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Isolation of a Neuraminidase Gene from *Actinomyces viscosus* T14V

MARIA K. YEUNG[1,2]* AND SHARON R. FERNANDEZ[1]

Departments of Pediatric Dentistry[1] and Microbiology,[2] The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78284

Received 29 April 1991/Accepted 4 August 1991

A genomic library of *Actinomyces viscosus* T14V DNA in λgt11 was screened for expression of neuraminidase activities. Four recombinant clones were detected that gave blue fluorescence upon incubation with a fluorogenic substrate, 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid. Of these, two were identical, and all of the neuraminidase-positive clones shared a common 3.4-kbp DNA region. Expression of the enzyme activities in *Escherichia coli* carrying the cloned DNA was independent of the *lacZ* promoter of the vector. Maxicell analysis revealed that the 3.4-kbp DNA insert directed synthesis of a protein with an apparent molecular mass of 100,000 Da. The protein from cell extracts of *E. coli* clones migrated as a single band that stained for enzyme activity after electrophoresis in a nondissociating polyacrylamide gel. Moreover, human erythrocytes incubated previously with cell lysates from neuraminidase-positive *E. coli* were hemagglutinated by *Actinomyces* spp. The enzyme expressed by *E. coli* was active on substrates containing α-2,3 and α-2,6 ketosidic linked sialyl residues. Similar substrate specificities were obtained for both the extracellular and cell-associated neuraminidases from *A. viscosus* T14V. The 3.4-kbp insert hybridized to DNA fragments in a Southern blot containing *A. viscosus* T14V chromosomal DNA that had been digested with various restriction endonucleases. Data from hybridization studies show that *A. viscosus* T14V contains a single copy of the neuraminidase gene.

The adherence of oral *Actinomyces* spp. to host tissues and other bacteria involves distinct fimbrial types present on the cell surface of these organisms (8). Fimbriae designated type 1 are implicated in attachment to the tooth surface (11), while those designated type 2 are associated with lectinlike interactions with mammalian cells (6, 34) and other bacteria (8). The latter interactions are reversible in the presence of lactose. The fimbria-mediated adherence of these organisms to eucaryotic cells, including buccal epithelial cells (6, 8), human erythrocytes (9, 15, 17), and polymorphonuclear leukocytes (34), is initiated by treatment of the mammalian cell types with neuraminidase. Exposure of the sialoglycoproteins to neuraminidase results in an unmasking of the galactose or galactosamine moieties that serve as receptors for the type 2 fimbriae of these organisms (5, 9). Since neuraminidase has been detected in saliva and plaque fluids (28, 30), it is likely that interactions between neuraminidase and salivary glycoproteins occur in vivo, thereby modulating bacterial adherence.

Results of previous studies showed that neuraminidase is produced by several indigenous plaque bacteria. These include strains of *Actinomyces* (15, 17, 25), *Streptococcus* (3), *Haemophilus* (37), and *Bacteroides* (19) species. However, the influence of enzyme production from each of the various bacteria on the oral ecology has not been investigated. For instance, it is not known whether the levels of enzyme synthesis by each of the different organisms are comparable or whether there is a selection for strains that produce higher levels of enzyme activity in localized areas within the oral environment. A recent study by Moncla and Braham (25) reported detection of neuraminidase from 79% of 71 *Actinomyces* spp. The results complemented a previous study by Costello et al. (15), who demonstrated neuraminidase activities in a large number of *Actinomyces* spp. (approximately 80% of all strains tested). Moreover, Costello (14a) indicated that about 10% of the total neuraminidase activity from *Actinomyces viscosus* T14V was found in the culture supernatant fluid, while >80% of the enzyme activity was cell associated. Interestingly, the soluble neuraminidase initiated hemagglutination by *Actinomyces* spp., while the cell-associated form of the enzyme did not (15). It is not known whether differences in substrate specificity between the two forms of the enzyme may have contributed to the different activities on human erythrocytes. A neuraminidase was isolated recently from *A. viscosus* DSM 43798 (36). Results from that study showed that the extracellular and cell-associated neuraminidase from this strain shared identical properties. However, hemagglutination of erythrocytes by this strain, as influenced by the two forms of the enzyme, was not presented.

This study focuses on the molecular cloning of a neuraminidase gene from *A. viscosus* T14V in *Escherichia coli*. The enzyme activity was detected with a fluorogenic substrate (29), using a rapid screening method similar to that described by Russo et al. (32). Isolation of the neuraminidase gene provides the basis for characterization of the gene and biosynthesis of the enzyme in *Actinomyces* spp. at the molecular level.

MATERIALS AND METHODS

Bacteria and plasmids. Table 1 summarizes the bacteria, plasmids, and bacteriophage used in this study. *E. coli* strains were grown in Luria broth (L-broth; 24) supplemented with antibiotics or nutrients as required for the particular strains. *Actinomyces* spp. were grown in caries-active medium supplemented with 0.2% glucose (15).

Construction and screening of *A. viscosus* T14V genomic library in *E. coli*. *A. viscosus* T14V chromosomal DNA was prepared as described previously (40). The genomic DNA was partially digested with *Sma*I (Bethesda Research Laboratories Inc., Gaithersburg, Md.) and fractionated through a 10 to 40% sucrose gradient as described in reference 24.

* Corresponding author.

TABLE 1. Bacterial strains, plasmids, and bacteriophage

Bacteria or plasmids	Genotype/remarks	Reference or source
Actinomyces spp.		
A. viscosus T14V	Presence of both type 1 and 2 fimbriae on cell surface	8
A. naeslundii WVU45	Presence of only type 2 fimbriae	10
E. coli		
Y1090	Δ*lacU169 proA*$^+$ Δ*lon araD139 strA supF* [*trpC22*::Tn*10*] (pMC9)	21
Y1089	ΔlacU169 proA$^+$ Δlon araD139 strA hf1A[*chr*::Tn*10*] (pMC9)	21
MV1184	*ara* Δ(*lac-pro*) *strA thi* (φ80 *lacZΔM15*) Δ(*st1-recA*)*306*::Tn*10*(*tet*^{r}) F′:*traD36 proAB lac*^{q}ZΔM15	Chiaki[a]
CSR603	F$^-$ *recA1 uvrA6 phr-1 thi-1 thr-1 leuB6 lacY1 galK2 ara-14 xyl-15 mtl-1 proA2 argE3 rpsL31 tsx-33 supE44 gyrA98* λ$^-$	33
Plasmid		
pSK$^+$	Ap^r; *lacZ*	Stratagene
pMY1415-5	3.4-kbp *Eco*RI DNA fragment from λ1415 cloned into the *Eco*RI site of pSK$^+$	This study
pMY1415-6	3.4-kbp *Eco*RI fragment from λ1415 inserted in the *Eco*RI site of pSK$^+$ in an opposite orientation to pMY1415-5	This study
pMY450-1	4.2-kbp *Eco*RI fragment from λ127 inserted into the *Eco*RI site of pSK$^+$	This study
pMY450-5	4.2-kbp *Eco*RI fragment from λ127 inserted in the *Eco*RI site of pSK$^+$ in an opposite orientation to pMY450-1	This study
Bacteriophage		
λgt11	λ*lac5* Δ*shn*dIIIλ2-3 *sr*Iλ3° c*Its857 sr*Iλ4° *nin5 sr*Iλ5° Sam100	21

[a] K. Chiaki, The University of Texas Health Science Center at San Antonio.

DNA fragments of 2 to 8 kbp were treated with *Eco*RI methylase (Promega Corp., Madison, Wis.) and then ligated with phosphorylated *Eco*RI linker (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.) by the procedures of Huynh et al. (21). The *Eco*RI fragments were ligated with λgt11 arms (digested with *Eco*RI and dephosphorylated; Bethesda Research Laboratories), and the ligation mixture was used to transfect *E. coli* Y1090 to generate a phage library.

The λgt11 recombinant library was examined for neuraminidase activities by using a fluorogenic neuraminidase substrate, 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNeuNAc, sodium salt; Sigma Chemical Co., St. Louis, Mo.) (29). Bacteriophage λ plaques were transferred to Whatman 3MM filter papers (Whatman Laboratory, Portland, Ore.) previously saturated with 100 μM MUNeuNAc in 0.17 M sodium acetate, pH 5.4. The filters were incubated at 37°C for 10 to 15 min and examined under long-wavelength UV. Positive clones were plaque purified three to four times by standard procedures (24).

Analysis of cell lysates from lambda lysogens. *E. coli* Y1089 was lysogenized with bacteriophage at a multiplicity of infection of 10, as described previously (21). Colonies that grew at 32°C but failed to grow at 42°C were assumed to be lysogens. A 5-ml culture of a lysogenic *E. coli* strain was induced at 44°C for 20 min followed by induction with 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma Chemical Co.) at 37°C for 1 h as described by Snyder et al. (35). Controls uninduced with IPTG were also prepared. Cells from IPTG-induced and uninduced cultures were suspended in 250 μl of Tris-buffered saline (0.15 M NaCl, 0.02 M Tris HCl [pH 7.8], 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.02% sodium azide) containing 0.2 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.) and lysed by multiple freezing and thawing at −70°C and room temperature, respectively. The viscosity of the cell lysate was reduced by sonication for 10 s. Expression of β-galactosidase fusion proteins was examined by Western blot (immunoblot) analysis. An aliquot of the phage lysate was subjected to electrophoresis in a 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel (23). The proteins were electroblotted to nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.) as described previously (39). The transfers were incubated in sequence, with blocking buffer (2), 5 μg of rabbit anti-β-galactosidase immunoglobulin G (Five prime three prime, Inc., West Chester, Pa.) per ml, and peroxidase-conjugated anti-rabbit immunoglobulin G (Bio-Rad Laboratories, Richmond, Calif.). The filters were developed in a solution containing 4-chloro-1-naphthol (Bio-Rad) and hydrogen peroxide.

Restriction mapping and subcloning. Bacteriophage DNA was prepared from a 50-ml culture infected previously with phage particles at a multiplicity of infection of 10 as described by Maniatis et al. (24). Aliquots of purified DNA were digested to completion with restriction endonucleases (Bethesda Research Laboratories) in various combinations for construction of a physical map. For subcloning of DNA inserts from bacteriophage into plasmids, 10 μg of phage DNA was digested with *Eco*RI and then electrophoresed in 0.7% agarose gels. The DNA fragments were eluted with GeneClean (Bio 101, La Jolla, Calif.) and subcloned into pBluescript SK$^+$ plasmid (Stratagene Cloning Systems, La Jolla, Calif.) digested previously with *Eco*RI and dephosphorylated. *E. coli* MV1184 was used as the host for transformation. Plasmid DNA was isolated by the method of Birnboim and Doly (4) and purified by CsCl-ethidium bromide gradient centrifugation (24).

Extracellular and cell-associated neuraminidase from *A. viscosus* T14V and *E. coli* strains. One liter of *A. viscosus* T14V was harvested during the stationary phase of growth. The bacterial cells were washed with phosphate-buffered saline (PBS) three times, and the cell density was adjusted to 5×10^9 cells per ml (A_{660} of 2.0). An aliquot of the washed cell suspension was assayed for cell-associated neuraminidase activities and bacterium-mediated hemagglutination. The culture supernatant was brought to 60% saturation with solid ammonium sulfate. The precipitated proteins were dissolved in a buffer containing 10 mM Tris HCl (pH 7.0),

150 mM NaCl, 4 mM $CaCl_2$, and 0.2 mM phenylmethylsulfonyl fluoride and dialyzed against 500 volumes of the same buffer at 4°C. A similar ammonium sulfate fractionation protocol was used to concentrate extracellular proteins from a 5-ml overnight culture of *E. coli*. The *E. coli* whole bacteria were washed twice in PBS and suspended in 1/10 of the culture volume in a buffer containing 50 mM sodium acetate, 150 mM NaCl, 4 mM $CaCl_2$, and 0.2 mM phenylmethylsulfonyl fluoride. Cell lysates were obtained by sonication on ice for 2 min with a Branson Sonifier (Branson Ultrasonics Corp., Danbury, Conn.). Cell debris was removed by centrifugation at 12,000 × *g* for 10 min.

Neuraminidase activities in the soluble cell extract or concentrated extracellular protein fractions were detected by a rapid spot filter assay (25). Aliquots of the samples also were analyzed in 7.5% nondissociating polyacrylamide gels. After electrophoresis, the gels were soaked in 0.17 M sodium acetate, pH 5.4, for 15 min and stained with MUNeuNAc in the same buffer at 37°C for 30 min.

Maxicell protein analysis. *E. coli* CSR603 carrying different plasmids (Table 1) was seeded in 10 ml of minimal growth medium (M9 minimal salt solution [24] supplemented with 1% Casamino Acids [Difco Laboratories, Detroit, Mich.] and 0.1 μg of thiamine per ml). At an A_{660} of 0.5, the culture was exposed to UV irradiation at 2.5 J in a Stratalinker (model 1800; Stratagene Cloning Systems). This condition was equivalent to the UV irradiation used by Sancar et al. (33). The irradiated culture was incubated in the presence of 100 μg of D-cycloserine (Sigma Chemical Co.) per ml for 12 h. The bacteria were incubated for 1 h at 37°C in 5 ml of sulfate-free minimal growth medium and in ^{35}S-methionine (Du Pont New England Nuclear, Boston, Mass.) at 5 μCi/ml for an additional hour. The labeled cells were lysed in 200 μl of SDS-containing sample buffer (23). The lysates were boiled at 100°C for 5 min and analyzed on 10% SDS-polyacrylamide gels. The gels were fixed, dried, and exposed to Kodak XAR-5 film (Eastman Kodak Co., Rochester, N.Y.).

Southern hybridization and DNA probe. *A. viscosus* T14V genomic DNA (2 μg) was digested to completion with several restriction endonucleases. The digested DNA fragments were separated by agarose gel electrophoresis and transferred to GeneScreen (New England Nuclear Corp.) under conditions recommended by the manufacturer. Membranes were prehybridized at 42°C for 2 to 4 h in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (24), 50% formamide, 10% dextran sulfate, 1× Denhardt's solution, 1% SDS, 1 M NaCl, 0.5% sodium pyrophosphate, and 200 μg of denatured herring sperm DNA (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml. Denatured radiolabeled DNA probe was added, and hybridization was continued for 18 to 20 h at the same temperature. Membranes were washed with 0.1× SSC containing 0.5% SDS at 65°C for 1 h with one change of buffer. The filters were air dried and exposed to Kodak XAR-5 film.

DNA was labeled with α-^{35}S-dCTP (1,000 to 1,500 Ci/mmol; New England Nuclear) by nick translation, using reagents from Bethesda Research Laboratories. The labeled DNA was purified and concentrated with NICK columns (Pharmacia LKB Biotechnology).

Protein and enzyme assays. Protein concentrations were determined with bicinchoninic acid reagents (Pierce Chemical Co., Rockford, Ill.). The neuraminidase activities of *E. coli* and *A. viscosus* protein fractions were monitored by the amount of sialic acid released from human α_1-acid glycoprotein (Sigma Chemical Co.), which served as the substrate for

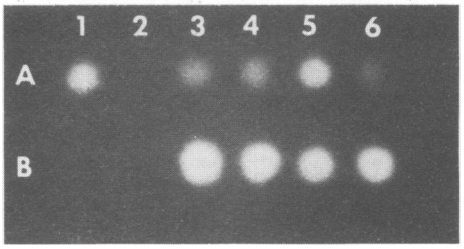

FIG. 1. Filter paper spot assay of lysates from *E. coli* recombinant clones. One microliter of *E. coli* phage lysate (row A) or sonicated extract (row B) was applied to Whatman 3MM paper saturated with 100 μM MUNeuNAc in 0.17 M sodium acetate, pH 5.4. The paper was incubated at 37°C for 10 min and examined under UV illumination. Positions: A1, *C. perfringens* neuraminidase; A2 through A6, phage lysate from *E. coli* carrying λgt11, λ127, λ1210, λ1415, and λ1720, respectively; B1 through B6, sonicated cell extracts from *E. coli* MV1184, MV1184(pSK$^+$), MV1184 (pMY1415-5), MV1184(pMY1415-6), MV1184(pMY450-1), and MV1184(pMY450-5), respectively.

A. viscosus neuraminidase (15). A typical assay mixture contained 50 to 100 μg of total protein incubated for 1 h at 37°C in a final volume of 500 μl of 50 mM sodium acetate–150 mM NaCl–4 mM $CaCl_2$–200 μg of α_1-acid glycoprotein. Free sialic acid liberated was determined colorimetrically by the thiobarbituric acid method of Aminoff (1). Interference due to the presence of 2-deoxyadenosine (1) also was monitored. Other substrates used in the present study included: bovine submaxillary mucin (BSM), *N*-acetylneuramin-lactose (human or bovine), colominic acid (all chemicals obtained from Sigma Chemical Co.), and de-O-acetylated BSM prepared by incubating BSM in 0.05 M sodium carbonate as described previously (20). To quantitate cell-associated neuraminidase, 150 μl of a washed cell suspension was used in the reaction mixture. Bacteria were removed after the incubation period by centrifugation, and the supernatant fluid was assayed for sialic acid contents.

RESULTS

Isolation of λgt11 clones that expressed neuraminidase activities. A fluorogenic neuraminidase substrate, MUNeuNAc, was used to screen the genomic library of *A. viscosus* T14V DNA in λgt11 for expression of enzyme activities. Less than 5% of the library consisted of nonrecombinant plaques, as determined by plating of the phages on a medium containing 5-bromo-4-chloro-3-indolyl-β-galactoside and IPTG. Of approximately 3 × 10^5 plaques examined, four positive clones were detected by their ability to convert the fluorogenic substrate to give a blue fluorescence under UV light. Figure 1 shows the sensitivity of the filter paper spot assay for monitoring enzyme production in neuraminidase-positive strains. Each spot in row A of Fig. 1 contained proteins present in approximately 10^7 phage particles from each of the lambda clones. The fluorescence intensity generated in these clones differed, with the strongest signal observed in λ1415 and a relatively weak signal seen in λ1210. Lysates from λgt11 did not react with the substrate. The relative intensity of each clone remained unchanged after three or four plaque purifications.

The proteins expressed in the neuraminidase-positive clones were not fusion proteins. Immunostaining of cell lysates from each of the recombinant lambda lysogens with anti-β-galactosidase antibody showed the presence of a

TABLE 2. Expression of neuraminidase activity in λgt11 lysogen

Lysogenic strain	Sp act (mean ± SD)[a]	
	IPTG induced	Uninduced
λgt11	<0.05	<0.05
λ127	104 ± 6[b]	109 ± 4[b]
λ1210	44 ± 2[b]	48 ± 3[b]
λ1415	364 ± 21[c]	112 ± 5[c]
λ1720	76 ± 4[b]	71 ± 9[b]

[a] Nanomoles of sialic acid released per hour per milligram of protein lysate from lambda lysogens. Data shown are average values from three lysogenic lysates.
[b] $P \geq 0.2$ for comparative data between the two conditions.
[c] $P = 0.0522$ for comparative data between the two conditions.

protein that migrated only slightly slower than the β-galactosidase detected in the cell extract of λgt11 and bona fide β-galactosidase (data not shown). Enzyme production by each neuraminidase-positive clone was quantitated by using human α_1-acid glycoprotein as substrate. The results were similar to those obtained by the qualitative assay in which MUNeuNAc was the substrate (cf. Fig. 1, row A, and Table 2, second column). IPTG induction had little or no effect on the expression of neuraminidase by λ127, λ1210, and λ1720 ($P \geq 0.2$ for comparison of data between induced and uninduced conditions for each group) (Table 2). In contrast, the enzyme activity detected in λ1415 was elevated in the presence of IPTG ($P = 0.052$) (Table 2). These results suggested that transcription and translation of the DNA insert in all of the neuraminidase-positive *E. coli* strains were independent of the λgt11 *lacZ* promoter.

Physical mapping and subcloning. On the basis of restriction endonuclease analyses, λ1210 and λ1720 contained the same 3.4-kbp DNA insert and were cloned in the same orientation with respect to the *lacZ* promoter of λgt11 (Fig. 2). While the size of the DNA insert in λ1415 was similar to that in λ1210 or λ1720, the DNA fragment was cloned in the opposite orientation. λ127 contained a 4.2-kbp DNA insert

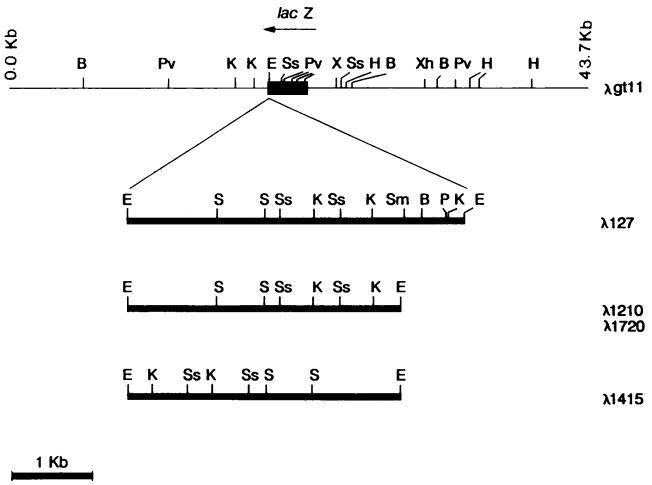

FIG. 2. Physical maps of lambda recombinant bacteriophages of neuraminidase-positive clones. Each of the recombinant phages contained *A. viscosus* T14V DNA (■) inserted at the unique *Eco*RI site of vector λgt11 (——). The arrow indicates the transcription direction of the *lacZ* gene (▩) of λgt11. Restriction enzymes: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*I, S, *Sal*I; Sm, *Sma*I; Ss, *Sst*I; X, *Xba*I; Xh, *Xho*I.

TABLE 3. Synthesis and secretion of neuraminidase by *E. coli* strains

Strain	Sp act (mean ± SD)[a]	
	Sonicated extract	Supernatant
MV1184	<0.02	<0.02
MV1184(pSK$^+$)	<0.05	<0.02
MV1184(pMY1415-5)	816 ± 60[b]	400 ± 71[c]
MV1184(pMY1415-6)	809 ± 75[b]	313 ± 13[c]
MV1184(pMY450-1)	645 ± 55[d]	421 ± 20[c]
MV1184(pMY450-5)	605 ± 20[d]	365 ± 63[c]

[a] Expressed as nanomoles of sialic acid released per hour per milligram of soluble sonicated extract or culture supernatant fluid prepared by 60% ammonium sulfate fractionation.
[b] $P = 0.3906$ for comparative data between the two groups.
[c] $P = 0.1080$ for comparative data between the four groups.
[d] $P = 0.1185$ for comparative data between the two groups.

that overlapped the 3.4-kbp insert in λ1210 and λ1720. DNA-DNA hybridization data showed that the 3.4-kbp DNA region was shared by all of the recombinant strains (data not shown). Thus, all of the neuraminidase-positive clones expressed the same neuraminidase activity. Since a higher level of neuraminidase activity was obtained from cell lysates of the λ1415 lysogen under IPTG induction (Table 2), it is likely that the *lac* promoter of the vector directs transcription of the neuraminidase gene present in this clone. Further, transcription of the cloned gene probably initiates near the rightmost *Eco*RI site on the physical map of λ1415 (Fig. 2).

The 3.4- and 4.2-kbp *Eco*RI DNA fragments from λ1415 and λ127, respectively, were cloned separately into the *Eco*RI site of pBluescript SK$^+$. Two pairs of clones were obtained: one pair contained the 3.4-kbp insert and the other contained the 4.2-kbp DNA insert in both orientations with respect to the *lacZ* promoter of the vector (Table 1). Neuraminidase activities were detected from *E. coli* carrying these plasmids, as monitored by the qualitative filter spot assay (Fig. 1, row B).

Characterization of neuraminidase-positive *E. coli* strains. *E. coli* harboring the recombinant plasmids listed in Table 1 were stable and expressed measurable levels of neuraminidase. Table 3 shows that *E. coli* carrying pMY1415-5 or pMY450-1 containing the 3.4- or 4.2-kbp DNA insert, respectively, synthesized and secreted neuraminidase at comparable levels. As expected, similar levels of expression were observed in *E. coli* carrying pMY1415-6 and pMY450-5, in which the inserts were in the reverse orientations (see calculated confidence intervals in Table 3). In a separate experiment, subcellular fractionation of *E. coli* carrying pMY1415-5 revealed that >80% of the total enzyme produced was associated with the cytoplasmic and membrane fractions, while 3.4% of the activity was retained in the periplasmic fraction. Extracellular neuraminidase accounted for 10% of the total activity (data not shown).

As shown in Fig. 3, the enzyme synthesized by *E. coli* MV1184(pMY1415-5) or MV1184(pMY450-1) was similar to the extracellular neuraminidase from *A. viscosus* T14V obtained by fractionation of the culture supernatant fluid with 60% ammonium sulfate. The protein exhibiting neuraminidase activity in soluble sonicated extracts of these strains migrated as one band in a nondenaturing polyacrylamide gel and stained for enzyme activity with the fluorogenic substrate (Fig. 3, lanes 2 and 3). The enzyme from *E. coli* clones ($R_f = 0.16$) migrated slightly faster than the *A. viscosus* extracellular neuraminidase ($R_f = 0.15$) and signif-

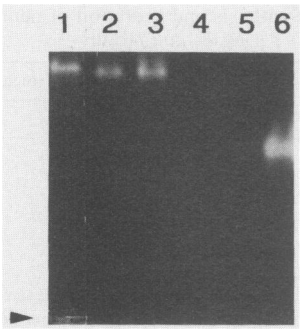

FIG. 3. Nondissociating gel electrophoresis of cell extracts of *E. coli* MV1184 carrying different plasmids. Sonicated extracts of *E. coli* strains were electrophoresed in a 7.5% nondissociating polyacrylamide gel. An aliquot of a partially purified cell-free neuraminidase of *A. viscosus* T14V was also analyzed. The gel was stained with MUNeuNAc in 0.17 M sodium acetate, pH 5.4, at 37°C for 30 min. Lanes: 1, *A. viscosus* T14V extracellular neuraminidase; 2, *E. coli* MV1184(pMY1415-5) cell extract; 3, *E. coli* MV1184 (pMY450-1) cell extract; 4, *E. coli* MV1184(pSK$^+$) cell extract; 5, *E. coli* MV1184 cell extract; 6, *C. perfringens* neuraminidase. The arrowhead marks the position of the electrophoretic dye front.

TABLE 4. Hemagglutination of *Actinomyces* spp.

Human erythrocytes treated with[a]:	Sialic acid released (μmols/ml, mean ± SD)	Hemagglutination[b] with:	
		A. viscosus T14V	*A. naeslundii* WVU45
C. perfringens neuraminidase	6.2 ± 0.1[c]	+	+
E. coli MV1184 (pMY1415-5)	5.8 ± 0.1[c]	+	+
E. coli MV1184 (pSK$^+$)	ND[d]	−	−
PBS	ND[d]	−	−

[a] Human erythrocytes were washed three times with PBS and adjusted to a 20% (vol/vol) packed-cell suspension. Fifty microliters of the washed erythrocytes was incubated in a final volume of 100 μl of assay mixture at 37°C for 1 h with 15 μg of *C. perfringens* neuraminidase or 50 μg of soluble *E. coli* cell extract. The mixtures were centrifuged, and the supernatant fluids were assayed for sialic acid.

[b] Neuraminidase-treated erythrocytes were washed with PBS three times and suspended as a 2% packed-cell suspension in PBS containing 1% bovine serum albumin. Fifty microliters of this suspension was mixed with 50 μl of washed cell suspensions (5×10^9 cells per ml) of *Actinomyces* spp., and hemagglutination was observed immediately.

[c] $P = 0.2647$ for comparative data between the two groups.

[d] ND, below detection level (<0.2).

icantly slower than the *Clostridium perfringens* neuraminidase ($R_f = 0.39$; Fig. 3, lane 6).

The proteins encoded by pMY1415-5 and pMY450-1 in the *E. coli* maxicell strain CSR603 were labeled with ^{35}S-methionine. Both plasmids directed the synthesis of a protein with an apparent molecular mass of 100,000 Da (Fig. 4, lanes 4 and 5), which was absent in maxicell proteins from *E. coli* carrying pSK$^+$ (Fig. 4, lane 3).

Neuraminidase-initiated hemagglutination and substrate specificities. The rate at which sialic acid was released from packed human erythrocytes by neuraminidase in 50 μg of the *E. coli* MV1184(pMY1415-5) soluble cell lysate was similar to that released by 15 μg of commercially purified *C. perfringens* neuraminidase ($P = 0.2647$) (Table 4). Immediate hemagglutination of the treated erythrocytes by *A. viscosus* T14V and *A. naeslundii* WUV45 was observed, and the interaction was reversible with the addition of 50 mM lactose. A hemagglutination score of 1+ was obtained when the cell suspensions were diluted eightfold with the reaction buffer. As expected, erythrocytes treated previously with cell lysates of *E. coli* MV1184(pSK$^+$) or MV1184 alone were not hemagglutinated by the *Actinomyces* cell suspensions.

Substrate specificities of neuraminidase isolated from *E. coli* clones were similar to the specificities of the enzyme isolated from *A. viscosus* T14V (Table 5). The enzyme was active on substrates containing α-2,3- and α-2,6-linked sialic acid. At least twice as much sialic acid was released from

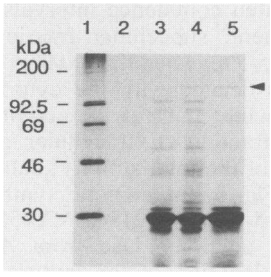

FIG. 4. SDS-polyacrylamide gel electrophoresis of ^{35}S-methionine-labeled proteins expressed in maxicells. Cell lysates from *E. coli* CSR603 (lane 2) and *E. coli* CSR603 carrying plasmids pSK$^+$ (lane 3), pMY450-1 (lane 4), and pMY1415-5 (lane 5) were electrophoresed in a 10% SDS-polyacrylamide gel followed by autoradiography. The molecular masses of ^{14}C-labeled protein markers (lane 1) are indicated. The arrowhead denotes the protein band present only in lanes 4 and 5.

TABLE 5. Substrate specificity of neuraminidase from *A. viscosus* T14V and *E. coli* recombinant clones[a]

Substrate	α-Ketosidic linkage	Sp act (± SD)		
		A. viscosus T14V		*E. coli* cell lysate[d]
		Extracellular[b]	Cell associated[c]	
BSM	α-2,6	42 ± 3	33 ± 3	74 ± 6
BSM, deacetylated[e]	α-2,6	99 ± 9	78 ± 8	150 ± 3
α_1-acid glycoprotein	α-2,6 & α-2,3	41 ± 3	65 ± 4	85 ± 8
N-acetylneuraminlactose (bovine)	α-2,3 (15%) α-2,6 (85%)	29 ± 3	51 ± 3	71 ± 8
N-Acetylneuraminlactose (human)	α-2,3 (85%) α-2,6 (15%)	20 ± 1	38 ± 5	49 ± 6
Colominic acid	α-2,8	3.3 ± 0.3	5.4 ± 0.1	3.1 ± 0.1

[a] Neuraminidases form different sources were incubated with each of the substrates at 37°C for 1 h. Specific activity is expressed as nanomoles of sialic acid released per hour per 100 μg of soluble protein preparation or from 8×10^8 bacterial cells. Substrates used in each incubation were as follows: BSM, 1 mg; α_1-acid glycoprotein, 200 μg; *N*-acetylneuraminlactose (bovine or human), 50 μg; colominic acid, 100 μg. Data are the average of three determinations. Sialic acid detected from substrates incubated with the assay buffer alone was as follows, in nanomoles: BSM, 8.6 ± 0.3; BSM (deacetylated), 11 ± 1; α_1-acid glycoprotein, 1.6 ± 0.1; *N*-acetylneuraminlactose (bovine), 2.4 ± 0.2; *N*-acetylneuraminlactose (human), 2.3 ± 0.3; colominic acid, 5.4 ± 0.4.

[b] Extracellular neuraminidase from *A. viscosus* T14V prepared by fractionation with ammonium sulfate.

[c] 8×10^8 washed cells of *A. viscosus* T14V were incubated with the soluble substrate. Bacteria were removed after the incubation period, and the sialic acid content in the supernatant fluid was determined.

[d] Sonicated cell lysate of *E. coli* MV1184(pMY1415-5).

[e] BSM treated previously with alkali to remove the O-acetyl group.

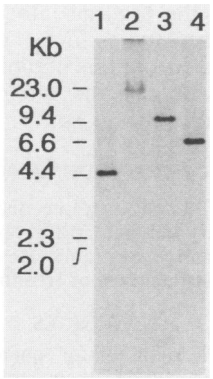

FIG. 5. Southern hybridization of *A. viscosus* T14V DNA with the 3.4-kbp DNA insert containing the cloned neuraminidase gene. *A. viscosus* T14V chromosomal DNA was digested with *Pst*I (lane 1), *Hin*dIII (lane 2), *Kpn*I (lane 3), and *Bam*HI (lane 4). The digested DNA was separated on a 1% agarose gel and transferred to GeneScreen. The filter was hybridized to an α-^{35}S-dCTP-labeled, 3.4-kbp *Eco*RI fragment from λ1415. Molecular sizes of lambda DNA digested with *Hin*dIII are indicated on the left.

BSM treated with alkali, which chemically removes the *O*-acetyl group from the substituted sialic acid residue (20). In contrast, enzyme from *A. viscosus* and from *E. coli* clones did not hydrolyze colominic acid, a homopolymer of α-2,8-linked sialic acid. The amount of sialic acid released (Table 5) was comparable to a background level obtained when this substrate was incubated with the assay buffer alone. The presence of sialic acid in colominic acid was confirmed by mild acid hydrolysis with 0.05 N sulfuric acid. Also, 127 nmol of sialic acid was released from the latter compound after incubation for 1 h with 15 μg of *C. perfringens* neuraminidase (data not shown). Although the cell-associated form of *A. viscosus* T14V neuraminidase did not initiate hemagglutination (15; this study), the substrate specificities were similar to those of the extracellular enzyme when soluble substrates were used in the reaction mixtures (Table 5). As expected, the enzyme activities of neuraminidase from *A. viscosus* T14V and from *E. coli* strains were inhibited in the presence of 2,3-dehydro-*N*-acetylneuraminic acid (15; unpublished data).

Copy number of *nanH* gene in *A. viscosus* T14V. The DNA inserts derived from each recombinant lambda clone were used separately as DNA probes in hybridization reactions with *A. viscosus* chromosomal DNA that had been digested with various restriction endonucleases. In all cases, identical patterns were obtained. The results argued against the possibilities that the neuraminidase-positive clones obtained from the library may have encoded isoenzymes of *A. viscosus* T14V neuraminidase. Figure 5 shows the results of a typical Southern analysis under conditions of high stringency, with the 3.4-kbp fragment from λ1415 used as a probe. The DNA probe hybridized to a single band present in the genomic DNA after digestion with *Bam*HI, *Hin*dIII, and *Pst*I. One major band of approximately 10 kbp was observed in the lane containing chromosomal DNA that had been digested with *Kpn*I. Upon prolonged exposure, two additional minor bands (approximately 1.0 and 0.75 kbp) were observed (Fig. 5, lane 3), consistent with the presence of two internal *Kpn*I sites in the DNA probe (Fig. 1, physical map of λ1415).

DISCUSSION

A structural gene that encodes the *A. viscosus* T14V neuraminidase was isolated by screening a genomic library of *Actinomyces* DNA constructed in the expression vector λgt11. On the basis of growth of bacteriophage plaques on a medium containing 5-bromo-4-chloro-3-indolyl-β-galactoside and IPTG, >95% of the library contained recombinant phages. Analysis of six random clones revealed that the average size of the inserts was between 3 and 5 kbp (data not shown). Thus, on a statistical basis (12), this library should have contained the entire genome of the organism. Four independent neuraminidase-positive clones were detected that gave fluorescence signals of varying intensities when reacted with a fluorogenic substrate. Restriction endonuclease analyses and DNA-DNA hybridization studies of the inserts derived from each of the neuraminidase-positive clones confirmed that they shared a common 3.4-kbp DNA region. Southern analyses revealed that each of the inserts hybridized to the same *A. viscosus* T14V DNA fragments generated by digesting the chromosomal DNA with various restriction enzymes. Thus, the DNA insert in each of the neuraminidase-positive clones encodes the same neuraminidase activity. Moreover, only one copy of the *nanH* gene is present in this organism.

Expression of the cloned gene in *E. coli* was controlled by a putative promoter present within the 3.4-kbp DNA insert. This was supported by the observation that comparable levels of neuraminidase activity were obtained from *E. coli* carrying the *nanH* gene in opposite orientations with respect to the *lacZ* promoter of the vector (Table 3). Thus, the *A. viscosus* T14V neuraminidase structural gene, *nanH*, together with its promoter must be located within the 3.4-kbp DNA fragment. It is noteworthy that *E. coli* maintained the *A. viscosus* T14V *nanH* gene stably on a high-copy-number plasmid and was capable of overproducing the enzyme. Moreover, these strains secreted the enzyme into the growth medium. Similar to *A. viscosus* T14V, 10% of the total enzyme synthesized by the *E. coli* clones was extracellular neuraminidase. Nevertheless, the enzyme activity per milliliter of culture obtained from *E. coli* was approximately four- to fivefold higher than that from *A. viscosus* T14V (unpublished data). Results from previous studies demonstrated that the synthesis of extracellular neuraminidase by various bacteria was inducible by sialic acid and other sialyl-containing glycoproteins (18, 32, 39). Additional studies in which sialic acid was added to the growth medium of *A. viscosus* T14V or neuraminidase-positive *E. coli* did not result in increased synthesis of extracellular neuraminidase (data not shown). This finding does not exclude the possibility that enzyme production can be induced in the presence of other sialyl-containing glycoproteins. In this regard, data from Teufel et al. showed a slight increase of neuraminidase production by *A. viscosus* DSM 43798 in a medium supplemented with sialoglycopeptides from *Collocalia* mucin (36). Secretion of the cloned enzyme by *E. coli* suggested that the regulatory sequences utilized by *A. viscosus* T14V for neuraminidase export also were recognized and efficiently processed in *E. coli*. Elucidation of the nucleotide base sequence of the 3.4-kbp insert containing the *nanH* gene will be essential to confirm the presence of the proposed regulatory sequences.

That the protein synthesized by neuraminidase-positive *E. coli* clones is authentic neuraminidase from *A. viscosus* T14V was supported by the finding that, in nondenaturing gels, the enzyme from these strains migrated only slightly

faster than the neuraminidase from the culture supernatant fluid of this organism. The substrate specificity of the enzyme expressed in *E. coli* was similar to that of the cell-free and cell-associated neuraminidase of *A. viscosus* T14V (Table 4). This finding is in agreement with that of Teufel et al. (36), who demonstrated identical substrate specificity between the two forms of the enzyme from *A. viscosus* DSM 43798. The *A. viscosus* T14V neuraminidase hydrolyzes sialic acid residues that are either α-2,3 or α-2,6 linked to galactose, *N*-acetylgalactosamine, or lactose. When *N*-acetylneuramin-lactose from bovine colostrum (containing 15% α-2,3 and 85% α-2,6 neuraminyl-lactose isomers) and from human milk (containing 85% α-2,6 and 15% α-2,3 neuraminyl-lactose isomers) were used as substrates, the results (Table 4) suggested that the 6′-neuraminyl-lactose isomer was more susceptible to this enzyme. Further studies with glycoproteins with unique ketosidic linkages will be required to determine the specific substrate preferences of this enzyme. The reduced rate of hydrolysis by the *A. viscosus* T14V neuraminidase on BSM was attributed to the presence of O-acetylated sialic acid residues in this substrate. This conclusion was based on the observation that increased levels of sialic acid were released from alkali-treated BSM versus untreated BSM (Table 4). The resistance of O-acetylated or N-substituted sialic acids to hydrolysis by other bacterial neuraminidases such as those from *C. perfringens* or *Vibrio cholerae* (14, 26) and from *A. viscosus* DSM 43798 (36) has been described before. Thus, with respect to hydrolytic properties, the *A. viscosus* T14V neuraminidase shares certain similarities with neuraminidases from *A. viscosus* DSM 43798 (36), *C. perfringens* (7), and other bacteria (18, 22, 38) that have broader ranges of substrate specificity. It is of interest that the properties of neuraminidase from *A. viscosus* T14V are not identical to those of *A. viscosus* DSM 43798. For instance, while neuraminidase from the latter strain hydrolyzes α-2,3 linkages at rates faster than α-2,6 ketosidic bonds and is apparently quite active on colominic acid containing α-2,8-linked sialic acid residues (36), the *A. viscosus* T14V neuraminidase does not exhibit similar characteristics (Table 5).

Neuraminidase has been isolated from a variety of microorganisms (13, 31) and is often considered a potential virulence factor contributing to the pathogenesis of the organisms. Most neuraminidase-producing organisms secrete large amounts of the enzyme into the growth medium (31). Exceptions to this general observation include *Klebsiella aerogenes* (27), *Pasteurella multocida* (16), *Bacteroides* spp. (19), and *Actinomyces* spp. (15, 17, 36), which possess predominately cell-associated neuraminidase. At present, it is not known whether the cell-bound neuraminidase of *Actinomyces* spp. is associated with the bacterial cell wall alone or is also anchored in the cytoplasmic membrane. Results from previous studies suggested that some of the cell-bound enzyme must be associated with the cell wall. This is based on the observations that the cell-associated enzyme was released when treated with lysozyme (14a, 36) and that, upon storage, a small fraction of the cell-associated enzyme was released from the cell suspension of *Actinomyces* spp. (15; unpublished data). It will be of interest to determine the factors that influence enzyme release from the bacteria, since only the soluble cell-free neuraminidase was capable of initiating bacterium-mediated hemagglutination (15). Further studies will be necessary to elucidate the relationship between the cell-free and cell-associated neuraminidases from *A. viscosus* T14V. Clearly, isolation of the *nanH* gene in the present study, and the finding that *E. coli* clones secreted the enzyme into the growth medium, should facilitate future studies focusing on the biochemical analysis of the enzyme from this organism. Data from these studies should provide insights into the biosynthesis of neuraminidase by *Actinomyces* spp.

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