

Isolation and Expression in *Escherichia coli* of *csIA* and *csIB*, Genes Coding for the Chondroitin Sulfate-Degrading Enzymes Chondroitinase AC and Chondroitinase B, Respectively, from *Flavobacterium heparinum*

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In medium supplemented with chondroitin sulfate, *Flavobacterium heparinum* synthesizes and exports two chondroitinases, chondroitinase AC (chondroitin AC lyase; EC 4.2.2.5) and chondroitinase B (chondroitin B lyase; no EC number), into its periplasmic space. Chondroitinase AC preferentially depolymerizes chondroitin sulfates A and C, whereas chondroitinase B degrades only dermatan sulfate (chondroitin sulfate B). The genes coding for both enzymes were isolated from *F. heparinum* and designated *csIA* (chondroitinase AC) and *csIB* (chondroitinase B). They were found to be separated by 5.5 kb on the chromosome of *F. heparinum*, transcribed in the same orientation, but not linked to any of the heparinase genes. In addition, the synthesis of both enzymes appeared to be coregulated. The *csIA* and *csIB* DNA sequences revealed open reading frames of 2,103 and 1,521 bp coding for peptides of 700 and 506 amino acid residues, respectively. Chondroitinase AC has a signal sequence of 22 residues, while chondroitinase B is composed of 25 residues. The mature forms of chondroitinases AC and B are comprised of 678 and 481 amino acid residues and have calculated molecular masses of 77,169 and 53,563 Da, respectively. Truncated *csIA* and *csIB* genes have been used to produce active, mature chondroitinases in the cytoplasm of *Escherichia coli*. Partially purified recombinant chondroitinases AC and B exhibit specific activities similar to those of chondroitinases AC and B from *F. heparinum*.

Glycosaminoglycans (GAGs) are a group of linear heterogeneous polysaccharides made up of repeating disaccharide units of glucosamine or galactosamine and uronic acid (3). They are divided into four main groups: chondroitin sulfates, including dermatan sulfate (chondroitin sulfate B); heparin and heparan sulfate; hyaluronic acid; and keratan sulfate (in which the uronic acid is replaced by galactose). As the carbohydrate components of proteoglycans, they are thought to be involved in the regulation of various cellular processes such as adhesion, differentiation, migration, and proliferation (3, 15, 16). Chondroitin sulfates are the most common forms of GAGs found in proteoglycans.

Flavobacterium heparinum (also classified as *Cytophaga heparina* [2], *Sphingobacterium heparinum* [41], and *Pedobacter heparinus* [39]), a gram-negative, nonpathogenic soil bacterium isolated by Payza and Korn (29), synthesizes five GAG lyases: three heparinases and two chondroitinases. All of these enzymes have been purified and characterized as heparinase I (HepI) (23, 44), HepII, and HepIII (23), all of which degrade either heparin, heparan sulfate, or both; chondroitinase AC

(ChnAC), a 75-kDa enzyme which degrades both chondroitin sulfates A and C; and ChnB, a dermatan sulfate-degrading 55-kDa enzyme (8, 26, 43).

Chondroitin lyase activities have also been reported in other bacteria. *Proteus vulgaris* synthesizes two chondroitinase ABC lyases, each with a different mode of action (10, 43). Chondroitin lyases I and II were identified in *Bacteroides thetaiotaomicron* (22). Chondroitin lyase activity was also reported in *Arthrobacter aurescens* (12, 13), *Porphyromonas gingivalis* W50 (37), *Streptococcus intermedius* (36), and several other bacteria, as reviewed by Linhardt et al. (21). The genes coding for chondroitin sulfate ABC endolyase and chondroitin lyase II from *P. vulgaris* and *B. thetaiotaomicron*, respectively, have been cloned and expressed in *Escherichia coli* with enzymatic activity (1, 9, 35). However, none of these chondroitin lyases harbor the unique substrate specificity of ChnB from *F. heparinum*, which can only degrade dermatan sulfate.

At the molecular level, very little is known about the regulation of GAG lyase expression in *F. heparinum*. Maximal expression levels of heparinases are obtained when heparin is used as the sole carbon source (6, 7), while optimal expression of chondroitinases is reached when chondroitin sulfates are used (25, 43). Sulfate repression and glucose effects have been observed in the regulation of heparinase expression (7). In addition, GAG lyase enzymes allow *F. heparinum* to use heparin, heparin sulfate, and the chondroitins as sole sources of carbon, nitrogen, and energy. GAG molecules are catabolized to smaller polysaccharides, mainly disaccharides, with 4,5-unsaturated uronic acid residues at their nonreducing ends (3). It is interesting that all five GAG lyases are located in the periplasmic space of *F. heparinum* (8, 46). Knowledge of the mechanism of enzyme induction, the enzymes involved in their

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TABLE 1. Bacterial strains, phages, and plasmids used in this study

Strain, phage, or plasmid	Genotype and relevant characteristics	Source or reference
Bacterial strains		
<i>E. coli</i> XL-1 Blue		Stratagene
<i>F. heparinum</i>		ATCC 13125
λ phages		
λ-DASH II		Stratagene
λIB7	λ-DASH II phage containing <i>csIA</i>	This study
λIB8	λ-DASH II phage containing <i>csIB</i>	This study
Plasmids		
pBluescript KS+		Stratagene
pIB11	400-bp amplicon of <i>csIA</i> generated with primers OAC-2 and OAC-4	This study
pIB12	400-bp amplicon of <i>csIA</i> generated with primers OAC-1 and OAC-3	This study
pIB13	800-bp amplicon of <i>csIA</i> generated with primers OAC-4 and OAC-3	This study
pIB14	300-bp amplicon of <i>csIB</i> generated with primers OB-1 and OB-2	This study
pIB15	pBluescript KS+ vector with 6-kb <i>Bam</i> HI DNA fragment from λIB7	This study
pIB16	pBluescript KS+ vector with 5-kb <i>Hind</i> III DNA fragment from λIB8	This study
pIBX1	<i>E. coli</i> expression vector	40
pIBX7	pIBX1 vector with <i>csIA</i>	This study
pIBX8	pIBX1 vector with <i>csIB</i>	This study

translocation across the cytoplasmic membrane, or the elements that control their gene expression in *F. heparinum* is limited.

Until recently, little was known about the structure of the chondroitinases from *F. heparinum*. Both chondroitinases have been crystallized (4, 19), and their structures have been resolved (J. Fethiere, B. Eggimann, and M. Cygler, submitted for publication; W. Huang, A. Matte, Y. Li, Y. S. Kim, R. J. Linhardt, H. Su, and M. Cygler, submitted for publication). It was shown that the two chondroitinases differ significantly in terms of their crystal forms and three-dimensional structures (4, 19; Fethiere et al. and Huang et al., submitted for publication).

F. heparinum, unlike most bacteria, is capable of posttranslational glycosylation. Four GAG lyases (HepI, HepII, ChnAC, and ChnB) are glycosylated (18). Thus far, documentation of glycosylation in bacteria has been limited to structural or secreted proteins (11). Structural analysis of the carbohydrate moieties of these GAG lyases using nuclear magnetic resonance and mass spectroscopy show them to be O linked to either serine or threonine with the consensus sequence Asp-Ser or Asp-Thr (14, 17). This is consistent with the O glycosylation consensus sequence of glycoproteins synthesized by *F. meningosepticum* (30).

In this paper, we report the isolation and characterization of the genes encoding the chondroitin lyase enzymes of *F. heparinum*. We also describe the relationship of these genes within the *F. heparinum* chromosome and the expression of active chondroitin lyases in *E. coli*.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The bacterial strains, plasmids, and λ phages used in this study are listed in Table 1.

Growth and induction conditions. The minimal medium and growth conditions used for *F. heparinum* were described by Zimmermann et al. (45). In the study of *csIA* and *csIB* gene expression in *F. heparinum* (see Table 4), growth was at 23°C for 24 h in minimal medium supplemented with (i) 0.2% (wt/vol) glucose, (ii) 0.2% (wt/vol) glucose and 1% (wt/vol) chondroitin sulfate A (Sigma Chemical Co., St. Louis, Mo.), or (iii) 1% (wt/vol) chondroitin sulfate A. Cells were then subcultured in the same medium for an additional 8 h of growth at 23°C.

E. coli was grown in Luria broth at the temperatures indicated below. λ phage was grown and amplified as recommended by the supplier (Stratagene, La Jolla, Calif.). Ampicillin was provided at a concentration of 200 µg/ml. Expression of the recombinant chondroitinases was achieved by growing *E. coli* XL-1 Blue harboring either plasmid pIBX7 (ChnAC) or pIBX8 (ChnB) at 37°C overnight in Luria broth containing ampicillin. Cells were subcultured in the same medium

and grown to an A_{600} of approximately 0.5. Cultures were induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Boehringer Mannheim, Montreal, Quebec, Canada) and were incubated at 25, 30, and 37°C for 5, 3.5, and 2 h, respectively.

Purification of chondroitinases. The chondroitinases were purified from *F. heparinum* as previously described (8). Recombinant chondroitinases were purified as previously described (8) but with the following modifications. Homogenates of *E. coli* cultures synthesizing the recombinant proteins were diluted with 10 mM sodium phosphate buffer (pH 7). The homogenate containing recombinant ChnAC (rChnAC) was applied to a CM-Sepharose Fast Flow column (16 mm [inside diameter] by 100 mm; Pharmacia, Mississauga, Ontario, Canada) and eluted at a flow rate of 6 ml/min with a linear gradient of 0 to 1 M sodium acetate in 10 mM sodium phosphate buffer (pH 7). rChnAC eluted from the column at about 400 to 500 mM sodium acetate. The homogenate containing rChnB was applied to a Cellufine Sulfate affinity chromatography column (16 mm [inside diameter] by 25 mm; Amicon Inc., Oakville, Ontario, Canada) and eluted at a flow rate of 2.5 ml/min with a linear gradient of 0 to 400 mM NaCl in 10 mM sodium phosphate buffer (pH 7). rChnB eluted from the column at about 100 to 200 mM NaCl.

Enzyme assays. Enzyme assays were performed as previously described (42) but with the following modifications. Pepstatin and phenylmethylsulfonyl fluoride were omitted from the phosphate-buffered saline (PBS). Fractions were analyzed for chondroitin sulfate A-, chondroitin sulfate C-, and dermatan sulfate-degrading activities in reaction buffers composed of either chondroitin sulfate A (Sigma Chemical Co.), chondroitin sulfate C (Sigma Chemical Co.), or dermatan sulfate (Celsus Laboratories Inc., Cincinnati, Ohio) at 500 µg/ml in 50 mM Tris, pH 8. For HepI expression (see Table 4), heparin-degrading activity was measured as described previously (44).

Clostripain digestion and peptide sequence analysis. The enzymes were subjected to digestion using clostripain (EC 3.4.22.8; Sigma Chemical Co.). Preactivated clostripain was added to either chondroitinase at 1 to 2% (wt/wt) in a buffer comprised of 25 mM sodium phosphate, 0.2 mM calcium acetate, and 2.5 mM dithiothreitol (Sigma Chemical Co.) at pH 7.5 ± 0.1 and incubated for 2 to 3 h at 37°C. The reaction mixtures were applied to a Brownlee C₈ reverse-phase high-pressure liquid chromatography column (2.1 mm [inside diameter] by 3.0 cm; Applied Biosystems, Mississauga, Ontario, Canada), and the individual peptide fragments were eluted at a flow rate of 0.2 ml/min with a linear gradient of 0 to 60% acetonitrile in 0.1% trifluoroacetic acid. Peptide fragments recovered from the reaction mixtures were subjected to amino acid sequence analysis using a 470A gas-phase protein sequencer (Applied Biosystems, Foster City, Calif.) at the Biotechnology Research Institute in Montreal, Quebec, Canada.

Molecular biology techniques. Isolation of chromosomal DNA, cloning, and DNA manipulation techniques were performed as described by Sambrook et al. (32). T4 DNA ligase and restriction endonucleases were purchased from New England Biolabs (Mississauga, Ontario, Canada). DNA fragments destined for ligation were first separated by agarose gel electrophoresis, and the DNA was extracted from the gel with a GeneClean I kit (Bio 101, Inc., La Jolla, Calif.). DNA of bacterial transformants was isolated for restriction analysis using the RPM kit (Bio 101).

DNA amplification. Oligonucleotide primer synthesis, preparation of genomic *F. heparinum* DNA for use as a template, and analysis of PCR-generated products were all performed as previously described (40). PCR amplification was performed as described by Mullis et al. (27) with the modifications described

TABLE 2. Primer sequences and their amino acid coordinates in ChnAC and ChnB

Enzyme and primer	Primer sequence ^a	Amino acids ^b
chnAC		
OAC-1	5' ggatcc GAR TAY TAY AAY ATH ATG CCN GT 3'	396–403
OAC-2	5' ggatcc ACN GGC ATD ATR TTR TAR TAY TC 3'	403–396
OAC-3	5' ggatcc TCN GGR AAR TAR TAN CCD ATN GCR TCR TG 3'	531–522
OAC-4	5' ggatcc TAY ATG GAY TTY AAY GTN GAR GG 3'	280–287
OAC-5	5' GTC CTT GCC AAT ACC AAC CAG 3'	602–608
OAC-6	5' CTG CGG AGT GGC AAT TTC 3'	135–130
OAC-7	5' TTT CAG TTC AAC CGT TGC ACC 3'	700–694
OAC-8	5' ggatcc ATG CAG CAG ACC GGT ACT GCA GAA 3'	23–29
OAC-9	5' ggatcc CCT AGA TTA CTA CCA TCA AAA 3'	3' of csIA
chnB		
OB-1	5' ggatcc CAR ATY GCC GAY GGN ACN TAT AAA GA 3'	48–56
OB-2	5' ggatcc GGC NSK ATT GGC TTC RTC AAA 3'	144–138
OB-4	5' ATC TTT ATA AGT CCC ATC GGC 3'	56–50
OB-5	5' ggatcc ATG CAG GTT GTT GCT TCA AAT GAA ACT 3'	26–33
OB-6	5' ggaatcaattc ACC GGG ATG ATC 3'	3' of csIB

^a Lowercase nucleotides represent the *Bam*HI and *Xmn*I restriction sites incorporated at the 5' ends of the primers for use in cloning. The nucleotide codes used are those recommended by the International Union of Pure and Applied Chemistry-IUB Biochemical Nomenclature Commission.

^b Amino acid coordinates are those of the precursor proteins.

previously (40). For the gene mapping experiment (see Fig. 5), intact λ IB8 was used and the PCR products were generated by using a *Taq* extender kit (Stratagene) as recommended by the manufacturer.

DNA hybridization. DNA for Southern blotting (38) was digested with the appropriate restriction endonuclease, separated by gel electrophoresis, and transferred to a nylon membrane (Hybond-N; Amersham, Oakville, Ontario, Canada). Plaque and dot blots, labelling of probes with [α -³²P]dATP, hybridizations, and washes were performed as previously described (40).

***F. heparinum* chromosomal DNA library screening.** A λ -Dash II chromosomal *F. heparinum* library was screened for the *csIA* and *csIB* genes as previously described (40).

Cloning of the *csIA* region. Degenerate oligonucleotide primers were designed by using the clostripain peptides. Primer pairs OAC-4 plus OAC-2 and OAC-1 plus OAC-3 both generated 400-bp PCR fragments, while primer pair OAC-4 plus OAC-3 generated an 800-bp PCR fragment (Table 2). These PCR fragments were cloned into the vector pBluescript KS+ to form plasmids pIB11, pIB12, and pIB13. The 800-bp insertion from pIB13 was used as a probe to screen the *F. heparinum* λ -Dash II genomic library. Phage λ IB7, containing a 6-kb *Bam*HI fragment, hybridized strongly to this insertion. This 6-kb fragment was isolated and cloned into pBluescript KS+ to produce plasmid pIB15.

Cloning of the *csIB* region. Partial guessmer and degenerate oligonucleotide primers were designed with *Bam*HI restriction sites incorporated at their 5' ends. Primer pair OB-1 plus OB-2 (Table 2) generated a 300-bp PCR fragment which was cloned into pBluescript KS+ to produce plasmid pIB14. This insertion was used to screen the *F. heparinum* λ -Dash II genomic library. Phage λ IB8 was found to hybridize strongly with the pIB14 insertion and contained a 5-kb *Hind*III fragment, which was cloned into pBluescript KS+ to produce plasmid pIB16.

DNA sequence analysis. DNA sequences were determined by the dideoxy-chain termination method of Sanger et al. (33).

Construction of plasmids pIBX7 and pIBX8. Inserts were obtained by DNA amplification. Oligonucleotides OAC-8 and OB-5 (Table 2), specific to *csIA* and *csIB*, respectively, were designed to contain *Bam*HI restriction sites at their 5' ends, followed by an ATG start site inserted immediately preceding the codon for the first amino acid of the mature protein (Q23 in the case of ChnAC; Q26 for ChnB). The other oligonucleotides, OAC-9 and OB-6 (Table 2), corresponding to *csIA* and *csIB*, respectively, were designed to hybridize downstream of the stop codon, followed by either a *Bam*HI (OAC-9) or an *Xmn*I (OB-6) restriction site at the 3' end. Amplicons of the expected sizes, about 2.1 and 1.5 kb for *csIA* and *csIB*, respectively, were inserted into the corresponding restriction sites of the expression vector pIBX1 (40). DNA sequence analysis was carried out to ensure that no errors were introduced during amplification.

Antibody production. Polyclonal antibodies against ChnAC and ChnB were raised in rabbits. A standard immunization procedure was employed, and ChnAC, ChnB, and HepI affinity columns were prepared as described by Su et al. (40). Anti-ChnAC or anti-ChnB antibodies were purified from sera preabsorbed on separate HepI columns to eliminate any cross-reactivity with other GAG lyases from *F. heparinum*. The unbound fractions were collected and purified over the respective ChnAC or ChnB antigen column. Bound antibodies (anti-ChnAC or anti-ChnB) were eluted in 0.1 M glycine (pH 2.0).

Protein and Western blot analysis. Protein concentrations were determined by the Bradford dye-binding procedure using the Bio-Rad protein assay (Bio-Rad Laboratories); bovine serum albumin was the standard used. Protein electro-

phoresis was performed on crude *E. coli* extracts by using precast, discontinuous sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gel electrophoresis (PAGE) gels (Bio-Rad Laboratories), while 10% polyacrylamide gels were used for the purified chondroitinases. Reducing gels were run in which 5% 2-mercaptoethanol (Sigma Chemical Co.) was added to the sample loading buffer. Quantities loaded per lane were equivalent to an optical density at 600 nm 0.15 U of or approximately 2 μ g for the crude and purified samples, respectively. Proteins were visualized with 0.1% Coomassie brilliant blue R-250 (Bio-Rad Laboratories). Western blot analysis was performed as previously described (40), except that the primary antibodies used were rabbit anti-ChnAC antibodies diluted 1:50,000 or rabbit anti-ChnB antibodies diluted 1:25,000.

Protein stability assay. Both recombinant *E. coli* and wild-type *F. heparinum* purified chondroitinases were subjected to heat inactivation as follows. The enzymes were diluted in PBS (10 mM sodium phosphate buffer [pH 7], 150 mM NaCl) to an initial total protein concentration of 50 μ g/ml. Samples were incubated at 37°C for 6 h and then diluted in PBS containing bovine serum albumin at 1 mg/ml and assayed for the appropriate activities.

Nucleotide sequence accession numbers. The GenBank accession numbers of the *csIA* and *csIB* sequences are U27583 and U27584, respectively.

RESULTS

Isolation of the *F. heparinum* gene for ChnAC, *csIA*. The amino acid sequences of three clostripain-digested ChnAC peptides were used as templates to design degenerate oligonucleotides (Table 2). *F. heparinum*-specific amplicons were obtained and cloned. DNA sequence analysis indicated that plasmid pIB13, which contained the 800-bp amplicon, consisted of the two 400-bp amplicons found in plasmids pIB11 and pIB12. The deduced amino acid sequence corresponded to *csIA*-derived peptide sequences (data not shown). Using the insertion from pIB13 as a probe, a 6-kb *Bam*HI fragment was isolated from a λ -Dash II *F. heparinum* chromosomal DNA library, cloned, and subjected to DNA sequence analysis.

A region of approximately 3.2 kb was sequenced, and a single continuous open reading frame (ORF) of 2,103 bp, encoding 700 amino acid residues, was deduced. The N-terminal peptide sequence of mature ChnAC, as reported by Gu et al. (8), lacks amino acids 1 to 22, implying a signal peptide which is 22 residues long. The mature ChnAC protein consisted of 678 amino acid residues with a calculated molecular mass of 77,169 Da. The G+C content of the *csIA* gene, 44.2 mol%, is similar to that reported for *F. heparinum* chromosomal DNA (43 mol%) (39).

Isolation of the *F. heparinum* gene for ChnB, *csIB*. Degenerate primers designed from clostripain-generated ChnB pep-

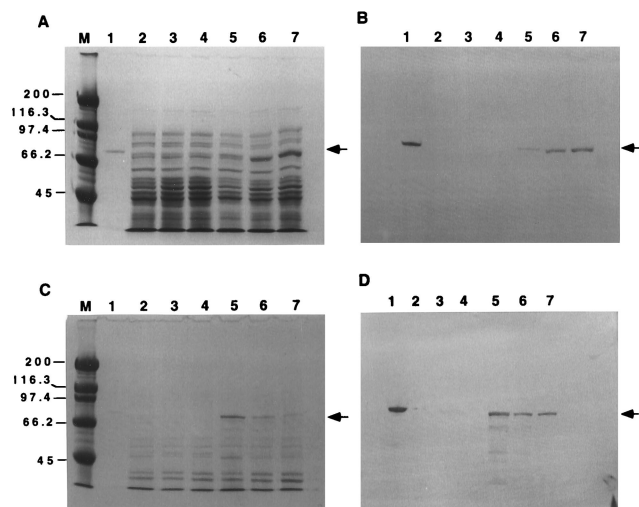


FIG. 1. SDS-PAGE and Western blot analysis of recombinant ChnAC in *E. coli* induced at various temperatures. Both soluble (A and B) and insoluble (C and D) fractions (0.15 OD₆₀₀ unit) were loaded onto SDS-7.5% PAGE reducing gels. Panels: A and C, Coomassie blue R-250-stained gels; B and D, Western blots of duplicates of gels A and C transferred to nitrocellulose membrane filters. Lanes: 1, wild-type *F. heparinum* ChnAC (1 µg) (positive control); 2 to 4, pIBX1 samples (negative control) induced at 37, 30, and 25°C, respectively; 5 to 7, pIBX7 samples induced at 37, 30, and 25°C, respectively; M, molecular size markers. An arrow indicates the position of ChnAC. The value on the left are molecular masses in kilodaltons.

tides (Table 2) were used to create a 300-bp PCR amplicon from *F. heparinum* chromosomal DNA which was cloned to form plasmid pIB14. DNA sequence analysis of this 300-bp amplicon confirmed that a portion of *csIB* had been cloned. A 5-kb *Hind*III fragment, isolated from a λ -Dash II *F. heparinum* chromosomal DNA library by using the insert from pIB14 as a probe, was cloned to create plasmid pIB16 and subjected to DNA sequence analysis.

A single 1,521-bp ORF coding for 506 amino acid residues was identified after sequencing of a portion of the insertion. The mature protein's N-terminal sequence, as reported by Gu et al. (8), mapped to the deduced peptide sequence of the *csIB* gene. The putative signal peptide is 25 amino acids long. Mature chnB is composed of 481 amino acid residues with a calculated molecular mass of 53,563 Da. The G+C content of this gene is 43.6 mol%.

Expression of *csIA* and *csIB* in *E. coli*. Due to the fact that the signal sequence from *F. heparinum* was not functional when the gene for HepI was expressed in *E. coli* (34), both ORFs were amplified by PCR and modified to include an ATG initiator codon immediately preceding the N-terminal residue of the mature protein. The DNA fragments were then cloned into expression vector pIBX1. After induction at various temperatures, the *E. coli* strains which contained plasmids pIBX7 and pIBX8 were analyzed for recombinant protein expression.

Protein electrophoresis indicated that both proteins were expressed in both soluble and insoluble forms at all induction temperatures. Intense bands of 70 and 55 kDa, corresponding to ChnAC and ChnB, respectively, were seen in *E. coli* fractions harboring plasmids pIBX7 (ChnAC) (Fig. 1A and C) and pIBX8 (ChnB) (Fig. 2A and B) but not in fractions with the expression plasmid pIBX1 (Fig. 1A and C and 2A and B). Western blot analysis of duplicate gels using antibodies specific for ChnAC (Fig. 1B and D) or ChnB (Fig. 2C and D) confirmed only those bands expressed by pIBX7 or pIBX8 as being ChnAC or ChnB. In the case of either recombinant protein,

TABLE 3. Chondroitin sulfate A-, chondroitin sulfate C-, and dermatan sulfate-degrading activities in soluble extracts from *E. coli* strains containing expression plasmids^a

Temp (°C) and substrate	Avg activity (mIU ml ⁻¹ A ₆₀₀ unit ⁻¹) ± SE ^b		
	pIBX1	pIBX7	pIBX8
37			
CS-A ^c	<3.0	17.6 ± 3.3	ND ^f
CS-C ^d	<3.0	11.7 ± 1.6	ND
DS ^e	<3.0	ND	13.7 ± 2.2
30			
CS-A	<3.0	36.7 ± 0.6	ND
CS-C	<3.0	26.0 ± 0.5	ND
DS	<3.0	ND	21.7 ± 2.4
25			
CS-A	<3.0	46.9 ± 3.0	ND
CS-C	<3.0	31.3 ± 1.4	ND
DS	<3.0	ND	62.5 ± 2.4

^a Values are based on three individual experiments.

^b One unit of chondroitin sulfate A-, chondroitin sulfate C-, or dermatan sulfate-degrading activity is defined as the amount of enzyme that will liberate 1 µmol of product per min at 30°C and pH 8.

^c CS-A, chondroitin sulfate A.

^d CS-C, chondroitin sulfate C.

^e DS, dermatan sulfate.

^f ND, not determined.

they were expressed mostly in the insoluble form at elevated induction temperatures whereas they were more predominant in the soluble form at lower temperatures.

Enzyme activity analysis of both recombinant proteins showed them to be biologically active (Table 3). However, activity was only detected in the soluble cell extracts and not in the insoluble forms (data not shown). The level of enzyme activity was inversely correlated to the induction temperature,

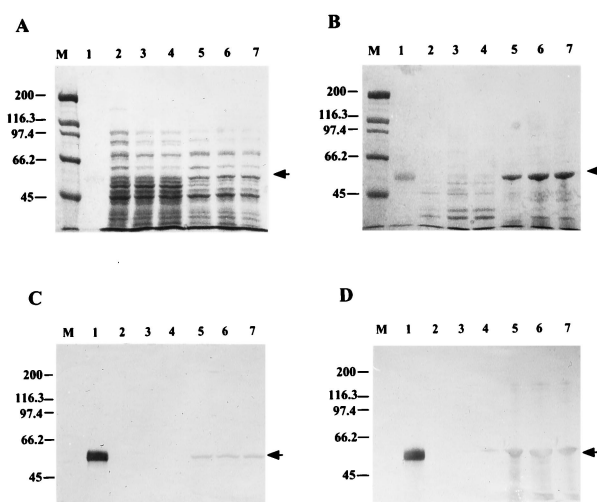


FIG. 2. SDS-PAGE and Western blot analysis of ChnB in *E. coli* induced at various temperatures. Both soluble (A and C) and insoluble (B and D) fractions (0.15 OD₆₀₀ unit) were loaded onto SDS-7.5% PAGE reducing gels. Panels: A and B, Coomassie blue R-250-stained gels; C and D, Western blots of duplicates of gels A and B transferred to nitrocellulose membrane filters. Lanes: 1, wild-type *F. heparinum* ChnB (1 µg) (positive control); 2 to 4, pIBX1 samples (negative control) induced at 25, 30, and 37°C, respectively; 5 to 7, pIBX8 samples induced at 25, 30, and 37°C, respectively; M, molecular size markers. An arrow indicates the position of ChnB. The values to the left are molecular masses in kilodaltons.

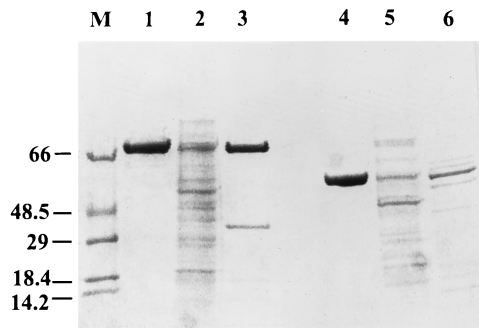


FIG. 3. SDS-PAGE analysis of purified chondroitinases from *E. coli* and *F. heparinum* stained with Coomassie blue R-250. Approximately 2 μ g of each sample was loaded onto SDS-10% PAGE reducing gels. Lanes: 1 to 3, ChnAC; 4 to 6, ChnB; 1 and 4, from *F. heparinum*; 2 and 5, *E. coli* homogenates; 3, CM-Sepharose-purified rChnAC; 6, Cellufine Sulfate affinity-purified rChnB; M, molecular weight markers. The values to the left are molecular masses in kilodaltons.

and the highest expression levels were reached at 25°C. For rChnAC, the ratio of chondroitin sulfate A-degrading activity to chondroitin sulfate C-degrading activity was approximately 1.5:1, which was similar to that observed for the native *F. heparinum* enzyme (8).

Purified rChnAC and rChnB were analyzed by protein electrophoresis, activity measurement, and stability assay. The sizes of rChnAC and rChnB were similar to those of ChnAC and ChnB from *F. heparinum*, respectively (Fig. 3, lanes 3 and 1 for ChnAC and lanes 6 and 4 for ChnB). The recombinant proteins also had specific activities similar to those of their wild-type counterparts (data not shown). However, rChnAC and ChnAC behaved differently when subjected to heat inactivation. Approximately 50% of rChnAC activity remained after its exposure to 37°C for 6 h, while ChnAC from *F. heparinum* retained full activity after similar treatment. rChnB and ChnB were also subjected to heat inactivation, and neither lost any activity.

Southern blot analysis. To investigate the relationship among the GAG lyase genes in *F. heparinum*, a Southern blot analysis of its chromosome was carried out in which *cslA*, *cslB*, and the heparinase genes *hepA* (34), *hepB* (40), and *hepC* (40) were used to probe PCR products corresponding to the 5' and 3' regions of *cslA* and *cslB*, as well as digests of λ IB7 and λ IB8. The *cslA* probe cross-hybridized to the amplicon corresponding to the region 5' of the *cslB* gene and also to a fragment from λ IB8 (data not shown). It was therefore concluded that *cslA* was 5' of *cslB* and that phage λ IB8 contained both genes. No cross-hybridization signals were observed with the heparinase genes (data not shown), suggesting that they are not proximally linked to the chondroitinase genes.

To determine the exact distance between the two genes and the orientation of the two ORFs with respect to one another, an experiment using PCR methodology and Southern blot analysis was performed. Oligonucleotides OAC-6 and OAC-5 were designed to correspond to the 5' and 3' ends of the *cslA* gene, respectively, and were used in a PCR in conjunction with oligonucleotide OB-4, corresponding to the 5' end of the *cslB* gene (Fig. 4). An amplicon was obtained only with primer pair OAC-5 plus OB-4 (Fig. 5A, lane 2). These results indicated that the *cslA* gene was 5' of the *cslB* gene and both genes were in the same orientation. The size of the PCR product, 5.5 kb, indicated the distance between the two genes. A PCR product of a similar size was also amplified by using *F. heparinum* chromosomal DNA as the template (data not shown). The

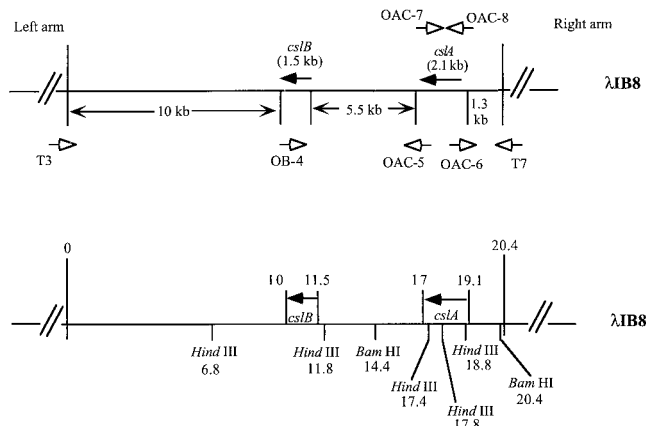


FIG. 4. Schematic diagram of λ IB8 containing both *cslA* and *cslB*. The directions of the ORFs of the two genes are indicated by labeled arrows with solid heads. The approximate sizes of the genomic regions on both sides of the genes are indicated between the arrows. The arrows with open heads indicate the locations of the PCR primers and the directions of synthesis from the primers. T3 and T7 indicate the locations of the primers derived from the T3 and T7 promoter regions in the lambda arms. OB-4 indicates the location of the primer derived from the DNA sequence at the end of the ORF of *cslB*. OAC-5, OAC-6, OAC-7, and OAC-8 indicate the locations of primers derived from 3' and 5' DNA sequences at the ends of the ORF of *cslA*. A partial restriction map is also provided, and positions are indicated in kilobases.

identity of these PCR products (Fig. 5A) was further confirmed by Southern hybridization experiments in which the *cslA* and *cslB* genes were used as probes (Fig. 5B and C).

Induction of *cslA* and *cslB* in *F. heparinum*. An experiment was conducted to analyze the synthesis of both chondroitinases when *F. heparinum* is grown in various media (Table 4). No

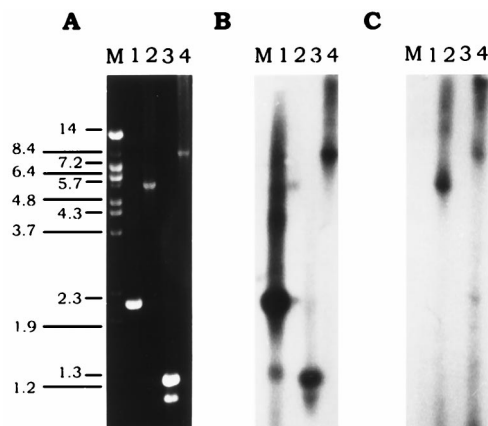


FIG. 5. Southern blots mapping the proximity and orientation of the *cslA* and *cslB* genes on the *F. heparinum* genome. (A) Ethidium bromide-stained agarose gel containing PCR products. (B) Autoradiograph of a Southern blot probed with a *Bam*HI fragment from pIBX7 containing the *cslA* ORF. (C) Autoradiograph of a Southern blot probed with a *Bam*HI-*Xmn*I fragment from pIBX8 containing the *cslB* ORF. Lanes: M, DNA molecular size markers (sizes [in kilobases] are indicated on the left); 1, 1 μ g of the PCR product of *cslA* obtained with primers OAC-7 and OAC-8; 2, 1 μ g of the PCR product, comprised of small portions of *cslA* and *cslB* and the intergenic region between the two genes, obtained with primers OAC-5 and OB-4; 3, 1 μ g of the PCR product, containing a small portion of *cslA* and the region 5' of *cslA*, obtained with primers OAC-6 and T7; 4, 1 μ g of the PCR product, composed of *cslB*, *cslA*, the intergenic region between the two genes, and the region 5' of *cslA*, obtained with primers T7 and OB-4. The DNA sequence of T3 is 5'-AATTAACCTCACTAAAGGG-3', and that of T7 is 5'-GTAATACGACTCACTATAGGG-3'. DNA sequences of the other primers are listed in Table 2. In all cases, λ IB8 was used as the template.

TABLE 4. Synthesis of ChnAC, ChnB, and HepI in *F. heparinum* grown in media containing various carbon sources^a

Enzyme	Avg activity (mIU ml ⁻¹ A ₆₀₀ unit ⁻¹) ^b ± SE when grown in:			
	Glu ^c	Glu + CS-A ^d	CS-A ^e	Ratio ^f
HepI	<5.0	20.8 ± 2.6	88.1 ± 3.3	4.2
ChnAC	<5.0	30.8 ± 0.9	79.5 ± 8.3	2.6
ChnB	<5.0	10.5 ± 1.5	29.4 ± 1.2	2.8

^a Values are based on three individual experiments.

^b One unit of heparin-, chondroitin sulfate A-, or dermatan sulfate-degrading activity is defined as the amount of enzyme that will liberate 1 μmol of product per min at 30°C and pH 8.

^c Glu, glucose-only medium.

^d Glu + CS-A, glucose medium with 1% chondroitin sulfate A.

^e CS-A, 1% chondroitin sulfate A medium.

^f Ratio of activity obtained in chondroitin sulfate A medium compared to medium containing glucose with chondroitin sulfate A.

ChnAC or ChnB activity was detected when the bacteria were grown in glucose-only medium, as was the case for HepI. They were expressed to detectable levels with the addition of the inducer chondroitin sulfate A to the medium. The highest level was reached when glucose was eliminated from the medium. A 2.5-fold increase in the expression levels of both chondroitinase enzymes was observed when the bacteria were grown in chondroitin sulfate A-only medium, compared to a 4-fold increase in HepI expression under similar conditions. The data suggest that the *csIA* and *csIB* genes were coregulated.

DISCUSSION

We have reported here the isolation and DNA sequence analysis of the *csIA* and *csIB* genes coding for ChnAC and ChnB, respectively. They were also expressed in *E. coli* with biological activity. This is the first report describing the cloning and expression of these enzymes from *F. heparinum*.

The calculated molecular masses of mature ChnAC and ChnB, derived from the DNA sequences, are 77,169 and 53,563 Da, respectively. Two carbohydrate moieties were identified in ChnAC and determined to be 1,194 and 1,080 Da. Only one was found in ChnB, and it had a molecular mass of 1,180 Da (17). Therefore, the total molecular mass of ChnAC is estimated to be 79,443 Da and that of ChnB is estimated to be 54,743 Da. These values closely reflect those of 79,557 and 54,779 Da as determined by mass spectroscopic analysis (17) for ChnAC and ChnB, respectively.

Signal sequences were observed in the coding regions of both genes, and their existence is consistent with their translocation to the periplasmic space of *F. heparinum*, as demonstrated by Linhardt's group and our laboratory (8). The leader peptides are typical of prokaryotic signal sequence regions (28). Moreover, the N termini of the mature proteins are also characteristic of exported bacterial proteins in that they lack a net positive charge (42). Glutamine is the +1 amino acid at the signal peptidase processing site in both chondroitinases and also is present in the heparinases from *F. heparinum* (40). This suggests that a similar translocation mechanism is involved.

A BLASTX search (www.ncbi.nlm.nih.gov/BLAST; version 2.0.6, released 16 September 1998) for both the *csIA* gene and its deduced peptide sequence was conducted. No significant homology with any previously published DNA sequences was found. However, a search using the deduced peptide sequence of the *csIA* gene revealed a high degree of homology between ChnAC and a putative secreted lyase from *Streptomyces coelicolor* (31) and a group of hyaluronate lyases from group B streptococci (20). In addition, homology was noted to the

chondroitin sulfate ABC endolyase from *P. vulgaris* and chondroitin lyase II from *B. thetaiotaomicron* in the C-terminal region of ChnAC, a region that has been suggested to be the active site of chondroitin sulfate ABC endolyase (35). Finding these sequence similarities was not surprising because all of these enzymes are also polysaccharide lyases. Therefore, ChnAC may have arisen from the same ancestor as these lyases and may have a similar three-dimensional structure and reaction mechanism.

A BLASTX search conducted for the *csIB* gene and its deduced amino acid sequence showed no significant homology to any other previously published DNA sequence. However, the deduced amino acid sequence revealed significant homology to a polysaccharide lyase, alginate lyase from *Pseudomonas* sp. (24), in the N-terminal region of both proteins. The ChnB protein might share structural similarities and an evolutionary relationship with the alginate lyase.

F. heparinum ChnAC and ChnB shared no homology at either the DNA or the peptide level. This dissimilarity is consistent with data from Cygler's laboratory (Fethiere et al. and Huang et al., submitted), in which the determined crystal structures of the two chondroitinases were very different. Detailed structure and function studies will be performed by using the sequence data reported herein.

The role of the glycosylation of these chondroitinase enzymes from *F. heparinum* remains unclear. The unglycosylated forms of both enzymes remain active, as was shown in the gene expression studies of both the *csIA* and *csIB* genes in *E. coli* (Table 3). Similar results were observed when the *hepA* and *hepB* genes were expressed in *E. coli* (34, 40). However, whether or not the absence of this glycosylation is of consequence to the specific activity or proper folding of these enzymes is uncertain. In addition, it has been suggested that protein glycosylation, especially O glycosylation, is related to protein stability (5) and perhaps this is the function of this posttranslational modification in *F. heparinum*. It seems that the nonglycosylated form of rChnAC produced by *E. coli* was not as stable as the native, glycosylated form from *F. heparinum*. This was not the case for rChnB from *E. coli*. The glycosylated and nonglycosylated forms had similar stabilities when subjected to heat denaturation. However, the results from this experiment are inconclusive because the enzymes purified from *E. coli* were less pure, with different modifications at their N termini. Further biochemical studies of the nonglycosylated forms of these enzymes from *F. heparinum* are needed to provide more conclusive answers to this question. We are currently working on creating a genetic system for *F. heparinum* which will be used to address these questions.

Expression of the chondroitinase enzymes in *E. coli* will provide an efficient means for their production. The ChnB activity measured in the *E. coli* cell extracts was higher than that in *F. heparinum*, as shown in Tables 3 and 4, even when *F. heparinum* was grown in a medium containing chondroitin sulfate A as the sole carbon source. Expression of ChnB reached 62.5 mIU ml⁻¹ A₆₀₀ unit⁻¹ in *E. coli*, compared to 29.4 mIU ml⁻¹ A₆₀₀ unit⁻¹ in *F. heparinum*. Slightly lower levels of expression of the *csIA* gene were found in *E. coli* than in *F. heparinum*, i.e., activities of 46.9 and 79.5 mIU ml⁻¹ A₆₀₀ unit⁻¹, respectively. However, the high biomass of *E. coli* fermentation and well-established techniques will offset these slightly lower specific activities observed in the expression of the *csIA* gene in *E. coli*. The findings of this study will provide an alternative source for the chondroitinase enzymes' production.

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