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The *exoA* Gene of *Streptococcus pneumoniae* and Its Product, a DNA Exonuclease with Apurinic Endonuclease Activity

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The gene encoding the major DNA exonuclease of *Streptococcus pneumoniae*, *exoA*, was cloned in a streptococcal host vector system. Its location was determined by subcloning and by insertion mutations. Transfer of a DNA segment containing the gene to an *Escherichia coli* expression vector showed that *exoA* was the structural gene for the enzyme and that it was adjacent to its promoter. DNA sequence determination indicated that the gene encoded a protein, ExoA, of molecular weight 31,263. Under hyperexpression conditions, the ExoA protein constituted 10% of total cellular protein. In addition to previously demonstrated 3′ to 5′ exonuclease and 3′-phosphatase activities, ExoA was shown to make single-strand breaks at apurinic sites in DNA. Its enzymatic activities are thus similar to those of exonuclease III of *E. coli* and other gram-negative bacteria. The nucleotide sequence of *exoA* revealed it to be homologous to *xth* of *E. coli*, with 26% identity of amino acid residues in the predicted proteins. So far, no null chromosomal mutants of *exoA* have been obtained, and the biological function of ExoA remains unknown.

Enzymes with DNase activity are found in all living cells. They vary in mode of action on DNA, and some bacterial cells, such as those of *Bacillus subtilis*, may contain as many as 81 distinct DNase proteins (7). The functions of the various DNases in cellular metabolism are incompletely understood, but roles have been demonstrated for some of them in a variety of processes, including DNA replication, repair, transfer, and recombination, as well as restriction of foreign DNA and nutrition. Because these enzymes have the potential to degrade the genetic material of the cell that makes them, their expression must be carefully controlled by the cell. Relatively few genes encoding DNases have been cloned. Further cloning and analysis of such genes should help reveal the function and regulation of their products.

Prior investigation of *Streptococcus pneumoniae* revealed a number of distinct DNase activities. One of two restriction endonucleases, *Dpn*I or *Dpn*II, is present in different strains of the species (33). A DNA polymerase I contains both 3′ to 5′ and 5′ to 3′ exonuclease activities (28a). The major endonuclease found in cell extracts (15), which produces oligonucleotides in vitro (15), corresponds to a 25-kilodalton (kDa) polypeptide (38). It is located in the cell membrane (21) and functions in DNA entry into the cell for genetic transformation (18). A major exonuclease was also found in cell extracts (15).

The major exonuclease is a cytosolic enzyme (21) that is 31 kDa in size (38). It is specific for double-strand DNA, and it acts at ends or nicks to hydrolyze one strand in the 3′ to 5′ direction to produce 5′-nucleotides (15). It can also release the 3′-terminal phosphate from DNA (15). In these respects, the major exonuclease is similar to exonuclease III of *Escherichia coli* (35, 49). The latter enzyme can also produce strand breaks at sites in double-strand DNA from which bases have been removed (48), an activity implicated in repair of alkylation damage to DNA (8). Mutants of *S. pneumoniae* deficient in the major exonuclease have been obtained, but they all retained considerable residual enzymatic activity (13, 19). They showed no defect in repair of UV damage to DNA (13).

To better characterize the major exonuclease and its cellular functions, the gene encoding it, named *exoA*, was cloned in the *S. pneumoniae* cloning system (42) with a DNase plate assay for screening clones (13). This procedure had proved effective in cloning the gene encoding DNA polymerase I of *S. pneumoniae* (31). The *exoA* gene was subcloned in an *E. coli* expression system. Enzymatic activities of the ExoA product of the gene, including hydrolytic activity on partially depurinated DNA, were examined. The nucleotide sequence of the *exoA* gene was determined. A mutant strain was checked for sensitivity to an alkylating agent, and an attempt was made to obtain a chromosomal mutant devoid of ExoA activity.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this work are listed in Table 1. Strains of *S. pneumoniae* are derivatives of the non-encapsulated wild-type strain R6. Mutations *end* and *exo* affect the genes encoding the major endonuclease and the major exonuclease, respectively, of *S. pneumoniae* (13, 19).

Growth and transformation of bacteria. Cultures of *S. pneumoniae* were grown and transformed as described previously (27). *E. coli* was grown in L broth and transformed by the method of Hanahan (12). Cultures were treated with plasmid DNA at 0.1 to 1.0 μg/ml. Transformants of *S. pneumoniae* were selected in agar medium with tetracycline or erythromycin at 1 μg/ml or chloramphenicol at 2.5 μg/ml. Transformants of *E. coli* were selected in medium containing ampicillin at 50 μg/ml.

DNA and plasmid preparation. Chromosomal DNA was prepared from *S. pneumoniae* by the procedure of Berns and Thomas (3). Labeled DNA was prepared from strain 470 as described previously (17), except that phenol instead of chloroform was used for deproteinization. Cultures were grown with [*methyl*-^{3}H]thymidine, and the DNA had a specific activity of 74,000 cpm/μg.

Purified plasmids were prepared by the procedure of Currier and Nester (9). Crude plasmid preparations, called

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype or relevant features	Reference[a]
S. pneumoniae		
470	*thy-7 ltr-1 vlt-3 str nov*	17
533	*sul-d sul-a str bry nov ery*	16
593	*end-1*	19
708	*end-1 exo-2 trt-1 hex-4 malM594*	28
E. coli		
BL21(DE3)	*hsdS gal* (*int*::P_{lacUV5} T7 *gene1* imm^{21} *nin5*)	43
LE392	*hsdR gal supE supF*	45
Plasmids		
pET-5	pBR322 derivative, phage T7 promoter, *amp*	36
pJS3	Derived from pLS1-pC194 composite, *cat*	2
pJDC9	pMB9 derivative, *ery* from pAMβ1	5
pLS1	pMV158 derivative, *tet*	20, 42
pLS10	*Bam*HI converter in pLS1 *Eco*RI site, *tet*	
pLS301	8-kb *Bgl*II chromosomal fragment in pLS10, *tet*	
pLS302	*Cla*I fragment deleted from pLS301, *tet*	
pLS305	*cat* from pJS3 in *Bam*HI site of pLS301, *tet cat*	
pLS306	0.7-kb *Hpa*II-*Bam*HI fragment of pLS302 replaced by *Cla*I-*Bam*HI fragment of pJDC9, *tet ery*	
pLS307	4 nucleotides removed at *Nhe*I site of pLS302	
pLS310	1.4-kb *Eco*RI fragment of pLS302 cloned in pET-5	
pLS311	Same as pLS310, fragment in opposite orientation	
pLS312	5.5-kb *Cla*I fragment of pLS301 inserted in pLS307 to restore chromosomal segment	

[a] From this work if no reference is indicated.

alkaline lysates, were obtained from *E. coli* by the method of Birnboim and Doly (4) and from *S. pneumoniae* by a modification of that method (42).

DNA manipulations. Restriction endonucleases were obtained commercially and used as specified by their suppliers. Analytical gel electrophoresis of plasmids and restriction fragments was carried out in 1% agarose or 5% polyacrylamide gels with staining by ethidium bromide at 1 μg/ml. Plasmid restriction fragments used in subcloning experiments were extracted from agarose gels containing ethidium bromide at 0.3 μg/ml, after the bands were revealed with long-wave UV light, by electrophoresis onto DEAE membranes (NA45; Schleicher & Schuell, Inc.) as indicated by the supplier.

Ligation was carried out as described before (42). Unless otherwise indicated, the DNA concentration in the ligation mixtures was approximately 50 ng/μl when joining two fragments and 5 ng/μl when circularizing a fragment.

Partially depurinated DNA was prepared by heating 12.5 μg of covalently closed circular pET-5 at 70°C for 15 min in 50 μl of 100 mM NaCl–20 mM sodium acetate (pH 4.8).

Analysis of plasmid-encoded proteins. The BL21(DE3) (pET-5) host-vector cloning system allows preferential labeling of protein products encoded by genes in the plasmid (43). The cell harbors a defective lambda prophage that contains the phage T7 RNA polymerase gene under the control of the *lac* repressor, and the vector contains a T7 RNA polymerase promoter site. In the presence of isopropylthiogalactoside (IPTG), which induces synthesis of the T7 polymerase, most of the protein synthesized after induction is plasmid encoded (43). To 5-ml cultures of BL21(DE3) carrying pET-5, pLS310, or pLS311, grown to an OD_{600} of 0.5 at 37°C in M9 medium containing 0.2 mg of ampicillin per ml, 0.5 μmol of IPTG was added. After 2 h the cultures were chilled, and the cells were centrifuged and suspended in 0.5 ml of 100 mM Tris hydrochloride (pH 8.0) containing 125 μg of egg white lysozyme. After 20 min at 0°C, lysis was obtained by addition of 10 μl of 10% Triton X-100. Samples were analyzed by electrophoresis in polyacrylamide gels containing DNA as described below.

Enzyme assays. The DNase colony assay was carried out in agar plates as described previously (13), but with herring testes DNA (Sigma Chemical Co.) as the substrate.

Assay of nuclease activity in sodium dodecyl sulfate (SDS)-polyacrylamide gels was carried out as described by Rosenthal and Lacks (38). For this DNase gel assay, bacterial extracts were prepared either directly by lysis with 1% SDS in the presence of phenylmethylsulfonyl fluoride or by addition of SDS to native protein extracts (see below). Samples were heated for 3 min at 100°C and then subjected to electrophoresis in polyacrylamide gradient gels containing native calf thymus DNA (Worthington Diagnostics) at 30 μg/ml. The electrophoresis buffer contained 0.05% SDS (Matheson, Coleman and Bell, product DX2490, lot 27 [23]). Electrophoresis was conducted at 20 to 25°C with a constant current of 30 mA for approximately 3 h. After removal of SDS, the gels were incubated at 30°C in 40 mM Tris hydrochloride (pH 7.6)–2 mM $MgCl_2$. They were periodically stained with ethidium bromide and photographed under UV illumination.

Cell extracts containing native proteins were prepared by lysis with Triton X-100 as indicated above for *E. coli* and described previously for *S. pneumoniae* (22). Exonuclease activity was determined by incubating samples of extract at 30°C in 40 μl of a mixture containing 10 mM Tris hydrochloride (pH 7.6), 3 mM β-mercaptoethanol, 2 mM $MgCl_2$, 16 μg of bovine serum albumin, and 0.2 μg of [^{3}H]DNA. Reactions were terminated by addition of 40 μl of 10% trichloroacetic acid; after 20 min at 0°C, the mixture was centrifuged and 60 μl of the supernatant fluid was taken for scintillation counting.

The AP-endonuclease (see below) assay was a modification of the procedure described by Clements et al. (6). Specifically, 250 ng of partially depurinated DNA (prepared from pET-5, see above) was incubated with different amounts of cell extracts in a total volume of 50 μl, containing 50 mM Tris hydrochloride (pH 8.0), 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 5 μg of bovine serum albumin, for 10 min at 37°C. Reactions were stopped by chilling, and 20-μl samples were subjected to electrophoresis in 0.8% agarose. The proportions of the plasmid in covalently closed and open circular forms were determined by densitometrically scanning negatives of gels photographed under UV light after staining with ethidium bromide.

Protein concentration in extracts was determined by the method of Lowry et al. (29). Approximate proportions of protein in bands of gels stained with Coomassie blue were determined by reflectance scanning with a densitometer (model 620; Bio-Rad Laboratories).

Sensitivity to MMS. The sensitivity of various strains of *S. pneumoniae* to methylmethane sulfonate (MMS) was determined from viable counts after treatment with the agent.

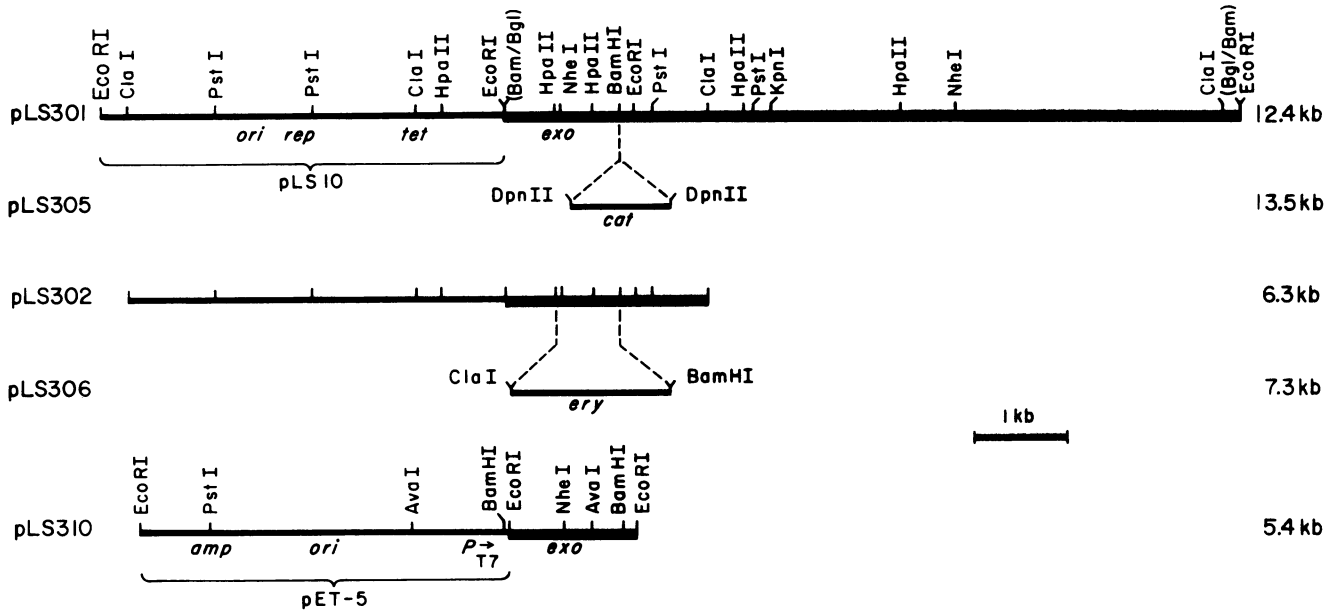

FIG. 1. Recombinant plasmids containing the *exoA* gene of *S. pneumoniae*. Restriction maps are shown linearized at either an *Eco*RI or a *Cla*I site. (Bam/Bgl) and (Bgl/Bam) indicate union of *Bam*HI- and *Bgl*II-cut fragments. Thin bar, Vector or antibiotic resistance factor; heavy bar, chromosomal DNA. Dashed line indicates insertion and deletion in pLS305 and pLS306. P_{T7} is a phage T7 RNA polymerase promoter; the arrow shows its direction of transcription.

Cultures in complete medium, at 5×10^7 CFU/ml, were incubated with 10 mM MMS for up to 60 min.

DNA sequence determination. Trace amounts of RNA were removed from plasmid samples by treatment with pancreatic RNase and gel filtration. After cleavage with restriction enzymes, the DNA fragments were treated with alkaline phosphatase and labeled at their 5′ ends with [γ-^{32}P]ATP and T4 polynucleotide kinase. Nucleotide sequences were determined by the chemical method of Maxam and Gilbert (32).

RESULTS

Cloning of the *exoA* gene in *S. pneumoniae*. The *exoA* gene encoding the major exonuclease of *S. pneumoniae* was cloned in a new vector, pLS10. This vector was constructed by insertion of the converter oligonucleotide 5′-AATTCG-GATCCG-3′ (Worthington) into the *Eco*RI site of pLS1 (20, 42) to introduce a *Bam*HI site, bracketed by *Eco*RI sites, into the plasmid. Chromosomal DNA from strain 533 was digested to completion with *Bgl*II, and the fragments were ligated to pLS10 cleaved with *Bam*HI. Ligations were performed at a total DNA concentration of 300 μg/ml, with a chromosome-to-vector DNA molar ratio of 5:1. The ligation mixture was used to transform the *end-1 exo-2* mutant strain 708. In order to increase the ratio of recombinant plasmids among the transformants, we made use of the facilitation of plasmid establishment as described previously (1); that is, plasmid DNA (alkaline lysate) from a transformed batch culture of 708 grown under selection with tetracycline to an OD_{650} of 0.4 was used to transform 708 again. The establishment of plasmids carrying chromosomal inserts is favored in this second transformation. Thus, as tested by restriction analysis, 80% of the Tc^r transformants that were sampled harbored plasmids with inserts, which ranged from 3 to 11 kilobases (kb) in size.

The Tc^r transformants were screened directly for nuclease activity in plates containing DNA and methyl green, as well as tetracycline, in the agar medium. Colonies of the recipient strain 708 do not give colorless zones in this plate assay after 36 h at 37°C. However, 9 of 1,500 transformants tested showed nuclease activity. To check the linkage of the nuclease phenotype to the plasmid, alkaline lysates from the nine clones were used to transform strain 708 again. Only one of the nine clones did not give transformants with nuclease activity; it could have arisen from a chromosomal transformation of the *end-1* or *exo-2* mutation, but it was not investigated further. In the other eight cases, the nuclease-positive phenotype was transferred to the recipient strain along with the Tc^r phenotype. However, approximately 2% of the Tc^r colonies did not show zones. Such behavior generally results from substitution of the wild-type allele in the donor plasmid by the recipient mutant allele during establishment of the plasmid by chromosomal facilitation (28). In this case it indicates that the nuclease-positive phenotype resulted from complementation of either the *end-1* or *exo-2* mutation in the chromosome by the wild-type allele in the plasmid. The restriction patterns of the plasmids in all eight nuclease-positive clones were identical (not shown). They could represent identical independent cloning events, or they could all be derived from a single first-round transformant. This recombinant plasmid, which carried a chromosomal insert of 8.0 kb, was called pLS301 (Fig. 1).

To determine which nuclease was expressed in cells carrying pLS301, extracts of the cells were analyzed by the DNase gel assay. The pattern of proteins with DNase activity in strains 708 and 593 with and without pLS301 is shown in Fig. 2. The nuclease-deficient strain 708 (lane 4) showed a single band of DNA degradation when it harbored pLS301 (lane 5). This band had the same mobility as the major exonuclease of *S. pneumoniae* present in the *end-1 exo*$^+$ strain 593 (lane 1); the mobility of the major endonuclease (observed in *end*$^+$ strains) is greater (38). Furthermore, the DNase band in 593 was enhanced for extracts of 593 cells carrying pLS301. Therefore, we conclude that the 8.0-kb chromosomal fragment cloned in pLS301 contains a

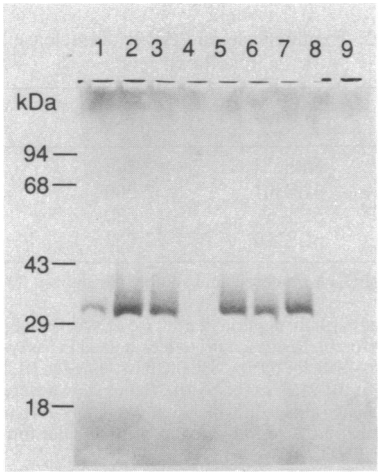

FIG. 2. DNase activity in strains with and without pLS301 or its derivatives. Approximately 3 μg of total cell protein from strains containing plasmids as indicated below was applied to a polyacrylamide gel containing DNA, subjected to electrophoresis in the presence of SDS, and assayed for DNase activity after renaturation by incubation at 30°C for 16 h. Host strain in lanes 1 to 3 was 593 (*end-1*); in lanes 4 to 9, it was 708 (*end-1 exo-2*). Lanes: 1, no plasmid; 2, pLS301; 3, pLS302; 4, no plasmid; 5, pLS301; 6, pLS302; 7, pLS305; 8, pLS306; 9, pLS312. Protein size standards, run in an adjacent lane and indicated at left, are, from top to bottom, rabbit phosphorylase, bovine serum albumin, chicken ovalbumin, bovine carbonic anhydrase, and bovine lactalbumin.

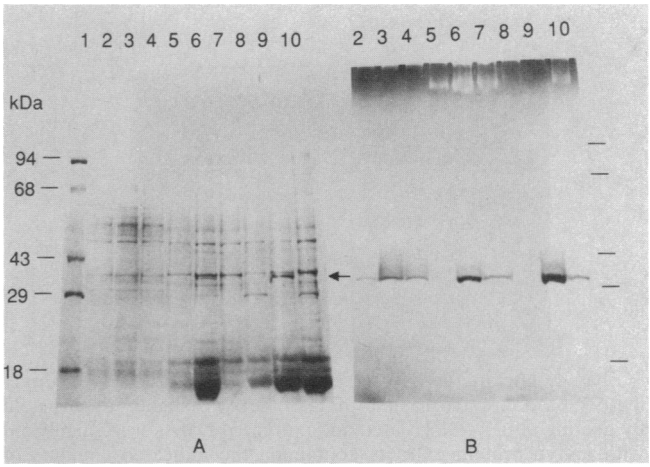

FIG. 3. Plasmid-specific expression and hyperproduction of ExoA in *E. coli*. (A) Gel stained for protein with Coomassie blue. (B) Parallel gel incubated at 30°C for 24 h for DNase activity. Approximately 6 μg of total cell protein from strains containing plasmids as indicated was applied to each of two gels containing DNA and subjected to electrophoresis in the presence of SDS. Lane 1, Protein size standards as in Fig. 2. Host strain in lanes 2 to 4 was *S. pneumoniae* 593; in lanes 5 to 7, it was *E. coli* BL21(DE3), not induced; in lanes 8 to 10 it was BL21(DE3) induced with IPTG. Plasmids: lane 2, none; lane 3, pLS301; lane 4, pLS302; lanes 5 and 8, pET-5; lanes 6 and 9, pLS310; lanes 7 and 10, pLS311. Arrow indicates ExoA protein.

gene able to complement *exo-2* and to increase the level of the major exonuclease activity in wild-type cells.

Identification of *exoA* as the structural gene for the major exonuclease. The structural gene for the cloned exonuclease was defined by subcloning and insertion mutagenesis in *S. pneumoniae* and by expression of the gene in *E. coli*. Partial digestion of pLS301 with *Cla*I yielded a 6.3-kb plasmid, pLS302, in which the chromosomal insert had been shortened to a 2.3-kb *Eco*RI-*Cla*I segment (Fig. 1). Cells harboring this plasmid showed the same pattern of nuclease activity as did cells harboring pLS301 (Fig. 2, lanes 3 and 6). This smaller plasmid could also pick up the *exo-2* allele by chromosomal facilitation, at a similar frequency to pLS301. Deletion of the 4.3-kb *Nhe*I fragment from pLS301 eliminated the nuclease band (data not shown). This was consistent with localization of a cloned nuclease gene in the 2.3-kb *Eco*RI-*Cla*I segment of both pLS301 and pLS302.

Further mapping of the gene responsible for nuclease production was achieved by insertions. A 1.1-kb *Dpn*II fragment from pJS3 carrying the *cat* gene was inserted into the *Bam*HI site of pLS301 to give the Tc^r Cm^r plasmid pLS305. Likewise, a 0.7-kb *Hpa*II-*Bam*HI fragment of pLS302 was replaced by the 1.7-kb *Cla*I-*Bam*HI *ery* fragment from pJDC9, to give the Tc^r Em^r plasmid pLS306 (Fig. 1). The nuclease patterns of pLS305 and pLS306 are depicted in Fig. 2. Although pLS305 produced the same nuclease band as its parental plasmid, with pLS306 no nuclease activity was detected. This indicates that the nuclease gene is located to the left of the *Bam*HI site in pLS301 (Fig. 1). This location was confirmed by a short deletion produced at the *Nhe*I site (Fig. 1) by cleavage by pLS302 with *Nhe*I, removal of the 4-nucleotide overhang with S1 endonuclease, and ligation to give pLS307. Interruption at this site blocked nuclease expression in pLS307 (data not shown) and pLS312 (Fig. 2, lane 9). The location of the nuclease gene, and mutations within it, near the left end of the cloned segments would explain the low frequencies (~2%) of marker substitution by chromosomal facilitation; the frequency of such substitution is dependent on the position of the marker in the insert (28).

To identify the product of the cloned gene, we subcloned the 1.4-kb *Eco*RI fragment from pLS302 into the *Eco*RI site of the *E. coli* expression vector pET-5. This fragment was inserted in both orientations to give the plasmids pLS310 (Fig. 1) and pLS311. To avoid problems encountered in direct transformation of BL21(DE3) (10), pLS310 and pLS311 were first established in LE392 and then transferred to BL21(DE3).

Protein and nuclease activity in cell extracts from BL21(DE3) carrying pET-5, pLS310, or pLS311 were examined after electrophoresis in SDS-polyacrylamide gels (Fig. 3). Protein staining revealed an extra polypeptide of approximately 31 kDa apparently encoded by both pLS310 and pLS311 (Fig. 3A, thin bands in lanes 6 and 7 indicated by arrow). When the cultures were induced with IPTG, however, the synthesis of this polypeptide increased the cells carrying pLS310 but not pLS311. The same cell extracts were loaded in a second gel to visualize bands with DNase activity. The results (Fig. 3B) clearly show that the bands at 31 kDa had DNase activity and that their mobility corresponded to the major exonuclease band observed in *S. pneumoniae* cell extracts. Furthermore, induced BL21(DE3) (pLS310) showed increased DNase activity. These results demonstrate that the gene cloned in pLS302, pLS310, and pLS311, which we have named *exoA*, is the structural gene for the major exonuclease of *S. pneumoniae*. Its response to induction of T7 RNA polymerase shows that the gene is transcribed from left to right in pLS310, as depicted in Fig. 1. Inasmuch as its protein product, ExoA, was synthesized from pLS311, where the gene was cloned in opposite orientation in pET-5, it appears that the cloned segment itself contains a bacterial promoter sequence. The greater synthesis without induction from pLS310, which is evident in Fig.

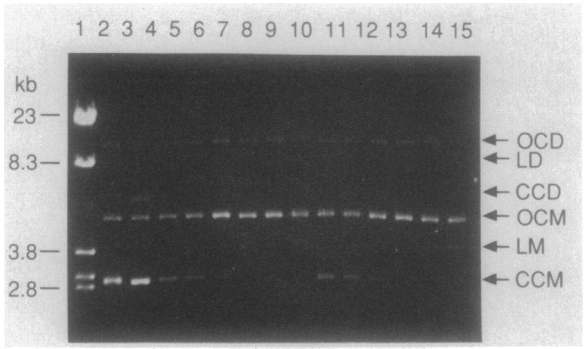

FIG. 4. Nicking activity of ExoA on depurinated DNA. Covalently closed circular plasmid pET-5 DNA was slightly depurinated by heating at pH 4.8. Treated and untreated DNAs were incubated with native protein extracts containing the indicated amounts of protein, and samples were subjected to electrophoresis in a 1% agarose gel, which was then stained with ethidium bromide. Lane 1, *Dpn*II-cut phage T7 DNA linear size standards. Substrate DNA in lanes: 2 and 3, untreated pET-5; 4 to 15, AP-DNA. Amount of protein from *S. pneumoniae* 593 in lanes: 2, 450 ng; 4, none; 5, 4.5 ng; 6, 15 ng; 7, 45 ng; 8, 150 ng; 9, 450 ng. Amount of protein from strain 593 containing pLS301 in lanes: 3, 45 ng; 10, 0.15 ng; 11, 0.45 ng; 12, 1.5 ng; 13, 4.5 ng; 14, 15 ng; 15, 45 ng. Symbols for plasmid forms on right: OCD, open circular dimer; LD, linear dimer; CCD, covalently closed dimer; OCM, open circular monomer; LM, linear monomer; CCM, closed circular monomer.

3, is attributable to a significant uninduced level of T7 RNA polymerase in BL21(DE3) (43).

ExoA is an apurinic-apyrimidinic DNA endonuclease. It has been reported that the major exonuclease of *S. pneumoniae* shares at least two characteristics with the *E. coli* exonuclease III: both have 3′-phosphatase and 3′ to 5′ exonuclease activities on double-strand DNA (15, 35), suggesting that these proteins may play similar roles. The major function of exonuclease III appears to be the repair of apurinic or apyrimidinic (AP) sites in DNA by the AP-endonuclease activity that cleaves the 5′ phosphodiester bond at AP sites in double-strand DNA (49). We tested for the possible AP-endonuclease activity of ExoA in cell extracts from *S. pneumoniae* and *E. coli* harboring pLS301 and pLS310, respectively. The AP-endonuclease activity was measured by the formation of nicked (relaxed) forms from supercoiled plasmids containing AP sites. This substrate was made by heating supercoiled pET-5 DNA at pH 4.8 under conditions expected to release two or three bases per molecule (6). The products obtained when the partially depurinated DNA was incubated with various amounts of extracts from 593 and 593(pLS301) are shown in Fig. 4. Inasmuch as untreated DNA was insensitive to large amounts of the extracts (lanes 2 and 3), the observed nicking activity was specific for DNA containing AP sites. A summary of the nicking activity data obtained from these assays is listed in Table 2. The presence of *exoA* in the multicopy plasmid gave a 9-fold increase in nicking activity relative to a single dose in the chromosome. Induction of BL21(DE3)(pLS310) with IPTG gave extracts with very high specific activity. Table 2 shows that AP-endonuclease activity was generally correlated to exonuclease activity in the cell extracts, which supports the idea that both reside in the same enzyme, ExoA.

If ExoA were the major AP-endonuclease of *S. pneumoniae*, it might be expected that cells carrying *exoA*$^+$ in multicopy dose would be less sensitive than *exoA* mutants to alkylating agents, such as MMS, that cause the appearance of AP sites in DNA (24). To test this, we compared the survival of the *exo-2* mutant strain 708 with and without pLS301 in the presence of MMS. The MMS sensitivity of the two strains was identical (data not shown). A possible explanation for this finding is that the high residual enzyme activity in 708, estimated for the exonuclease to be 20% of the wild-type level (19), is sufficient to provide the necessary amount of AP-site repair. Extracts of 708 in fact showed AP-endonuclease activities that were 20 to 30% of wild-type levels (data not shown).

TABLE 2. Exonuclease and AP-endonuclease activities in cell extracts

Strain	Plasmid	Exonuclease activity[a]	AP-endonuclease activity[b]
593	None	240	22
	pLS301	2,200	180
BL21(DE3)[c]	pET-5	80	9
	pLS310	22,000	8,700

[a] Nanomoles of DNA rendered acid soluble per hour at 30°C per milligram of protein.

[b] Nanomoles of partially depurinated pET-5 DNA nicked per hour at 37°C per milligram of protein. Ranges of protein examined in assays were 4.5 to 450 ng for 593, 0.15 to 45 ng for 593(pLS301), 10 to 30 ng for BL21(DE3)(pET-5), and 0.01 to 10 ng for BL21(DE3)(pLS310). Values shown were computed from densitometric scans of assays in which approximately 70% of the covalently closed plasmid substrate was converted to open circular form.

[c] Induced with IPTG.

All of the chromosomal *exoA* mutants previously obtained show significant residual exonuclease activity (13). To obtain a strain completely deficient in ExoA, we tried to transfer to the chromosome the mutant *exoA* allele of pLS306 containing the *ery* gene insertion (Fig. 1). Plasmid pLS306 cleaved with *Cla*I was used to transform strain 593, and Em^r clones were selected in plates containing DNA and methyl green. Because the vector portion of the plasmid was cut twice, the frequency of establishment of the plasmid should be very low, and the majority of Em^r clones obtained were expected to result from integration of the Em^r marker into the chromosome at the *exoA* locus by a recombination process mediated by linear synapsis (14) with sequences flanking the marker. However, the Em^r transformation frequency was very low (10^{-4} times that of a Str^r chromosomal DNA transformation run in parallel) and similar to the Tc^r frequency. All of the Em^r clones checked carried a 7.3-kb plasmid, and none showed a nuclease-deficient phenotype. They must have resulted from plasmid establishment, presumably from residual intact plasmid DNA. This failure to introduce the mutant allele into the chromosome suggests that either *exoA* is essential or a gene located downstream from it, possibly in the same operon, is essential for cell viability.

Nucleotide sequence of *exoA*. The nucleotide sequence of a 1-kb segment between *Eco*RI and *Ava*I sites in the *Eco*RI fragment cloned in pLS310 was determined according to the strategy shown in Fig. 5. Open reading frames predicted from the sequence are also shown in Fig. 5. The only extensive open reading frame in either direction enters the fragment from the left and reads from left to right. It includes the *Nhe*I site, at which a 4-nucleotide deletion blocked ExoA activity. Although this reading frame is open at its 5′ end, it is unlikely that the coding sequence extends upstream from the *Eco*RI site as ExoA is synthesized active and complete in both pLS310 and pLS311. The sequence data were submitted to GenBank under the accession number J04234.

The DNA sequence is shown in Fig. 6 along with the predicted amino acid sequence of the protein. The nucleotide sequence begins at the *Bgl*II site originally present in the

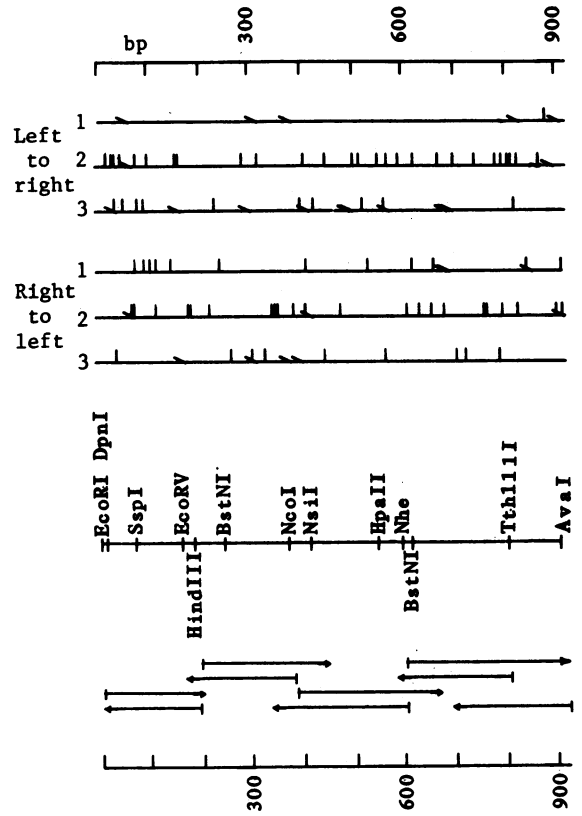

FIG. 5. DNA sequencing strategy and open reading frames in the vicinity of the *exoA* gene. Arrows at bottom show segments sequenced; vertical bars indicate sites of end labeling; arrowheads indicate extent of sequence determined. Reading frames in all three phases are depicted above for both directions: vertical bars indicate terminator codons; oblique marks indicate potential ATG start codons.

S. pneumoniae chromosomal DNA. The ExoA protein presumably starts from the first ATG codon, which is located at nucleotide 54. A putative ribosome-binding sequence (41) is found immediately upstream. The predicted protein contains 275 amino acid residues and has a molecular weight of 31,263. Three possible −10 promoter sites, which deviate by no more than a single base from the procaryotic consensus (TATAAT [37]), were present 5′ to the coding region at nucleotide positions 15, 20, and 27. None of these potential −10 sites had associated with it, in either vector, a good match to the −35 consensus sequence, TTGACA (37). We lack the sequence information to see whether a −35 site is present in the chromosome for the first two potential −10 sites.

The amino acid sequence of ExoA was compared with the recently published sequence of exonuclease III from *E. coli* (40). An alignment of the two sequences by the ALIGN program obtained from the Protein Identification Resource (Georgetown University, Washington, D.C.) is shown in Fig. 7. The proteins appear to be homologous, with 26% of the amino acid residues of ExoA identical in exonuclease III. Except for the region between residues 90 and 150 of ExoA, the similarity extends throughout the polypeptide chains. Overall, however, the extent of similarity is less than the 30 to 40% identity previously found between other equivalent proteins of *S. pneumoniae* and *E. coli* (28a, 30, 34).

DISCUSSION

Substantial amounts of the ExoA protein can be produced with the cloned *exoA* gene of *S. pneumoniae*. When the gene was present in a multicopy plasmid, production of the enzyme was 10-fold higher than when it was present as a single copy in the chromosome, and when it was present in the *E. coli* BL21(DE3)(pET-5) expression system, another 10-fold amplification was achieved (Table 2). In the latter case, it is estimated that the enzyme constituted approximately 10% of the total protein present (Fig. 3A).

In addition to the 3′ to 5′ double-strand DNA exonuclease

```
5'-AGATCTTTTTTTGGTATAATAAAATCTATAATCTGAATGAAAAAGGTAACTTTATGAAACTTATCTCATGGAATATTGATTCCCTAAACGCTGCCCTAAC  100
                                          S.D.      fMetLysLeuIleSerTrpAsnIleAspSerLeuAsnAlaAlaLeuThr

TAGTGACTCAGCTCGTGCCAAATTGTCCCAAGAAGTCCTACAAACCTTGGTCGCTGAAAATGCTGATATCATTGCTATCCAAGAAACCAAGCTTTCTGCC  200
 SerAspSerAlaArgAlaLysLeuSerGlnGluValLeuGlnThrLeuValAlaGluAsnAlaAspIleIleAlaIleGlnGluThrLysLeuSerAla

AAAGGACCTACAAAGAAACACGTGGAAATTTTAGAAGAACTCTTCCCAGGCTACGAAAACACGTGGCGTTCTTCCCAAGAGCCTGCCCGTAAAGGCTATG  300
LysGlyProThrLysLysHisValGluIleLeuGluGluLeuPheProGlyTyrGluAsnThrTrpArgSerSerGlnGluProAlaArgLysGlyTyr'

CTGGAACCATGTTCCTTTATAAGAAAGAACTTACACCTACTATCAGCTTCCCAGAAATCGGTGCCCCTTCTACCATGGACTTGGAAGGTCGTATCATCAC  400
AlaGlyThrMetPheLeuTyrLysLysGluLeuThrProThrIleSerPheProGluIleGlyAlaProSerThrMetAspLeuGluGlyArgIleIleThr

TCTAGAATTTGATGCATTTTTCGTAACCCAAGTTTACACTCCAAACGCTGGTGACGGTCTCAAACGCTTGGAAGAACGCCAAGTCTGGGATGCCAAATAT  500
 LeuGluPheAspAlaPhePheValThrGlnValTyrThrProAsnAlaGlyAspGlyLeuLysArgLeuGluGluArgGlnValTrpAspAlaLysTyr

GCTGAGTATTTGGCTGAACTAGACAAAGAAAAACCAGTCCTTGCGACCGGTGACTACAACGTAGCCCACAATGAAATCGACCTTGCAAATCCTGCTAGCA  600
AlaGluTyrLeuAlaGluLeuAspLysGluLysProValLeuAlaThrGlyAspTyrAsnValAlaHisAsnGluIleAspLeuAlaAsnProAlaSer

ACCGCCGTTCACCTGGATTTACTGACGAGGAACGTGCTGGATTTACCAACCTATTGGCAACTGGATTTACTGATACCTTCCGCCATGTTCATGGCGATGT  700
AsnArgArgSerProGlyPheThrAspGluGluArgAlaGlyPheThrAsnLeuLeuAlaThrGlyPheThrAspThrPheArgHisValHisGlyAspVal

TCCTGAACGCTACACTTGGTGGGCACAACGCAGCAAAACTTCTAAAATCAACAATACAGGCTGGAGAATCGACTACTGGCTCACAAGTAACCGTATCGCT  800
 ProGluArgTyrThrTrpTrpAlaGlnArgSerLysThrSerLysIleAsnAsnThrGlyTrpArgIleAspTyrTrpLeuThrSerAsnArgIleAla

GACAAGGTCACTAAATCTGATATGATTGACTCAGGTGCTCGCCAAGACCATACACCGATTGTCTTGGAAATTGATTTGTAAGGATTTTCTATATGGACTA  900
AspLysValThrLysSerAspMetIleAspSerGlyAlaArgGlnAspHisThrProIleValLeuGluIleAspLeu

CAATGCGGTCATTCCCGAG-3'
```

FIG. 6. Sequence of the *exoA* gene and the ExoA protein. The nucleotide sequence of one DNA strand is shown beginning at a *Bgl*II site and ending at an *Ava*I site. The predicted amino acid sequence of the ExoA polypeptide is shown. The putative Shine-Dalgarno (S.D.) sequence for ribosome binding of the mRNA is underlined.

```
ExoA: MKLISWNIDSLNAALTSDSARAKLSQEVLQTLVAENADIIAIQETKLSAKGPTKKHVEILEELFPGYENIWRSSQEPARKGYAGTMFLYKKELTPTISFP 100
Exo3: MKFVSFNINGL---------RARPHQ-LEAIVEKHQPDVMGLQETKVHDDMFP------LEEVANVGYNVFYHGQ----KGHYGVALLTKE----TPIAV

ExoA: EIGAPSTMDLEGRIITLEFDAFFVTQVYTPNA--------GDGLKRLEERQVWDAKYAEYLAELDKEKPVLATGDYNVAHNEIDLANPASNRRSPGFTD- 200
Exo3: RRGFPGDDEEAQRRIIMAEIPSLLGNVTVINGYFPQGESRDHPIKFPAKAQFYQNLQNYLETELKRDNPVLIMGDMNISPTDLDIGIGEENRKRWLRTGK

ExoA: -----EERAGFTNLLATGFTDTFRHVHGDVPERYTWWAQRSKTSKINNTGWRIDYWLTSNRIADKVTKSDMIDSGARQDHTPIVLEIDL
Exo3: CSFLPEEREWMDRLMSWGLVDTFRHANPQTADRFSWFDYRSKGFDDNR-GLRIDLLLASQPLAECCVETG-IDYEIRSMEKPSDHAPVWATFRR
```

FIG. 7. Comparison of ExoA of *S. pneumoniae* and exonuclease III of *E. coli*. The amino acid sequences of ExoA (upper) and exonuclease III (Exo3, lower) are aligned to give maximum correspondence. Dashes indicate gaps produced by this alignment in one or the other sequence. Numbers at right correspond to positions in the alignment and not to the polypeptide sequence. Amino termini of the polypeptides are at top left. Asterisks indicate identical amino acid residues.

and 3′-DNA phosphatase activity characterized previously (15), the ExoA protein was shown to have AP-endonuclease activity. In these respects, the protein is very similar to exonuclease III of *E. coli* (35, 48). The two proteins are also similar in size, 275 amino acid residues for ExoA and 265 for exonuclease III. Thus, it is not surprising that the protein sequences predicted for the two enzymes show considerable similarity. The *exoA* and *xth* genes that encode them apparently derived from a common ancestral gene in a progenitor of both gram-positive and gram-negative bacteria. A phosphatase-exonuclease of *Haemophilus influenzae* (11), which like *E. coli* is gram negative, was previously shown also to have AP-endonuclease activity (6). In addition to the similarity of the proteins encoded by *exoA* and *xth*, it is perhaps noteworthy that the putative promoter of *exoA*, like the demonstrated promoter of *xth* (40), may lack the common −35 consensus site.

Not much is known about repair of AP sites in *S. pneumoniae*. In *E. coli* such sites arise either from DNA damage directly (for example, by spontaneous depurination after alkylation of DNA [24]), or indirectly (for example, by removal of misincorporated uracil by uracil DNA glycosylase [25]). The first step in restoration of an AP site in *E. coli* is a single-strand break catalyzed by an AP-endonuclease, which can be exonuclease III or endonuclease IV (47). The latter is encoded by *nfo*, the sequence of which was recently reported (39). The *nfo* product shows no homology to either *xth* or *exoA*.

A couple of observations suggest that *exoA* may be essential for cell viability in *S. pneumoniae*. All mutants obtained so far still show appreciable exonuclease activity in crude extracts (13), although the activity is not completely retained on purification of the protein (19) and is not renaturable after SDS gel electrophoresis (38). We were unable to move an insertion-deletion mutation of *exoA* into the chromosome. Such null mutations may render the cell inviable. A possible explanation for the indispensability of ExoA may be that it is the only AP-endonuclease in *S. pneumoniae* and that at least one such enzyme is essential for viability. However, *E. coli xth nth nfo* triple mutants that lack the three known AP-endonucleases of *E. coli* are still viable (8). Perhaps the situation in *S. pneumoniae* is comparable to that of a *dut* mutant of *E. coli*. In that case, the *xth* product is essential (44), presumably because it is the major AP-endonuclease and the minor AP-endonucleases are not able to cope with the demand caused by the excessive incorporation of uracil into DNA in the *dut* mutant (46).

A DNase from *Streptococcus sanguis* was recently cloned in *E. coli* by Lindler and Macrina (26). *S. sanguis* is a gram-positive species closely related to *S. pneumoniae*, and the cloned gene, *don*, hybridized with *S. pneumoniae* chromosomal DNA (26). On the basis of similar properties, including a size of 34 kDa estimated from gel mobility, it was suggested that the *don* product is homologous to the major exonuclease of *S. pneumoniae* (26). In support of this, we found an *Nco*I site (Fig. 5) and a *Bam*HI site (Fig. 1) in similar positions within and near the *exoA* and *don* genes, respectively. However, some results suggest differences between the genes. Although the *don* product was not tested directly for AP-endonuclease activity, it failed to complement *xth* in an *xth dut* double mutant of *E. coli* to restore viability (26). Furthermore, it was possible to construct a null chromosomal mutant for *don* in *S. sanguis* by an approach similar to the one we tried for *exoA* in *S. pneumoniae*. That *don* mutant was very sensitive to MMS and UV irradiation (26). Further comparison of the *exoA*, *don*, and *xth* genes should be of interest.

The *exoA* gene appears to be the first gene in an operon. Its promoter is immediately upstream from it. Preliminary determination of the nucleotide sequence downstream from *exoA* shows no evidence of a transcription terminator or promoter between it and the next open reading frame. In this respect *exoA* appears to differ from *xth*, which is followed by a putative transcription terminator (40). A potential ribosome-binding sequence is present at the start of the next open reading frame. Because the *ery* insertion-deletion may affect a downstream gene, the failure to introduce it into the chromosome may reflect the essentiality of a gene in the operon other than *exoA*. Elucidation of the functions of the other genes in the operon and their relationship to the exonuclease and AP-endonuclease activities of ExoA will be the object of future work.

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