chromosomal DNA with the RECA-4 and RECA-5 primers. Results of RT-PCR from total RNA of *B. anthracis* using RECA-4 and RECA-5 are also shown, in the absence (lane 2) or presence (lane 3) of reverse transcriptase. Lane M, DNA size markers. (B) Labeling of intron RNA with [α - 32 P]GTP. Total RNAs were extracted from strains with various plasmids as described in Materials and Methods and incubated with [α - 32 P]GTP at 37°C in a splicing buffer. Lane 1, BL21/pBluescript SK(+) incubated for 60 min; lane 2 to 5, BL21/pT7*recA* [int⁺] incubated for 0 (lane 2), 10 (lane 3), 30 (lane 4), and 60 (lane 5) min; lane 6, BL21/pT7*recA* [int⁻] incubated for 60 min. After the reaction, samples were analyzed by 5% acrylamide–8 M urea gel electrophoresis and exposed to an X-ray film. The size of detected band was approximately the same as that of the intron (327 bp). A 32 P-labeled DNA ladder was used as a marker (M).

The putative LexA binding site, located 72 to 83 bp upstream of the start codon is exactly the same as that of *B. subtilis* *recA*.

In vivo and in vitro splicings of the IVS. We examined whether the IVS of the putative *recA* gene can be excised from the primary transcript. Using a pair of primers, RECA-4 and RECA-5 (Fig. 1), RT-PCR was carried out for total RNA from *B. anthracis*, resulting in a product smaller than that from the genomic DNA (Fig. 2A). Essentially the same result was obtained for total RNA extracted from *E. coli* containing *B. anthracis* *recA* (DH10 β /pUC*recA*[int⁺], data not shown). The size difference matches the size of the IVS, indicating that the IVS was excised, which was also confirmed by DNA sequencing. The spliced form of the mRNA contains an ORF encoding a protein of 343 amino acids with 85% identity to the RecA protein of *B. subtilis*.

Using the clone of *recA* whose transcription was under the control of T7 promoter, we obtained RNA in vitro using T7 RNA polymerase from a pT7*recA*[int⁺] plasmid linearized with *Xba*I (Materials and Methods). When RT-PCR was performed with RECA-4 and RECA-5 primers, the result was similar to that of the in vivo splicing experiment with total RNA from *B. anthracis* (data not shown), indicating that the splicing of the *recA* gene of *B. anthracis* (*recA*_{BA}) occurs by a self-splicing mechanism.

Secondary structure of the *recA* intron. Prediction of secondary structure for the *recA* intron revealed the distinctive features of a group I intron (Fig. 3). Group I introns contain regions of base pairing (P1 to P10), as well as the conserved sequence elements (P, Q, R, and S), which are necessary for proper folding and excision (2). The *B. anthracis* *recA* intron is spliced after the exonic u residue in the P1 stem that forms a pair with G, and the intron ends with G as in most group I introns. In addition, the 3'-terminal guanine of the intron is followed by three residues (CCA) capable of forming a base pair (P10) with residues (UGG) in the 3' side of P1. Thus, the nucleotides on the 3' side of P1 may serve as an internal guide sequence, promoting an alignment between the 3' and 5' splice sites for ligation of the two exons (4). The predicted structure of this intron resembles that of the *bmrE*-I1 group I intron,

inserted in the ribonucleotide reductase gene of the SP β -related prophage from *Bacillus* sp. strain BSG40 (19). About 36% of 327 nucleotides of the *recA* intron in the catalytic core, stem-loops, and single-stranded regions are identical (Fig. 3). Structural differences lie in the extra pairings of P1a and P1b, besides the absence of an ORF in the *recA* intron after P6a.

Group I-specific splicing of the *recA* gene. To test whether the splicing of the *recA* intron is mechanistically similar to that of group I introns, we carried out an in vitro splicing reaction with 32 P-labeled GTP. Total RNAs were extracted from IPTG-induced cells of BL21(DE3) containing pBluescript SK(+), pT7*recA*[int⁺], or pT7*recA*[int⁻], and incubated with [α - 32 P]GTP. After separation by urea-acrylamide gel electrophoresis, a labeled RNA product whose density increased over incubation time was detected from the sample containing the intron (Fig. 2B). The size of that band correlated well with that of the predicted intron (328 nucleotides including GTP). The same result was obtained with the pUC*recA*[int⁺] clone, but the band intensity was weak (data not shown) due to a relatively low copy number of the precursor RNA. From this result, we concluded that the IVS of the *recA*_{BA} gene is a self-splicing group I intron.

Complementation of a *recA* defect in *E. coli* by the RecA from *B. anthracis*. In order to assess the level of RecA_{BA} expression in *E. coli*, we performed Western blotting with an anti-RecA antiserum raised against the *E. coli* RecA protein. As shown in Fig. 4A, the RecA_{BA} proteins synthesized in *E. coli* (MG1655 Δ *recA*::Cm) from the plasmids pUC*recA*[int⁺] and pUC*recA*[int⁻] were identical in size and amount regardless of the presence of the intron. The size of RecA_{BA} produced in *E. coli* was similar to that of RecA_{EC}.

To further investigate the functionality of RecA_{BA}, we carried out a complementation test to assess its ability to protect against DNA damage caused by UV irradiation. As shown in Fig. 4B, *recA*_{BA} was able to complement the *recA* defect, and the degree of complementation for UV treatment was indistinguishable in the intron-positive and intron-negative *recA* genes. This indicates that the *recA*_{BA} gene expresses a func-

FIG. 3. Predicted secondary structure of the *recA* intron. The arrows indicate the boundaries between exons (lowercase) and intron (uppercase). The conserved base-paired regions (P1 to P9), extra pairings (P1a, P1b, P3.1, P3.2, P6a, and P7.2), and conserved sequence elements (R and S) are shown. The boxed residues, UGG near P1 and CCA near P9, can make a pair (P10) to promote an alignment between the 3' and 5' splice sites required for the ligation of exons. The G site (black box) is responsible for the binding of the guanosine cofactor. The shaded boxes represent residues identical with the *bnrE*-I1 intron from the *Bacillus* strain BSG40 (18).

tional RecA protein and that this expression is unaffected by the presence of an intron.

DISCUSSION

During the cloning and sequencing of the *recA* gene in *B. anthracis*, we found an IVS which turned out to be a group I self-splicing intron. The *in vivo* splicings of *B. anthracis* and *E. coli* were demonstrated by an RT-PCR experiment. It was also shown that the excised intron during splicing is labeled with [α - 32 P]GTP (Fig. 2B). These experimental observations are consistent with the prediction of a group I-like secondary structure for the IVS. The high similarity of predicted secondary structure and sequence of the *B. anthracis recA* intron to those of the *bnrE*-I1 intron from *Bacillus* sp. strain BSG40 may suggest a lateral transfer of these introns, most likely by transposition (7). The first identified microbial group I intron was the one in the thymidylate synthetase gene (*td*) of bacteriophage T4 (3). About 17 introns in protein-coding genes of bacteriophages have been reported (7), besides 74 introns in tRNAs and rRNA of bacteria. However, the group I introns have so far never been found in a protein-coding gene of a bacterial genome.

Most group I introns found on the bacterial genomes are contained in a prophage, not in the genome. Although the group I intron of the thymidylate synthase gene was observed in *Bacillus mojavensis* s87-18 (8), it was not clearly stated whether it was from a prophage or the genome. One could imagine that the *recA* gene presented here might be from a

prophage, and thus, another genomic copy of *recA* gene may exist. In a search for *recA* sequence from *B. anthracis* (Ames strain) genome sequences (www.tigr.org), we found a contig (6264) containing the *recA* gene with its intron. Analysis of the surrounding nucleotide sequences revealed the gene order *pgsA-cinA-recA-ymdA*, which is also preserved in the *B. subtilis* genome, except for the *pbpX* gene, which is inserted between *recA* and *ymdA* in *B. subtilis*. The orientations of these genes are identical, implying that the *recA* gene is a genomic copy in *B. anthracis*. Thus, the *recA* intron characterized here would be the first example of a group I intron in a protein-coding gene of a bacterial genome.

As previously indicated, there are certain characteristics shared by the group I introns from bacteriophages. Most of them are found in protein-coding genes associated with DNA metabolism and contain an ORF for an endonuclease involved in intron mobility. The group I introns found in some tRNAs or rRNAs do not usually encode a homing endonuclease, although there are a few exceptions. The *recA* intron from *B. anthracis* appears to be associated in some way with DNA metabolism similar to the phage introns but does not contain a homing endonuclease, as found in the group I introns of tRNAs or rRNAs. Since intron mobility depends on the presence of a homing endonuclease (17), the *recA* intron of *B. anthracis* would not be mobile by this mechanism.

The presence of an intron in the *B. anthracis recA* gene seems particularly interesting because some *recA* genes from mycobacteria are also interrupted by an intein, a protein intron that is removed after translation. There are two types of my-

FIG. 4. Complementation of *recA* deletion in *E. coli* by expression of *B. anthracis* *recA*. (A) *Bacillus* RecA expressed in *E. coli*. Total proteins were analyzed by immunoblotting with an anti-RecA_{EC} antiserum after sodium dodecyl sulfate-polyacrylamide (12%) gel electrophoresis and transfer to a nitrocellulose paper. Lane 1, MG1655; lane 2, MG1655Δ*recA*; lane 3, MG1655Δ*recA*/pUC19; lane 4, MG1655Δ*recA*/pUC*recA* [int⁺]; lane 5, MG1655Δ*recA*/pUC*recA* [int⁻]. The size of the *B. anthracis* RecA protein was about 37 kDa, which is similar to that of *E. coli* RecA. (B) Complementation of *E. coli* *recA* defect by cloned *B. anthracis* *recA*. Strains used were MG1655 (●), MG1655Δ*recA* (○), MG1655Δ*recA*/pUC19 (▼), MG1655Δ*recA*/pUC*recA* [int⁺] (▽), and MG1655Δ*recA*/pUC*recA* [int⁻] (■). The numbers of colonies were counted after UV irradiation and plating of the samples onto LB agar plates. The plates were incubated for 20 h at 37°C.

cobacterial inteins in terms of their insertion sites. More than eight species of mycobacteria, including *M. leprae*, have insertions at an identical site (RecA-b) (33), which is located immediately downstream of glycine 205 in *M. leprae*, while the intein from *M. tuberculosis* is found in another location (RecA-a) further downstream. It was proposed that the inteins were acquired independently (6, 33). It is striking that the insertion site of the *B. anthracis* *recA* intron is nearly identical to the RecA-b site of mycobacteria, with only one amino acid shift. Their origins might be evolutionarily related. The insertion of an intron and intein at almost the same location with one codon difference has previously been reported for the *nrdE* gene of SPβ-related prophage of *Bacillus* (19). Currently, no role for the RecA inteins has been assigned, and the presence of an intein does not affect RecA activity (10), pathogenicity, or the survival of the host organism. The presence of an

intron in the *B. anthracis* *recA* gene does not itself affect the expression or activity of RecA. It is possible that the *recA* intron might play some role during evolution, providing an advantage in adaptation or survival, although its function in current species seems dispensable.

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