

# Cloning and sequencing of a *Clostridium perfringens* sialidase gene

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*Escherichia coli* was transformed with pUC vectors containing Sau3A restriction fragments (RF) of *Clostridium perfringens* DNA. Two clones expressed sialidase activity when assayed with the fluorogenic substrate 4-methylumbelliferyl- $\alpha$ -D-N-acetylneuraminic acid. A synthetic oligonucleotide representing the N-terminus of the expressed enzyme hybridized with the clostridial insert and with a corresponding 2.1 kb Sau3A RF of the *C. perfringens* genome. The insert reduced to 1.4 kb, which still encoded active sialidase, has been sequenced. The structural gene encodes 382 amino acids representing an  $M_r$  of 42 770. A hydrophobic leader sequence is absent. Upstream from the initiation codon ATG, a GA-rich region is found and considered as the Shine-Dalgarno sequence. Homology with the N-terminus of the *Vibrio cholerae* sialidase gene and with viral sialidase sequences was not found.

Sialidase; Gene sequence; Gene expression; (*Clostridium perfringens*)

## 1. INTRODUCTION

Turnover of cell surface glycoconjugates in metazoan animals of the deuterostomate lineage is initiated by the cleavage of terminal sialic acids. The enzyme responsible (sialidase = neuraminidase, EC 3.2.1.18), obligatory in this phylogenetic branch, is also expressed in some viruses, bacteria and protozoa, which have close contact with animal hosts [1]. This may be due to the observation that sialidase-altered cell surfaces allow more direct contact of pathogens with the host and are advantageous to further damage of the cells by lytic enzymes [2]. In order to obtain information on the phylogeny and relatedness of sialidases, we

started investigations on bacteria of the genus *Clostridium*, which secrete large amounts of soluble enzymes. The sialidase genes might be easily accessible using DNA probes which represent the N-terminus of isolated and sequenced enzyme proteins, or by looking for sialidase expression of *E. coli* clones transformed with clostridial DNA RF. The latter was observed after cloning of Sau3A RF of a *C. perfringens* strain DNA in *E. coli*. Unexpectedly, the highly expressed gene product does not represent the previously described sialidase of this species [3], although it is encoded by the cloned clostridial insert. The functionally complete gene and the predicted amino acid sequence of this sialidase are presented here.

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**Abbreviations:** RF, restriction fragments; MU-Neu5Ac, 4-methylumbelliferyl- $\alpha$ -D-N-acetylneuraminic acids

The nucleotide sequence presented here has been submitted to the EMBL/GenBank database under the accession number Y00963

## 2. MATERIALS AND METHODS

*C. perfringens* strain A99, isolated from human gas gangrene infection, was a gift from Dr R. Hobrecht (Untersuchungsinstitut I der Bundeswehr, Kronshagen, FRG). *C. perfringens* type strain DSM 756 was purchased from Deutsche Sammlung für Mikroorganismen (Göttingen). Restriction enzymes, T<sub>4</sub> kinase, and Bal31 nuclease for insert reduction were from Gibco-BRL (Eggenstein, FRG). Vectors for cloning (pUC18, pUC19) and sequencing (M13mp18, mp19) as well as

*E. coli* strain JM101 were purchased from Pharmacia-LKB (Freiburg). Radiochemicals for hybridization ( $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ ) and sequencing ( $[\alpha\text{-}^{35}\text{S}]\text{thio-dATP}$ ), and nylon sheets (Hybond<sup>N</sup>) for Southern blotting [4] were obtained from Amersham Buchler (Braunschweig).

Clostridial cells were harvested by centrifugation ( $10000 \times g$ , 20 min,  $4^\circ\text{C}$ ) after 1:10 inoculation and multiplication for 6 h at  $37^\circ\text{C}$  in Todd-Hewitt broth (Difco, Detroit), supported by 0.1 mM sialoglycopeptides isolated from edible bird nest substance [3]. The sedimented cells were treated with 10 mg lysozyme (Serva, Heidelberg) and 0.1 mg proteinase K (Boehringer, Mannheim) per ml saline-EDTA (0.15 M NaCl, 0.1 M EDTA; pH 6.8) for 30 min at  $37^\circ\text{C}$ . Cells were lysed by addition of SDS to a final concentration of 2%. DNA was purified according to [5]. Cloning, plasmid isolation, electroelution, blotting and hybridization were performed as in [6].

Clones expressing sialidase activity were detected by spraying the colonies with 0.1 mM MU-Neu5Ac in 0.1 M acetate buffer, pH 6. Positive clones were visualised by the blue-white fluorescence of released MU under UV light of 360 nm.

Purified sialidase protein (unpublished) from one active clone was applied onto a 10% PAGE and electroblotted onto siliconized glass fiber (Glassybond, Biometra, Göttingen) for 3.5 h at 200 mA and  $4^\circ\text{C}$ . The 40 kDa band was excised and sequenced on a 470A gas-phase sequencer (Applied Biosystems, Pfungstadt, FRG) as described [7].

DNA probes were synthesized applying an automated DNA synthesizer (380B Applied Biosystems).

Nucleotide sequences were determined by the chain termination method [8] using an M13 sequencing kit (Boehringer). The sialidase sequence obtained was compared with sequences of the MicroGenie program (Microsoft available from Beckman, München).

### 3. RESULTS AND DISCUSSION

Two of about 3000 clones of *E. coli* JM101, transformed with pUC18 bearing Sau3A RF from *C. perfringens* A99-DNA, expressed sialidase ac-

tivity as determined with MU-Neu5Ac. The responsible inserts have a size of 2.1 kb which could be reduced by digestion with *Hind*III (in the direction of the promotor) and *Bal*31 (in the direction of the C-terminus of the encoded protein) to a 1.4 kb fragment, which still encoded an active sialidase. During insert reduction, the expressed enzyme protein remained constant in size, indicating that the procedure had not influenced the structural gene.

Subcloning of the reduced insert in pUC18 and pUC19 demonstrated that the expression is independent of the *lacZ'* promotor of the vector.

A DNA probe (17 nucleotides) representing the N-terminus of the cloned sialidase hybridizes with the insert and with 2.1 kb *Sau*3A fragments of DNA from both *C. perfringens* strains, but not with restricted *E. coli* DNA.

Fig.1 presents the restriction map of the reduced insert and the strategy for the subcloning of *Rsa*I- or *Bal*31-reduced insert fragments in M13 vectors for subsequent sequencing. The strategy resulted in high diversity of M13 subclones which frequently overlapped, thus assuring us of the determined sequence. The nucleotide sequence and the predicted amino acid sequence are depicted in fig.2. The nucleotide composition shows a low mol% G + C of 29 confirming its origin from *C. perfringens*, a species with a genomic mol% G + C of 24–27 [9]. The region upstream from ATG exhibits dyad symmetry between –82 and –119 including a TATAAT and a CAAT box which may represent the promotor. The Shine-Dalgarno sequence (–9

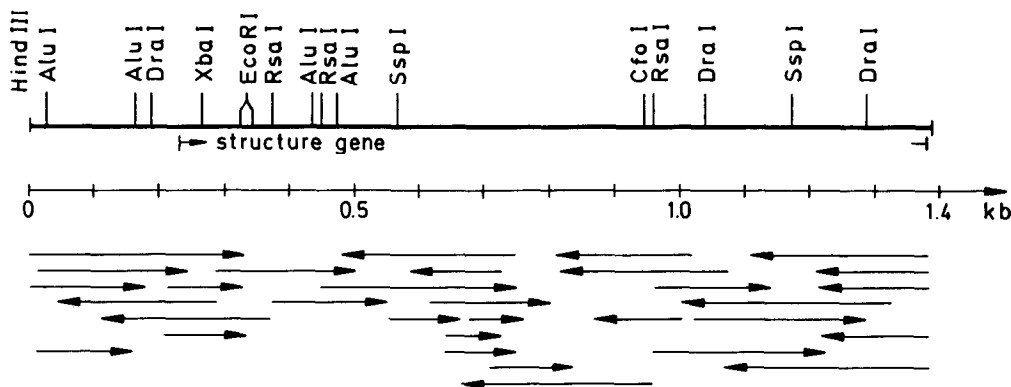


Fig.1. Restriction map of a *Clostridium perfringens* DNA fragment after maximum reduction but retaining sialidase activity in *E. coli*. Arrows indicate the direction and length of sequences obtained from insert fragment subclones.

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                                -230                                -211
                                CTTATACCTT CTTAAAGTTA
                                -141
TAAAAGCTAA AATTTTATAG AAAACTCCAA AAACAATTAT ATTTGCCTTA TTCCATAGTA AAGAATTCA
                                -71
AATACTGTTA TAATTTATTT GAAAACTTC TATAATTTTC AATATAGAAG TTTTAAATAG TTAGGTTTCT
                                -1
AAAGCTATTT ATAAGAAAAA TAACTTTTAA ATTAATGGGT AAATATGAAT TTATGGAGGA GATTATATTT
+1
ATG TGT AAC AAA AAC AAT ACC TTT GAA AAG AAT CTA GAT ATA AGC CAT AAA CCA GAA CCA
F-Met Cys Asn Lys Asn Asn Thr Phe Glu Lys Asn Leu Asp Ile Ser His Lys Pro Glu Pro
                                +60
CTA ATA CTA TTT AAC AAG GAT AAT AAC ATA TGG AAT TCA AAG TAT TTT AGA ATT CCC AAT
Leu Ile Leu Phe Asn Lys Asp Asn Asn Ile Trp Asn Ser Lys Tyr Phe Arg Ile Pro Asn
                                +120
ATA CAA TTA TTA AAT GAT GGT ACA ATT TTA ACC TTT TCA GAT ATT CGT TAT AAT GGT CCT
Ile Gln Leu Leu Asn Asp Gly Thr Ile Leu Thr Phe Ser Asp Ile Arg Tyr Asn Gly Pro
                                +180
GAT GAC CAT GCT TAT ATA GAC ATA GCT TCT GCA CGT AGT ACT GAT TTT GGA AAG ACA TGG
Asp Asp His Ala Tyr Ile Asp Ile Ala Ser Ala Arg Ser Thr Asp Phe Gly Lys Thr Trp
                                +240
AGC TAT AAC ATA GCA ATG AAA AAT AAT CGT ATT GAC TCT ACT TAT TCT CGT GTA ATG GAC
Ser Tyr Asn Ile Ala Met Lys Asn Asn Arg Ile Asp Ser Thr Tyr Ser Arg Val Met Asp
                                +300
TCC ACA ACA GTT ATT ACA AAT ACA GGT AGA ATA ATA TTA ATT GCA GGC TCA TGG AAT ACA
Ser Thr Thr Val Ile Thr Asn Thr Gly Arg Ile Ile Leu Ile Ala Gly Ser Trp Asn Thr
                                +360
AAT GGA AAC TGG GCA ATG ACT ACT TCT ACA AGA AGA AGT GAT TGG TCT GTC CAA ATG ATT
Asn Gly Asn Trp Ala Met Thr Thr Ser Thr Arg Arg Ser Asp Trp Ser Val Gln Met Ile
                                +420
TAT TCT GAT GAC AAT GGA TTA ACT TGG TCT AAT AAA ATA GAT TTA ACT AAG GAC TCT TCA
Tyr Ser Asp Asp Asn Gly Leu Thr Trp Ser Asn Lys Ile Asp Leu Thr Lys Asp Ser Ser
                                +480
AAA GTA AAA AAT CAA CCA AGT AAT ACA ATT GGA TGG CTA GGA GGA GTT GGC TCA GGT ATT
Lys Val Lys Asn Gln Pro Ser Asn Thr Ile Gly Trp Leu Gly Gly Val Gly Ser Gly Ile
                                +540
GTA ATG GAT GAT GGA ACA ATA GTT ATG CCA GCA CAA ATT TCC TTA AGA GAA AAT AAT GAA
Val Met Asp Asp Gly Thr Ile Val Met Pro Ala Gln Ile Ser Leu Arg Glu Asn Asn Glu
                                +600
AAT AAC TAT TAT TCA TTA ATT ATC TAT TCA AAG GAT AAT GGT GAA ACA TGG ACA ATG GGA
Asn Asn Tyr Tyr Ser Leu Ile Ile Tyr Ser Lys Asp Asn Gly Glu Thr Trp Thr Met Gly
                                +660
AAC AAG GTT CCT AAT TCA AAC ACC TCC GAA AAT ATG GTA ATA GAA TTA GAT GGC GCT TTA
Asn Lys Val Pro Asn Ser Asn Thr Ser Glu Asn Met Val Ile Glu Leu Asp Gly Ala Leu
                                +720
ATT ATG AGT ACA AGA TAT GAT TAC TCT GGC TAT AGG GCA GCA TAC ATC TCT CAT GAT TTA
Ile Met Ser Thr Arg Tyr Asp Tyr Ser Gly Tyr Arg Ala Ala Tyr Ile Ser His Asp Leu
                                +780
GGA ACC ACT TGG GAA ATA TAT GAA CCT TTA AAC GGT AAA ATT TTA ACT GGT AAG GGC TCT
Gly Thr Thr Trp Glu Ile Tyr Glu Pro Leu Asn Gly Lys Ile Leu Thr Gly Lys Gly Ser
                                +840
GGA TGC CAA GGT TCC TTT ATT AAG GCT ACT ACT TCA AAT GGA CAT AGA ATA GGA TTA ATT
Gly Cys Gln Gly Ser Phe Ile Lys Ala Thr Thr Ser Asn Gly His Arg Ile Gly Leu Ile
                                +900
TCA GCA CCT AAA AAC ACT AAA GGT GAA TAT ATA AGA GAC AAT ATT GCC GTT TAT ATG ATT
Ser Ala Pro Lys Asn Thr Lys Gly Glu Tyr Ile Arg Asp Asn Ile Ala Val Tyr Met Ile
                                +960
GAC TTT GAT GAT TTA TCT AAA GGA GTT CAA GAA ATA TGC ATT CCT TAT CCT GAA GAC GGT
Asp Phe Asp Asp Leu Ser Lys Gly Val Gln Glu Ile Cys Ile Pro Tyr Pro Glu Asp Gly
                                +1020
AAC AAA TTA GGT GGT GGC TAT TCT TGT CTA TCA TTT AAA AAT AAC CAT CTA GGC ATT GTT
Asn Lys Leu Gly Gly Gly Tyr Ser Cys Leu Ser Phe Lys Asn Asn His Leu Gly Ile Val
                                +1080
TAT GAA GCC AAT GGA AAT ATA GAA TAT CAA GAC TTA ACA CCT TAT TAC TCA CTA ATT AAT
Tyr Glu Ala Asn Gly Asn Ile Glu Tyr Gln Asp Leu Thr Pro Tyr Tyr Ser Leu Ile Asn
                                +1140
AAA CAA TAA TAA AAT
Lys Gln Stop
                                +1155

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Fig.2. The nucleotide sequence and the predicted amino acid sequence of a *Clostridium perfringens* DNA fragment encoding an active sialidase, as confirmed by gene expression in *E. coli*. The arrows denote a region with dyad symmetry probably representing the promotor. The predicted Shine-Dalgarno sequence is framed. The amino acid sequence obtained from N-terminal sequencing of the expressed enzyme is indicated by a broken line.

to -16) is comparable with the ribosome-binding site of *C. thermocellum* cel C gene [10], *C. tetani* toxin gene [11], or *C. pasteurianum* Mo-pterin binding protein gene [12].

The sequence of 20 amino acids obtained from the N-terminus of the expressed sialidase protein was found to be encoded by the insert. Although three ATG codons are in correct reading frame, the ATG nearest to the known amino acid sequence probably represents the start codon which typically follows a Shine-Dalgarno sequence. The six amino acids between this ATG and the protein sequence do not show the common hydrophobicity of a leader. This indicates the absence of a leader including the possibility that the sequence obtained by Edman degradation does not represent the real N-terminus, as the enzyme protein may have been partially degraded during purification.

The predicted insert region encoding a 42770 Da protein of 382 amino acids shows no highly significant homology with sequences presented by the MicroGenie program. Surprisingly, this was also valid for virus sialidase genes [13]. No homology was found with the N-terminal gene regions of *V. cholerae* sialidase [14], and *C. sordellii* sialidase (unpublished). This indicates that the N-terminus of bacterial sialidases is not highly conserved.

This work presents the rare case that the existence of an as yet unknown enzyme is disclosed after cloning and expression of the corresponding gene; after this finding a second sialidase with low activity as possible product of this gene could be detected in *C. perfringens* culture medium (work in progress). To our knowledge, these results represent the first description of a complete sequence of a bacterial sialidase gene.

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