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Enzymatic and Molecular Properties of the *Clostridium tertium* Sialidase

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Clostridium tertium **metabolizes sialoglycoconjugates *via* a secreted sialidase [EC 3.2.1.18] and an intracellular acylneuraminate pyruvate lyase [EC 4.1.3.3]. The sialidase was enriched 1,900-fold from the culture medium with a specific activity of 0.7 U per mg protein. It exhibits a temperature optimum of 50°C and tolerates mercury ions at relatively high concentrations (50% inhibition at 5.2 mM Hg^{2+}). The sialidase gene was detected on two restriction fragments (*Hin*cII, *Hin*dIII) of chromosomal DNA and their correct recombination resulted in an active enzyme expressed by *Escherichia coli*. The structural gene is represented by 2,319 bp encoding a protein of 773 amino acids with a molecular mass of 85.4 kDa. The first 28 amino acids possess the character of a signal peptide. The protein reveals a FRIP-region and four Asp-boxes common in all bacterial sialidases. It has 42.6% identical amino acids when compared with the sialidase of *Clostridium septicum* and 64.8% with a sialidase gene amplified from *Macrobdella decora*. A further open reading frame was detected 30 bp downstream from the sialidase gene, which exhibits significant homology with acylneuraminate pyruvate lyases. For the first time, both genes were found in close proximity on a bacterial chromosome, probably being part of one operon.**

Key words: ***Clostridium tertium*****, sialidase gene, sialidase properties, phylogeny of sialidases, putative acylneuraminate pyruvate lyase gene.**

Sialidases (exo-α-sialidases, neuraminidases, EC 3.2.1.18) hydrolyse sialic acids that are α-glycosidically and terminally linked to the carbohydrate chains of glycoproteins or glycolipids or to oligosaccharides. Sialic acids are derivatives of neuraminic acid, an amino sugar with nine carbon atoms in pyranose configuration. These molecules occur in the deuterostomate lineage from echinoderms to man and are involved in many biological events, for example, cell recognition, adhesion or repulsion events, and the masking of cell surface structures (*1, 2*). Sialidases are key enzymes in sialic acid metabolism and are involved in the turnover of sialoglycoconjugates in animals. They are also widely but irregularily distributed among viruses, bacteria, fungi, and protozoa, which in most cases lack sialic acid-containing substrates.

As many of these sialidase producers prove to be pathogens, this enzyme is thought to enhance pathogenicity, *e.g.*, by acting as a spreading factor (*3*). This was shown for the sialidases of *Corynebacterium diphtheriae* (*4*) and some Clostridia (*5, 6*). The pathogenicity of viral sialidases was described by Huang *et al.* (*7*) and Maeda *et al.* (*8*). Since the sialidases of many microorganisms are secreted, they may provide the bacteria with nutrients and thus support their growth, because the sialic acids liberated can provide carbon and energy. Correspondingly, mutants of *Bacteroides fragilis* that lack a functional *nanH* exhibit a reduced growth rate (*9*), and *Micromonospora viridifaciens* was shown to produce sialidase only when either the substrate colominic acid or the product *N*-acetylneuraminic acid (Neu5Ac) was added to the culture medium (*10*). The occurrence of acylneuraminate pyruvate lyase ("aldolase," EC 4.1.3.3), which is involved in the further sialic acid catabolism in, *e.g.*, *Clostridium perfringens*, again indicates a nutritional importance of the sialidase. Both enzymes of *C. perfringens* are induced by free or glycoconjugate-bound sialic acids (*11*). Sialidase and acylneuraminate pyruvate lyase were also found in *Clostridium tertium* (*12*).

The genes for sialidases are irregularily distributed among microorganisms, and therefore closely related species like *Clostridium sordellii* and *C. bifermentans* (*13*), or even single strains of one species, *e.g.*, *C. butyricum* (*14*) and *Salmonella typhimurium* (*15*), are differentiated by their ability to produce sialidase. Therefore, the production of sialidase seems not to be essential for these microorganisms, but might be advantageous for nutrition, especially after the consumption of other carbon sources, and for virulence.

The facultative anaerobic, Gram-positive and sialidase-producing (*16*), rod-like bacterium *C. tertium* was shown to be only slightly pathogenic in man, but may aggravate persisting infections in animals (*17-19*). *C. tertium* can be isolated from soil, but also from the intestinal tract of animals and man.

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Abbreviation: *nanH*, sialidase gene.

At the beginning of this work, sialidase genes were cloned and sequenced from different clostridial species, namely, *C. perfringens* (*20, 21*), *C. septicum* (*22*), and *C. sordellii* (*23*). Two kinds of conserved sequence motifs were determined. One is a motif named "Asp-box," represented by the amino acid sequence SXDXGXTW, in which aspartate is highly conserved. In bacterial sialidases, this motif is repeated four or five times (*24-26*), while in eucaryotic sialidases it appears at least twice (*27-31*). The second conserved sequence motif is positioned near the N-terminus of the enzyme and was named FRIP, due to the exhibition of the conserved amino acids arginine and proline (*32*). Information on both sequences was valuable for the cloning strategy of the *C. tertium* sialidase.

The sialidase of *C. tertium* was tested for its biochemical properties after enrichment and found to show peculiar characteristics when compared with other clostridial sialidases. To elucidate whether similar characteristics of various sialidases could be traced back to similarities on a molecular level, and to explain some unique properties of the sialidase of *C. tertium*, the corresponding gene of strain DSM 2485 was cloned and sequenced. It was aligned with other clostridial sialidases to demonstrate the relationship among these enzymes, and to localize the amino acid exchanges, which may be responsible for its high tolerance to mercury ions.

RESULTS

Sialidase Production—Highest sialidase amounts were produced during 24 h of growth at 37°C (100%) by the *C. tertium* strains tested; they were lower at 40°C (83%) and at 20°C (17%). Prolonged incubation of the cultures for 48 or 72 h reduced the differences in yield at the three temperatures but led to an overall reduction of enzyme activity (about 80%) in the culture medium. Of the three strains tested, DSM 2485 produced the highest amounts of sialidase (7.9 mU/ml) in BHI supplemented with SGP, followed by strain DSM 662 in CMM (5.3 mU/ml) and DSM 748 in HI+SGP or BHI+SGP (2.7 mU/ml). In THB, or media without SGP, activities did not exceed 0.5 mU/ml.

Activity of Acylneuraminate Pyruvate Lyase—Culture of *C. tertium* DSM 2485 cells for 24 h at 37°C followed by enzymatic lysis of the harvested cells yielded 104 mU of lyase activity per gram of wet cells.

Sialidase Enrichment—The enzyme was completely secreted into the medium, as no increase in sialidase activity was measured in the supernatant of cells disrupted by treatment with glass beads. The sialidase of strain DSM 2485 was enriched 10-fold from culture supernatant by ion-exchange chromatography as a first step, resulting in a specific activity of 4 mU/mg and a yield of 11%. A further 48-fold enrichment was obtained by gel-filtration on Sephadex G 75. This step yielded 6% of initial enzyme activity with a specific activity of 190 mU/mg. Subsequent FPLC-gel-filtration led to 4-fold enrichment with an overall yield of 1.2% and a specific activity of 700 mU/mg protein. Commonly, the sialidase was enriched 1,900-fold and yielded 95 mU from 1 liter of culture supernatant by three purification steps.

Properties of the Sialidase—The molecular size of 56,000 of the enzyme was determined by HPLC-gel-filtration. A similar value of 52 kDa for the molecular mass was obtained by SDS-PAGE. Silver-staining of the gel revealed two further minor protein bands of 29 and 14 kDa to be present in the preparation.

The sialidase activity slightly increased with temperature (5% at 5°C), until reaching its maximum value at 50°C (100%). Higher temperatures led to a steep decline in activity (1% residual activity at 60°C).

The enzyme was highly active over a broad pH-range from 4.5 to 8, exhibiting a maximum at pH 5.5. Below pH 4.5, the activity markedly decreased (8% at pH 4.0).

The purified sialidase was not significantly reduced in activity during ten cycles of freezing and thawing. The common sialidase inhibitor Neu2en5Ac reduced enzyme activity only to 50% at a concentration of 5 mM. The addition of the sialidase reaction product Neu5Ac at 3 mM resulted in 91% of residual activity. The divalent cations Ca^{2+}, Mg^{2+}, and Co^{2+} slightly increased sialidase activity up to 120% when applied at concentrations of 0.5 to 10 mM. Cu^{2+} at a concentration of 2 mM and Hg^{2+} at 5.2 mM reduced the sialidase activity to 50%. Zn^{2+} inhibited the activity insignificantly (5% at 3 mM). EDTA also had no significant effect (105% at 1 mM).

Among the natural substrates hydrolyzed by the *C. tertium* sialidase, sialic acids were released in the highest rate from native BSM (100%), followed by colominic acid (87%), sialyl-α2,3-lactose (84%), gangliosides+Triton CF-54 (62%), saponified (de-*O*-acetylated) BSM (60%), sialyl-

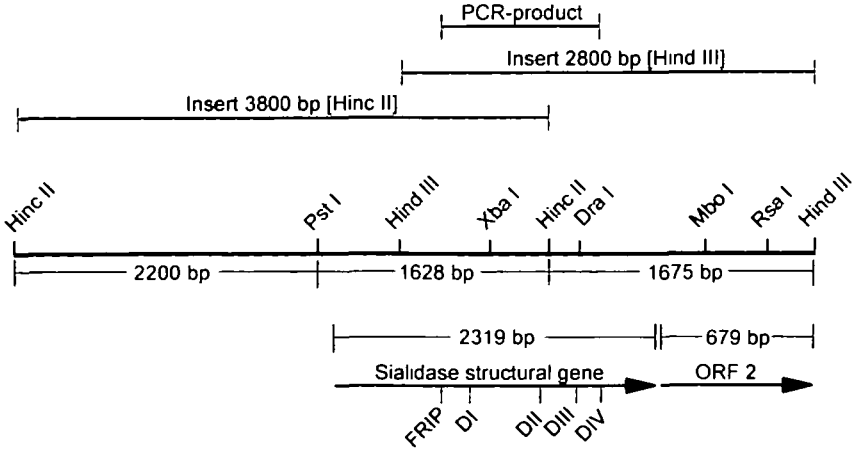

Fig. 1. **Restriction map of two overlapping restriction fragments cloned from chromosomal DNA of *Clostridium tertium* DSM 2485, which bear the complete sialidase gene and a second open reading frame (ORF 2) predicted to encode the main part of an acylneuraminate lyase gene.** The locations of the sialidase-typical sequence motifs (FRIP-region and Asp-boxes D I-IV) used to generate a PCR-product are indicated.

α2,6-lactose (43%), fetuin (16%), and gangliosides without detergent (10%).

Cloning of the Sialidase Gene—The yield of DNA from 1 g wet *C. tertium* cells was 1.68 mg. The purity of the isolated DNA is described by the relative absorption A_{260}: A_{230}:A_{280}, which was 1:0.51:0.57. DNA-amplification by PCR with the oligonucleotides *A* and *B* led to two products with sizes of 670 and 900 bp, respectively. Blunt-end ligation with *Hinc*II-digested pUC-vectors and subsequent sequencing suggested these products to be part of a sialidase gene, as Asp-boxes were found in the sequences (Fig. 1), as well as further amino acids conserved in clostridial sialidase proteins (Fig. 2). The 670-bp product spanned the sialidase gene from the FRIP-region down to Asp-box III. The 900-bp product spanned the gene from the FRIP-region down to Asp-box IV and contained the entire sequence of

```
C.t.  M-YslIkksIsTiAl-----------STL----------------AItsLpTysvSSqtTeEygarky  40
C.se. MN-------------------------------KKkImsIL-V--SAf--LiT-NLSSNiIFADIkenv  32
C.p.l MNYkgItl-IlTaAmvisgnyvlvkgSTLdsgknnsGyeI-KVnnS------e-NLSSlGeykDI----  57
C.p.s M-------------------------------------------------CNkNnTFE------   9
C.so. M-------------------------------KKfI-kILKVlsmAI-vLsa--CNiNGIFA------  27

C.t.  fINnnıE-Nik-nIenKs--fdlIQnlnTkıLekEnıEtlsgtvvdftk-------------------  85
C.se. yINqysEgNrsqpIaeKlvprseIQasaTsaLtgEgpEkaidgntstlwhtpwagvdiqinpqsltlklg 102

C.se. ktrnissicvtprqegtngmitdykıysgddviaegkwksdssdkyvvfdnpistdnirıeaistvgden 172

C.t.  ---------EatsnstiPngLiiEkSNinItaGkG----YDLssemgSEYvKaLEkGTIIVsykSTsNNs 142
C.se. nkhasiaevEvyeladtPvkLa-ESnNkvInnGNGgNyegDi-----SEi-slLEEGTaIiRF--T-NN- 231
C.p.l ------------------nL--ESS-------NasNitYDL-----ekY-KnLdEGTIvVRFnSkdse-  92

C.t.  ıQSLvSIgNNtsgNrdrHFHiYiTNtGeVGmELRNtdsvlkytlG-rpaAvrSiyKn-NlvfNTIAFKAd 210
C.se. gsSLfSISNNertN-e-HFHvYI-NgGaıGyELRkQ-------SG--NLAtgSvnKALNaGiNTIAFKAE 289
C.p.l mQSLlgISNsktkN-g-yFnfYvTNs-rVGfELRNQknegntqSGteNLvhmykdvALNdGdNTvAlKiE 159

C.t.  psNKqYKLFaNGEllaTlntdvykFiNdItGvNnvmLGgTvRdGvia-YpFgGtIGnvkIYNEiLtDeaL 279
C.se. -KgKGYsiyLNGEkIlTsssitaNFLstlEGLNtlsLGKTdRpsgSNEYNFtGeIdıfe.YskPLaD..L 358
C.p.l -KNKGYKLFLNGkıIkevkdtntsFLNnIEnLdsafıGKTnRyGqSNEYNFkGnIGImnj TEPLgvdYL 228

C.t.  KaeTGaTTygKNi-FyaGdstK------------SNYFRIPSLlsLrsGTVvsaaDARYGGtHDsksN-I 335
C.se. KerTGETTs-KdLpFpEGAVKTEPVDiFtPGeLgSNNFRIPALyTTKDGTVLASIDvRkGGGHDAPNN-I 426
C.p.l lskTGETka-KeevlvEGAVKTEPVDlFhPGfLNSsNyRIPALfkTKeGTliASIDARrhGGaDAPNNdI 297
C.p.s ----------KNLd-ishkPeplilFNKdnNiWNSkYFRIPniQlLnDGTiLtFSDIRYNGpdDHAY--I  66
C.so. ----------sNLn-ttnePqKttVFNKndNtWNaqYFRIPSLQTLaDGTmLAFSDIRYNGaeDHAY--I  84

C.t.  DIAfsKSlDGGiIWknptIpLqfnDYvarnidwprdsigknvqiqGsaSfIDpvLLeDkETkRlFifada 405
C.se. DTGikRSTDGGvTWDEGkIIL---DY------------------PGaSSaIDTsLLQDDETGRIFLiVTH 475
C.p.l DTAvrRSeDGGKTWDEGqIIm---DY------------------PdkSSviDTtLiQDDETGRIFLlVTH 346
C.p.s DIAsARSTDfGKTWs-------------------------------------------------------  81
C.so. DIGaAKSTDnGqIWD-------------------------------------------------------  99
                    1
C.t.  mPaGıGssNAsTGSGYKdIaGKkYmKLrwhqDgs-STYnySiRENGViYnDvTnlpTEYkiDgdyN-LYK 473
C.se. FaeGYGFgNskTGSGYveIeGKrYLKLlgaNDtI-yTV----RE-GVVY-DSnGeaTNYTvD-nnNeLYe 537
C.p.l FPlkYGFwNAglGSGfKnIsGKeYLcLydssgke-fTV----REN-VVY-DkdGnkTEYTtn-aLgdLfK 408
C.p.s ----------------------Y-niaMkNnRIDSTY--S-R---VM--DSTTViTN-TGRIILIAGSW 118
C.so. ----------------------Y-KtvMeNDRIDSTf--S-R---VM--DSTTVvTd-TGRIILIAGSW 136

C.t.  NGıallykqydynfsgttlletatnidvNmNVfykdSlfKVfpTtYLdMkYSDDeGETWSNl-NIvssfK 542
C.se. NGnr-------------------------IGNVllSnSPLKVmGTSfLsLIYSDDDGqTWSDPIDLNKeVK 583
C.p.l NGTK-----------------------IdNinSSTaPLKakGTSYinLVYSDDDGkTWSePqNINfqVK 454
C.p.s N-T-------------------------NGNWAmtTStr-RSDWSVQ-MIYSDDNGlTWSNKIDLTKdss 160
C.so. N--K-------------------------NGNWASSTtsL-RSDWSVQ-MVYSDDNGETWSDKvDLTtnka 178
                                                          2
C.t.  pensKFLvl-GPGvGkQIskGqYKGRLiVPlYsSsYa-ElGFm-----YSDDhGqTWN--yvAaDNR--- 600
C.se. tDWMrFLGT-GPGkGhQIKtGrYaGRLlfPVY---lTNasGFQSSAVIYSDDNGaTWNIGEtAtDgRlmd 649
C.p.l KDWMKFLGi-aPGrGiQIKnGehKGRIVVPVY---YTNEkGkQSSAVIYSDDsGKnWTIGEspnDNRkle 520
C.p.s KvKNQPSNTIGWLgGVGSGIVMdDGTIVMPaQISLRENNeNNYYSliIYSKDNGETWTMGNKVPnsn--- 227
C.so. riKNQPSNTIGWLaGVGSGIVMsDGTIVMPiQIaLRENNaNNYYSsVIYSKDNGETWTMGNKVPDpk--- 245
                                                          3
C.t.  -------nTgT----------TaEaQiVEMPDGsLKsylR-TG--SGviAevTSinGGeTW-sDrVtvPn 649
C.se. NGdrasaeTiTtntSggvgQLT-ECQVVEMPNGQLKMFMRNTGgnSGrvrIATSFDGGATW-EDdVvrDe 717
C.p.l NGkiinskTl----SddapQLT-ECQVVEMPNGQLKlFMRNl---SGYlnIATSFDGGATWdEt-VekDt 581
C.p.s ---------------------TSENMVIEL-DGALIMStRyDy--SGYRAaYIShDlGtTW-E--iYePL 270
C.so. ---------------------TSENMVIEL-DGALIMSsRNDG--knYRAsYISyDmGsTW-E--VYdPL 238
                                                          4
C.t.  mhttSY----GtQLSVINYaglIDGKe-AIIlSAPDsssa---RrNGkiwIGLIsDt--Gas--GInKYs 707
C.se. NiKEPY-----CQLSVINYSQKIDGKD-AIIFAiPDaNypn--RvNGTVRvGLIten--GsYENGEPrYd 777
C.p.l NvlEPY-----CQLSVINYSQKvDGKD-AvIFSnP--Nars--RsNGTVRIGLInqv--GtYENGEPKYe 639
C.p.s NgKIlTGkGSGCQGSFIKa-TtsnGHRiGlI-SAPKNTKGeYiRDNIaVYMIDFDDLSKGvqE1CIP--- 335
C.so. hnKISTGnGSGCQGSFIKv-TakDGHRlGfI-SAPKNTKGgYvRDNITVYMIDFDDLSKGirElCsP--- 353

C.t.  IEWKYcysVdSSnmGysYSCLTELPNGdIGLLYEkydSwsrneLhlkNILKYe----------------- 760
C.se. IEerYNKvVaPGtYG--YSCLSEmPNGeIGLfYEGrGSrqMSftrM-NI-dYLkadllqdvpaaniksyt 843
C.p.l fdWKYNKlVkPGyYa--YSCLTELsNGnIGLLYEGtpSeEMSyieM-N-LKYL----------------- 688
C.p.s ----YPEDGNklGGG--YSCLSFk-NnhLGIvYEANGNIEYqDLTp-YY-SlI----------------- 379
C.so. ----YPEDGNSSGGG--YSCLSFn-dGkLsILYEANGNIEYkDLTd-YYLS-I----------------- 397

C.se. tnsenniydpgdkislnvtfdqtvsligdrtitadiggkevlltlanskggseytfegtvpadisngnyt 913

C.se. itikgksglkivnvvnkvtditedrntglnvqvgeevqsvdktllqdlvdstsnlikedyteeswilyek 983

C.t.  ----------------------tfsiNelKQpisn*  773
C.se. alevankflvneiavqeevdaakptlENAyK*      1014
C.p.l ------------------------esgANK*       694
C.p.s --------------------------NKQ*        382
C.so. -------------------------EN-NKklk*     404
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Fig. 2. **Alignment of five clostridial sialidases.** The sialidase proteins of *Clostridium tertium* (C.t.), *Clostridium septicum* (C.se.), *Clostridium perfringens* (C.p.l, large and C.p.s, small isoenzyme), and *Clostridium sordellii* (C.so.) were aligned. Residues are numbered from each N-terminus. Capital letters are used for identical amino acids in each column. The FRIP-region and the Asp-boxes are underlined, and the Asp-boxes are numbered 1-4. Stops are represented by stars. Gaps are indicated by hyphens.

the 670-bp product. The hybridization of chromosomal DNA with a specific oligonucleotide probe *C*, representing a stretch of the sequenced PCR-products, led to the detection and cloning of a 2.8-kb *Hin*dIII fragment and a 3.8-kb *Hin*cII fragment with an overlap of 1,100 bp. Both clones were sialidase-negative. Isolation and digestion of the 3.8-kb fragment with *Hin*dIII and combinational ligation with the 2.8-kb *Hin*dIII fragment in a pUC-vector resulted in a 5.5-kb insert (Fig. 1). This insert revealed the entire sialidase structural gene sequence of 2,319 bp, which was placed in the EMBL data base under the accession number Y08695. The rearrangement of the gene by correct ligation of its fragments led to *Escherichia coli* clones that expressed sialidase activity. The recombinant enzyme exhibited a comparable size on FPLC-gel-filtration to that of the natural sialidase, as well as the same high temperature optimum.

Analysis of the Sialidase Gene—The G+C-content of the *C. tertium* sialidase gene was 25.9 mol%. Upstream from the sialidase structural gene, a non-coding region was observed that exhibits stop-codons in all three reading frames, as well as an inverted repeat. A ribosome-binding site could be predicted 3-12 bases upstream from the start-codon. The entire gene encompasses 2,319 bp, corresponding to 773 amino acids and a calculated molecular mass of 85.4 kDa for the unprocessed protein. The start-methionine is followed by 4 hydrophobic, 2 negatively charged, and 22 further hydrophobic amino acids. The amino acid sequence of the sialidase of *C. tertium* is shown in an alignment with four further clostridial sialidase proteins (Fig. 2). The percentage of identical amino acids in each pair of sialidases compared is shown in Table I.

The Predicted Acylneuraminate Pyruvate Lyase Gene—Unexpectedly, 30 bp downstream from the sialidase gene a further open reading frame (ORF 2) was detected on the *Hin*dIII fragment (Fig. 1). The interspace region exhibits no terminating sequences but encodes a typical ribosome-binding site 4-13 bp upstream from the start ATG of ORF 2. The *Hin*dIII fragment contains 679 bp of ORF 2 encoding 226 amino acids of a protein which shows significant (29.2-33.5%) homology with acylneuraminate pyruvate lyases (Table II). The mol% G+C was calculated as 30.0. No signal peptide was found.

DISCUSSION

Sialidase Production and Enrichment—The amount of sialidase (7.9 mU/ml) in the culture supernatant is in the range observed for other clostridial sialidases (*33*). For the first time, the production of sialidase was correlated with the temperature of cultivation. The result is not surprising, as the highest amounts were produced at 37°C, the common temperature for optimum growth of Clostridia, and not at lower or higher temperatures, although the *C. tertium* sialidase shows maximum activity at 50°C.

The results of the enrichment steps (1.2% yield and a specific activity of 0.7 U/mg protein) indicate that the enzyme is less efficiently separated from contaminating proteins by methods that have successfully been applied for the purification of other sialidases (*33-37*). The particularly high loss of activity during ion-exchange chromatography suggests that significant quantities of the enzyme may have irreversibly been bound to the resin. The impurities observed after SDS-PAGE (data not shown) and silver staining indicate that the specific activity might be slightly higher for the pure enzyme.

Sialidase Properties—The molecular masses of the native and the denatured enzyme proteins are very similar, which points to a monomeric form. This behaviour corresponds to the large sialidase isoenzyme of *C. perfringens* (*33*), while the otherwise very similar sialidase of *C. septicum* is a trimer (*36*). The higher value of 85.4 kDa calculated for the protein deduced from gene information when compared to the average value of 54 kDa obtained from SDS-PAGE and gel filtration indicates a further processing of the sialidase.

The values obtained for the relation between temperature and activity describe a typical denaturation curve. Similar behaviour was observed for the large sialidase isoenzyme of *C. perfringens* (*33*) with a comparably high optimum at 55°C. For most sialidases, the optimum temperature is around 37°C, and the activity change with temperature is typically a bell-shaped curve. The reason for this thermostability was not immediately apparent from comparison of the primary structures, and further studies are needed.

Maximum activity at an acidic pH is a common property of conventional sialidases, in which they differ from the trans-sialidases of trypanosomes, which show an optimum at neutral pH (*38*).

The sialidase of *C. tertium* is stable against freezing and thawing, as are the enzymes of *C. chauvoei* (*35*), *C. septicum* (*36*), and *C. sordellii* (*37*). The inhibition of sialidase activity by Neu5Ac2en and Neu5Ac is in the range known for most sialidases (*39*). The slightly positive effect of most divalent cations on activity may be due to ionic strength and may not be specific, as a similar result was measured with the chelator EDTA. However, the concentration needed for the inhibition of *C. tertium* sialidase activity by mercury ions was amazingly high. It was 100-fold higher than was determined for the *C. septicum* sialidase (*36*), which at that time was the most tolerant

TABLE I. **Similarities among clostridial sialidase proteins.** Percentages of identical amino acids after alignment and normalizing protein sequences to 100% are shown.

	C. tertium	*C. septicum*	*C. perfringens* L	*C. sordellii*
C. septicum	42.6			
C. perfringens L	36.5	56.0		
C. sordellii	31.0	22.9	29.0	
C. perfringens S	22.7	35.0	28.7	71.3

L, large isoenzyme; S, small isoenzyme.

TABLE II. **Similarities among six acylneuraminate lyase proteins produced by the genera *Clostridium*, *Escherichia*, *Haemophilus*, *Trichomonas*, and *Homo*.** Percentages of identical amino acids after alignment and normalizing protein sequences to 100% are shown.

	C. tertium	*C. perfringens*	*E. coli*	*H. influenzae*	*T. vaginalis*
C. perfringens	32.26				
E. coli	33.48	37.19			
H. influenzae	31.05	73.26	35.86		
T. vaginalis	29.73	68.06	36.55	79.52	
H. sapiens	29.23	25.34	26.53	28.08	27.40

clostridial sialidase known. The mercury tolerance of the *C. tertium* sialidase could be traced back to the amino acid position 657 (Fig. 2) in the primary structure, at which a cysteine residue occurring in the four Hg^{2+}-sensitive sialidases is replaced by threonine. A second argument comes from recent direct-site mutagenesis experiments with the small sialidase of *C. perfringens* (*40*). The change of cysteine 282 to serine at the homologous position increased the tolerance of this sialidase to mercury ions 140-fold.

The enzyme hydrolyses a broad spectrum of substrates at a high rate, which is a common property of the sialidases with higher molecular mass (*33*). The preferred cleavage of *O*-acetylated sialic acids of mucins (native BSM) compared to the saponified form is so far unexplained.

Sialidase Gene—The central part of the gene encoding the sialidase of *C. tertium* was successfully cloned after performance of PCR with degenerate primers against the conserved sequences FRIP and Asp-box III (Fig. 1). This strategy was used for the first time with success for the amplification of a sialidase-coding region. Sequencing of the PCR-products allowed the construction of a specific oligonucleotide *C*, which led to the cloning of two overlapping fragments of a chromosomal multiple digest. The presence of the complete gene is indicated by an active enzyme produced by *E. coli* cells transformed with the combined fragments and by the alignment (Fig. 2), demonstrating high similarities with the large sialidases of *C. perfringens* and *C. septicum*. Gene expression points to the presence of a promoter, which is used by the highly unrelated *E. coli* species. Secretion of the sialidase by *C. tertium* is explained by the putative signal peptide detected by its hydrophobic character. The Asp-boxes typical of all microbial sialidases (*25*) occur fourfold in the protein. As is shown in the alignment of the five clostridial sialidase proteins (Fig. 2), further motifs could be identified as being conserved, *e.g.*, the FRIP-region stretching from amino acids 305 to 307, as well as Arg 305, which is postulated to be involved in substrate binding (*41*), and a glycine-rich region between Asp-boxes II and III. Additional stretches of amino acids of the large sialidases are thought to be responsible for the extended substrate specificity, compared to the small sialidases of *C. sordellii* and *C. perfringens* (*42*). The *C. tertium* sialidase protein exhibits such additional, unique stretches, even compared to the large enzymes of *C. septicum* and *C. perfringens*, *e.g.*, the amino acids spanning positions 362 to 379 and positions 478 to 501. These sequences, together with the change of Cys to Thr 657, make the *C. tertium* enzyme unique among all other known clostridial sialidases. This is also indicated by its relationship with other clostridial sialidase proteins, shown by its separation near the root in the diagram (Fig. 3). As shown in Table I, the enzyme of *C. tertium* is related to the sialidase of *C. septicum* (42.6% identical amino acids), followed by the large enzyme derived from *C. perfringens* (36.5%). The latter two enzymes are more closely related with each other through 56% identical amino acids than with the *C. tertium* sialidase. The very closely related sialidases from *C. perfringens* (small isoenzyme) and *C. sordellii* (71.3% identical amino acids) are even more distinct from the *C. tertium* enzyme, with which they have respectively only 22.7% and 31% identical amino acids. The *C. tertium* sialidase shows highest similarities of 64.7% with the protein and 59.6% with the gene of a recently published sialidase amplified from the leech *Macrobdella decora* (*43*). This similarity implies a recent gene transfer among pro- and eucaryotic organisms, or the presence of a sialidase-producing bacterium in this leech, the DNA of which has been copurified and might be highly related to *C. tertium*.

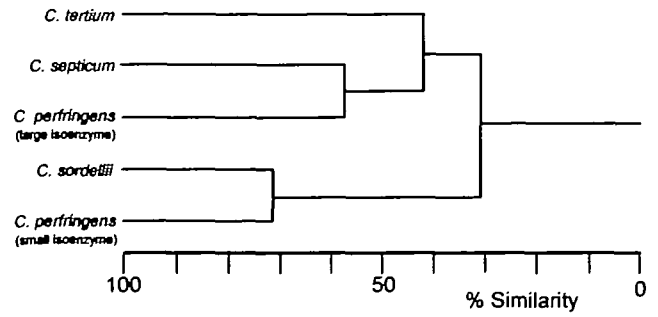

Fig. 3. **Relationship of clostridial sialidases.** Similarity values were combined by the average-linkage method of Anderberg (*67*).

The low G+C-content of 25.9 mol% of the sialidase gene agrees well with the value of 24–26 mol% described for the chromosomal DNA of *C. tertium* (*44*), thus indicating that the gene has persisted in this species for a long period since its possible acquisition by horizontal gene transfer, as is postulated for sialidase genes (*32*).

The protein deduced from the partial ORF 2 perfectly matches with acylneuraminate pyruvate lyases from *E. coli* (*45*; P06995), *Haemophilus influenzae* (*46*; L44787), *Trichomonas vaginalis* (*47*; U35878), *C. perfringens* (*48*; Y 12876), and a fragmentary sequence from *Homo sapiens* (*49*; W79930). The alignment (Fig. 4) of the *C. tertium* sequence with these lyase sequences shows a close relationship, which is indicated by 42 amino acids being identical in all, and further amino acids being conserved in most primary structures of this enzyme at respective positions. Similarity values calculated from pairwise comparisons of the protein sequences are shown in Table II. No close relationship of the *C. tertium* protein to one of the other lyases could be determined, but its similarity values are even higher than those calculated for the human lyase. The data were used to form a dendrogram (Fig. 5), which shows a cluster of three highly related lyases (*H. influenzae, C. perfringens, T. vaginalis*), with those of *E. coli, H. sapiens*, and the *C. tertium* ORF2 branching at lower similarities. As these facts are not in agreement with the relationship of the organisms, horizontal gene transfer is indicated also for acylneuraminate pyruvate lyases. Interestingly, the fragmentary *H. sapiens* sequence shows its highest similarity with that of *C. tertium* (Table II). The latter has a branching point at the lowest similarity value compared to the proteins of the other microorganisms. Both findings additionally indicate that the *C. tertium* lyase represents an ancient form of this enzyme type.

During comparisons of *C. tertium* and *M. decora* sialidases, a second open reading frame was also detected on the latter sequence published by Chou *et al.* (*43*). This ORF exhibits 79% identity of deduced amino acids and 80% of nucleic acids with the postulated *C. tertium* acylneuraminate pyruvate lyase. The distance between the two genes was 152 bp, a putative ribosome-binding site was found 8–16 bp upstream from the start-ATG of ORF 2. This finding

```
C.t.                                MRNLEKYKGIIPAFYACYDDEGKISPERTQMFTQYLIDKG-VKG  43
C.p.                                   MKGIYSALLVSFDKDGNINEKGLREIIRHNIDVCKIDG  38
E.c.                                 MATNLRGVMAALLTPFDQQQALDKASLRRLVQFNIQQG-IDG  41
H.i.                                  MRDLKGIFSALLVSFNEDGTINEKGLRQIIRHNIDKMKVDG  41
T.v.  MFVFLAISMAKSAAAEATTGPKGKSAKSLKGLFSALLVSFNEDGTINEKGLREIVRYNIDKMKIDG  66

C.t.  LYVCGSSGECIYQSKEERKITLENVMKVARGKITIIAHVGCNNTRDSEELAEHAESIGVDAIASIP 109
C.p.  LYVGGSTGENFMLSTDEKKRIFEIAMDEAKGQVKLIAQVGSVNLKEAVELAKFTTDLGYDAISAVT 104
E.c.  LYVGGSTGEAFVQSLSEREQVLEIVAEEGKGKIKLIAHVGCVTTAESQQLAAASAKRYGFDAVSAVT 107
H.i.  LYVGGSTGENFMLSTEEKKEIFRIAKDEAKDQIALIAQVGSVNLKEAVELGKYATELGYDCLSAVT 107
T.v.  LYVGGSTGENFELSTEEKKQIFRIAKDEAKDQVALIAQVGSINIHESIELGKYATELGYNCLSAVT 132
H.s.                                                    ...QQEIGADGIAVIA

C.t.  PIYFHLPDYSIAEYWNDISNAA-PNTDFIIYNIPQLAGVGLGIN--LYKQMLKNPRVIGVKNSSMP 172
C.p.  PFYYKFDFNEIKHYYETIINSVD-N-KLIIYSIPFLTGVNMSIE--QFAELFENDKIIGVKFTAAD 166
E.c.  PFYYPFSFEEHCDHYRAIIDSAD-GLPMVVYNIPALSGVKLTLD--QINTLVTLPGVGALKQTSGD 170
H.i.  PFYYKFSFPEIKHYYDTIIAETG-N-NMIVYSIPFLTGVNMGIE--QFGELYKNPKVLGVKFTAGD 169
T.v.  PFYYKFTFPEIKNYYNTIVNATG-M-NMIVYSIPALTGVSMTAD--QFGELFENPKIIGVKFTAGD 194
H.s.  PFFLKPWTKDILINFLKEVAAAAPALPFYYYHIPALTGVKIRAEELLDGILDKIPTFQGLKFSDTD

C.t.  VQDIQMFKDISGDESVVF-NGPDEQFVAGRIMGADGGIGGTYAVMPELFLAADKA...          226
C.p.  FYLLERMRKAFPDKLIFA--GFDEMMLPATVLGVDGAIGSTFNVNGVRARQIFEAAQKGDIETALE 230
E.c.  LYQMEQIRREHPD-LVLY-NGYDEIFASGLLAGADGGIGSTYNIMGWRYQGIVKALKEGDIQTAQK 234
H.i.  FYLLERLKKAYPNHLIWA--GFDEMMLPAASLGVDGAIGSTFNVNGVRARQIFELTKAGKLAEALE 233
T.v.  FYLLERVKRAYPDHLIWA--GFDEMMLPACSLGIDGAIGSTFNVNAKRARQIFELSKAGKYDEALE 258
H.s.  LLDFGQCVDQNRQQQFAFLFGVDEQLLSALVMGATGAVGSTYNYLGKKTNQIVGGF*TKGLLFRNE

C.p.  VQHVTNDLITDILNNGLYQTIKLILQEQGVDAGY-CRQPMKE-ATEEMIAKAKEINKKYF*       288
E.c.  LQTECNKVIDLLIKTGVFRGLKTVLHYMDVVSVPLCRKPFGPVDEKYQPEL-KALAQQLMQERG*  297
H.i.  IQHVTNDLIEGILANGLYLTIKELLKLEGVDAGY-CREPMTSKATEEQLAKAKDLKAKFLS*      293
T.v.  VQHVTNDLIAGILSNGLYLTIKELMRLDGVDAGY-CREPMTKALTPAQVAFAKQLKEKYLL*     318
H.s.  LSVLYPEI...
```

Fig. 4. **Alignment of the *C. tertium* protein encoded by ORF 2 and five acylneuraminate lyases of bacterial, trichomonal, and human origin.** C. t., *Clostridium tertium*; C. p., *C. perfringens*; E. c., *E. coli*; H.i., *Haemophilus influenzae*; T. v., *Trichomonas vaginalis*; H.s., *Homo sapiens* putative lyase. Conserved amino acids are marked by bold print. Gaps are indicated by hyphens. Stars represent stop codons. Points give notice of incomplete sequence.

indicates that the sequence amplified from *M. decora* is of bacterial origin, and that the unknown bacterial species might be closely related to *C. tertium*.

The sialidase of *C. tertium* looks well adapted to its environment (soil and intestine of animals), as can be predicted from the ranges and optima of its temperature and pH dependence, its relatively high tolerance of heavy metal ions, the broad spectrum of substrates hydrolysed, and the different kinds of glycosidic linkages recognized. Therefore, and also based on the good correspondence of the G+C-content of the sialidase and the acylneuraminate pyruvate lyase gene with that of the bacterial genome, it can be postulated that both genes of *C. tertium* were obtained at an early stage of evolution. Another argument for this point comes from the short distance between the two genes representing a functional unit in sialic acid catabolism. The combination of the genes in one operon can be considered the result of a long-lasting optimization process leading to an evolutionary advantage for the bacterium.

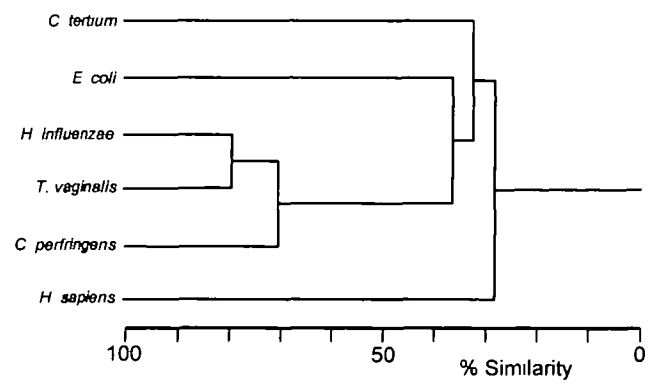

Fig. 5. **Relationship of six acylneuraminate lyase proteins calculated from similarity values by the average-linkage method of Anderberg (*67*).**

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—The *C. tertium* strains DSM 2485, DSM 748, and DSM 662 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM, Göttingen). The bacteria were multiplied and tested for maximum sialidase production in the complex media Todd Hewitt Broth (THB), Cooked Meat Medium (CMM), Heart Infusion (HI), or Brain Heart Infusion (BHI; Difco, Detroit), which were supplemented for sialidase induction with sialoglycopeptides (SGP) at a final concentration of 0.1 mM bound sialic acids. The SGP were prepared from edible bird nest substance (*50*). The strains were grown under anaerobic conditions in screw-cap flasks at 20, 37, or 40°C. Purity controls were performed by plating the cells on THB+1.8% agar, which was incubated at 37°C in a Gaspack Anaerobic System (BBL Microbiology Systems, Beckton Dickinson & Co., USA).

Escherichia coli JM 101 (*51*) was purchased from Pharmacia Biosystems (Freiburg) and was propagated at 37°C in Luria Bertani (LB) broth, or on LB+1.8% agar. Alternatively, 2×YT broth containing 16 g/liter Bacto-Tryptone (Difco), 10 g/liter yeast extract (Serva, Heidelberg), and 5 g/liter NaCl (Merck, Darmstadt) was used. Media were supplemented with 100 μg/ml ampicillin for vector selection and maintenance.

Vectors—Vectors for cloning (pUC18, pUC19) and sequencing (M13mp18, M13mp19) were purchased from Gibco BRL (Eggenstein), Boehringer (Mannheim), or New England Biolabs (NEB; Schwalbach).

Assays—Sialidase activity was measured fluorimetrically (*52*) by cleavage of the synthetic substrate 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid (MU-Neu5Ac; Dr. Pallmann GmbH, München). In the standard assay, 80 μl of

reaction buffer (0.2 M sodium acetate buffer, pH 5.5), 10 μl enzyme solution, and 10 μl substrate solution (1 mM in water) were thoroughly mixed and incubated for 10 min at 37 or 50°C. The reaction was terminated by the addition of 900 μl of buffer (0.133 M glycine, 0.04 M Na_2CO_3, 0.06 M NaCl, pH 10). The released MU was measured at 450 nm in a filter-spectrofluorimeter (M 1000, Perkin-Elmer, Ueberlingen) with excitation at 365 nm. The instrument was calibrated with MU-standard solutions. One unit of enzyme activity was defined as 1 μM substrate hydrolysed per min.

Escherichia coli clones expressing sialidase activity were identified by their blue-white fluorescence under UV-light (360 nm) after being sprayed with 1 mM MU-Neu5Ac-solution in water.

The substrate spectrum of the sialidase was investigated with bovine submandibular gland mucin (BSM) used in natural and saponified (de-*O*-acetylated) form (*53*), bovine brain gangliosides (*54*) applied with or without 1% Triton CF-54 (Serva), sialyl-α2,3- and α2,6-lactoses prepared as described (*55*), colominic acid as an α2,8-linked polymer of sialic acids (Sigma, Deisenhofen), and fetuin (Sigma). The assay contained 10 μl of sialidase solution (representing 0.14 mU), 50 μl of 0.2 M sodium acetate buffer, pH 5.5, and 40 μl of the respective substrate at a final concentration of 1 mM bound sialic acids, which was determined by the microadaptation of the orcinol/Fe^{3+}/HCl-reaction (*56*). The amount of sialic acids released from these substrates was quantified with the microadaptation of the periodic acid/thiobarbituric acid assay (*57*). Samples containing free Neu5Ac in concentrations from 10 to 80 μg/ml were used to establish standard curves.

For the liberation and determination of acylneuraminate pyruvate lyase activity, a 1 liter culture of *C. tertium* DSM 2485 was harvested by centrifugation (10,000 × *g*, 20 min, 4°C) after 24 h of growth in BHI supplemented with SGP at 37°C under anaerobic conditions. After storage at −20°C the cells were resuspended in 30 mM Tris-HCl, pH 8.0, supplemented with 10 mM EDTA and with lysozyme; RNase T1 (Sigma), and DNase I (Boehringer) were added as described by Nees (*58*). The cell suspension was stirred for 30 min at 20°C, then sonicated for 30 min at 4°C. After centrifugation (20,000 × *g*, 1 h, 2°C), the supernatant was heat-precipitated and dialysed (*48*), then introduced into the assay for acylneuraminate pyruvate lyase (*59*).

The protein content in the culture supernatant was determined with the Bio-Rad protein assay according to the manufacturer's manual (Bio-Rad, Richmond). During sialidase isolation, the protein content was measured by absorption at 280 nm in a spectrophotometer. Absorption of 1 was set at 1 mg protein per ml (path = 1 cm).

Sialidase Isolation—The localization of the sialidase produced by the *C. tertium* cells was investigated by successive destruction of the cells. A suspension of 1 g of wet cells in 10 ml of 0.2 M sodium acetate buffer, pH 5.5, was repeatedly shaken for 30 s and 4,000 rpm with glass beads (0.1 mm in diameter) in a cell mill (Braun, Melsungen). During the intervals the suspension was stored on ice and samples were taken for the determination of sialidase activity by the standard assay.

As a first step of sialidase enrichment, the cells were separated from the culture medium (1 liter) by centrifugation (10,000 × *g*, 20 min, 4°C) and the pH of the supernatant was adjusted to 4.5. The solution was applied to an S-Sepharose fast flow column (Pharmacia, 4 × 15 cm) and the sialidase eluted with a linear gradient of 0–0.5 M KCl in 0.2 M sodium acetate buffer, pH 4.5. Active fractions were collected and concentrated to 2 ml by pressure dialysis at 4°C on ultrafilters (cutoff MW 10,000; Sartorius, Göttingen) in a 100-ml cell (Amicon, Witten). The concentrate was filtered on a Sephadex G-75 column (3.4 × 95 cm), which was equilibrated and run with 0.2 M sodium acetate buffer, pH 5.5. Fractions containing sialidase activity of 5 mU and more per ml were pooled and concentrated. As a third step, 100-μl aliquots of the sialidase solution were filtered on a Superose-12 column (HR10/30, Pharmacia) run by HPLC (LKB, Bromma) in the same buffer with a flow rate of 0.5 ml per min and a fraction volume of 0.25 ml. Fractions exhibiting a higher activity than 8 mU per ml were pooled and concentrated.

Sialidase Characterization—The size of the native protein was determined by HPLC-gel-filtration described above, with bovine serum albumin (MW 67,000) and ovalbumin (MW 45,000) as calibration standards. The molecular mass and the purity of the preparations was investigated by SDS-PAGE (*60*). Standards were bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carboanhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), and lactalbumin (14 kDa). Proteins were silver-stained (*61*).

The temperature dependence of the sialidase activity was measured by the standard assay at pH 5.5 for 13 temperatures between 4 and 60°C. The enzyme (0.06 mU at 37°C) was preincubated for 15 min at each temperature.

The sialidase activity (0.07 mU at pH 5.5) was measured at different pH values from 3.0 to 8.0 in steps of 0.5 by the standard assay at 37°C.

The stability of the enzyme was determined by ten cycles of freezing and thawing of a solution of 0.47 mU sialidase in 100 μl of 0.2 M sodium acetate buffer, pH 5.5. After each step, a sample was measured by the standard assay.

The sialidase inhibitors 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (Neu2en5Ac, Boehringer) and free *N*-acetylneuraminic acid (Neu5Ac) were introduced into the standard assay (8 mU of enzyme activity) in final concentrations of 0.01, 0.05, 0.1, 0.5, 1, 2, 3, 5, and 10 mM. Under the same conditions, heavy metal ions and EDTA were tested for their influence on sialidase activity. Final concentrations were 10 nM–0.1 mM for Hg^{2+}, 0.1–10 mM for Cu^{2+}, 1–30 mM for Ca^{2+}, Mg^{2+} and Mn^{2+}, 0.5–10 mM for Co^{2+} and Zn^{2+}, and 1 mM for EDTA.

DNA Methods—Chromosomal DNA of *C. tertium* DSM 2485 was isolated according to Marmur (*62*), modified by Gebers *et al.* (*63*). Cloning, preparation of plasmid DNA, ligation, agarose gel electrophoresis, and elution of DNA fragments were performed as described by Sambrook *et al.* (*64*). Bacterial cells were transformed by electroporation in a Gene-Pulser™ (Bio-Rad, München). Enzymes for molecular biology were purchased from Gibco BRL (Eggenstein), Boehringer Mannheim (Mannheim), or NEB (Schwalbach).

Design and Labeling of Oligonucleotides—Two oligonucleotides were derived from conserved sequences of clostridial sialidase genes (*1*) and were used as primers. Primer *A* represents the most conserved part of the FRIP-region of known clostridial sialidase genes, primer *B* is complementary to the most highly conserved Asp-box III. The latter was converted into a reverse sequence to allow the perfor-

mance of PCR in combination with primer *A*. The primers showed a different grade of degeneration at less conserved nucleotide positions. The oligonucleotides exhibited the following sequence: *A* = 5′T(G/T)(A/G) AAT (A/G/T) C(A/C/T) A(A/G)(T/G) (A/T)AT T(A/T)(C/T) AGA ATA CC 3′, *B* = 5′TTC (T/C)AT TGT CCA (A/T)GT (T/C) T(T/C) ACC ATT ATC3′. A third oligonucleotide *C* = 5′GTT GTA TCA GCT GCT GAT GC3′ represents a 20 bp sequence of the PCR-product derived later. It was used as a probe to detect fragments of restricted *C. tertium* DNA after Southern blotting and hybridization. All oligonucleotides were obtained from Eurogentec (Seraing). Oligonucleotide *C* was labeled according to Sambrook *et al.* (*64*) with $\gamma(^{32}P)$-dATP (Amersham, Braunschweig) using T4-polynucleotide kinase obtained from NEB.

Polymerase Chain Reaction—Amplification of the sialidase-encoding DNA region flanked by FRIP and Asp-box III was performed as follows: Hotstart for 5 min at 96°C, followed by 5 cycles of denaturation (95°C, 30 s, ramp time 60 s), annealing (37°C, 30 s, ramp time 60 s), and extension (72°C, 60 s). These first cycles were followed by 30 cycles of denaturation (95°C, 30 s, ramp time 60 s), annealing (46°C, 30 s, ramp time 150 s), and extension (72°C, 60 s). Finally, one terminal extension at 72°C for 300 s was carried out, followed by constant cooling to 15°C. *Taq* polymerase was obtained from Amersham. The reaction mixture contained 60 ng *C. tertium* DNA, 1 μl dNTP-Mix (10 mM), 5 μl *Taq* buffer (10-fold concentrated), 1 μl primer *A* (20 μM stock solution), 5 μl primer *B* (20 μM stock solution), 1 μl $MgCl_2$ solution (50 μM stock solution), water ad 47.5 μl, and 2.5 U *Taq* polymerase in a volume of 2.5 μl.

Blotting and Hybridization—DNA was transferred from agarose gels to nylon sheets (Hybond-N™, Amersham) using a Hybaid™ Vacuum Blotter (Biometra, Göttingen). *E. coli* colonies were transferred to nylon filters as described (*23*). The DNA was denatured and immobilized, and hybridization was performed according to Wallace *et al.* (*65*). Nylon sheets with immobilized DNA were prehybridized for at least 1 h at the appropriate temperature in sixfold (6×) concentrated standard saline citrate buffer (SSC) containing 5×Denhardts solution (*64*), 0.5% SDS, and 0.5 mg/ml yeast-RNA to reduce nonspecific binding of the probe. Hybridization was done overnight under identical conditions with probe *C* at 55°C. The filters were washed three times in 6×SSC for 20 min at 55°C, wrapped in plastic foil and exposed for 5 to 20 h on X-ray films (Fuji RX-100).

Sequencing and Analysis—Sequence analysis was carried out with the dideoxy chain-termination procedure of Sanger *et al.* (*66*). Single-stranded M13mp18 and M13-mp19 templates were prepared and sequenced with a Sequenase DNA-sequencing kit (USB, Bad Homburg) as recommended by the producer, with [α-^{35}S]dATP (Amersham) as a label. The products were separated at 56°C in a wedge-shaped polyacrylamide gel (8%, 7 M urea) by applying the LKB Makrophor sequencing system.

Sequence data were analyzed with the GENMON program, version 4.3 (GBF, Braunschweig) and compared with the EMBL 29.0/Swissprotein 20.0-database of this program. The amino acid sequences were aligned by eye. The percentage of identical amino acids between each pair of proteins was calculated by setting the number of comparable amino acids at 100%. The similarity values were calculated by applying the average-linkage method of Anderberg (*67*).

The sialidase sequence was placed in the EMBL database under the accession number Y08695.

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