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Characterization of a new serotype g isolate of *Aggregatibacter actinomycetemcomitans*

K. Takada[1], M. Saito[1], O. Tsuzukibashi[2], Y. Kawashima[1], S. Ishida[1] and M. Hirasawa[1]

1 Department of Microbiology and Immunology, Nihon University School of Dentistry at Matsudo, Chiba, Japan
2 Laboratory Medicine for Dentistry, Nihon University School of Dentistry at Matsudo, Chiba, Japan

Correspondence: Kazuko Takada, Department of Microbiology and Immunology, Nihon University School of Dentistry at Matsudo, 2-870-1 Sakaecho-nishi, Matsudo, Chiba 271-8587, Japan Tel.: + 81 47 360 9625; fax: + 81 47 360 9343; E-mail: takada.kazuko@nihon-u.ac.jp

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SUMMARY

Aggregatibacter actinomycetemcomitans **is usually isolated from the oral cavity where it is associated with active periodontitis. The species can be divided into six serotypes (a–f) according to their surface carbohydrate antigens. However, some clinical isolates cannot be grouped within these six serotypes. Gram-negative, facultative anaerobic, catalase-positive coccobacilli were isolated from a patient with periodontitis and identified by employing genetic, biochemical and serological analyses. Phenotypic data identified the isolate as *A. actinomycetemcomitans.* Serotype-specific polysaccharide antigen from the isolate was untypeable by immunodiffusion testing in comparison with reference *A. actinomycetemcomitans* serotype a to f strains. Biofilm formation by the isolate was strong but cytotoxic activity was low. Gas chromatography/mass spectroscopy analysis of partially methylated alditol acetates from surface polysaccharide showed the presence of 2,4-di-*O*-methyl-rhamnose and 2,3,6-tri-*O*-methyl-glucose, with a 1 : 1 M ratio. The ^{1}H- and ^{13}C-nuclear magnetic resonance spectra of the antigen showed that both constituent glycoses had α-anomeric configuration. It is proposed that the untyped strain is a new *A. actinomycetemcomitans* serotype, designated serotype g.**

INTRODUCTION

Aggregatibacter actinomycetemcomitans is an important pathogen related to aggressively progressive periodontal breakdown in adolescents and adults (Slots *et al.*, 1980; Zambon, 1985). It is a gram-negative, facultative anaerobic coccobacillus, and is a member of the family *Pasteurellaceae*. Granular growth in broth is common and X and V factors are not required (Nørskov-Lauritsen & Kilian, 2006). On primary isolation, the colonies have an internal, opaque pattern described as star-like, which corresponds with fimbrial production. The organism produces an RTX (repeats in toxin) leukotoxin. *A. actinomycetemcomitans* is classified into six distinct serotypes (a to f) based on the composition of the polysaccharides present on the surface of the organism, which function as immunodominant antigens. Several studies have focused on the relationship between serotype distribution, racial and geographic populations, and periodontal status (Zambon *et al.*, 1983; Saarela *et al.*, 1992; Gmür *et al.*, 1993; Asikainen *et al.*, 1995; Yamamoto *et al.*,1997; Paju *et al.*, 2000; Kaplan *et al.*, 2002; Tsuzukibashi *et al.*, 2008).

In this paper, we describe the genetic, biochemical and serological characteristics of a new isolate, and have structurally analysed its serotype-specific antigen. We propose that the isolate, strain NUM4039, be assigned to a new *A. actinomycetemcomitans* serotype, designated g.

METHODS

Bacterial strains

This study used reference strains ATCC29523, ATCC43718 (Y4), ATCC33384^T, IDH781, OMZ541 and NUM-Aa 5005, representing *A. actinomycetemcomitans* serotypes a, b, c, d, e and f, respectively, and ATCC700685 (JP2-like, highly leukotoxic). The new isolate was obtained from periodontal pockets of ≥6 mm depth from a patient with chronic periodontitis. The strains were maintained on Bact^{TM} brain–heart infusion (BHI; Becton, Dickinson and Co., Sparks, MD) supplemented with 1% Bact^{TM} yeast extract (BHIY; Becton Dickinson) and 1.5% agar. The organisms were cultured for 48 h at 37°C in 5% CO_2.

Isolate identification

A pure culture of the isolate was identified by Gram staining and by biochemical tests using the API NH, API 50CH and API ZYM systems (bioMerieux, March L'Etoile, France) according to the manufacturer's instructions.

Biofilm formation

Adherence of *A. actinomycetemcomitans* was examined qualitatively using the method described by Tomich *et al.* (2006), with some modifications. Briefly, a stationary-phase culture was used to inoculate the wells of a 24-well microtiter dish containing 1 ml *A. actinomycetemcomitans* growth medium (AAGM; Fine *et al.*, 1999). The strains were grown for 20 h, after which the medium was removed and wells were washed five times with water. Biofilms were stained with 0.2 ml of 0.5 mg ml^{-1} ethidium bromide per well, and washed five times with water. Biofilms were visualized under ultraviolet illumination.

Genetic analysis

Genomic DNA was extracted from the organisms using a Promega Genome kit (Promega, Madison, WI). The G+C content of the DNA was determined by high-performance liquid chromatography (HPLC), as previously described (Takada *et al.*, 2008). To determine the phylogenetic affinity of the isolate, the 16S ribosomal RNA (rRNA) gene was amplified by polymerase chain reaction (PCR) and directly sequenced using a Big Dye Terminator v.1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA), and an automatic DNA sequencer (ABI PRISM model 373A; Applied Biosystems) as previously described (Takada *et al.*, 2008). Database searches identified the closest known relatives of the new isolate. Their 16S rRNA gene sequences were compared with those available at DNA Database of Japan, European Molecular Biology Laboratory and GenBank.

Polymerase chain reaction

The PCR was performed directly from a single colony suspended in 25 μl distilled water and boiled for 5 min, with 5 μl suspension used as template. The PCR conditions and primers for 16S rRNA, leukotoxin A, leukotoxin promoter, major fimbrial subunit (*flp-1*) and *O*-polysaccharide (O-PS) genes of *A. actinomycetemcomitans* in this study were described previously (Tønjum & Hass, 1993; Ashimoto *et al.*, 1996; Haubek *et al.*, 1996; Kaplan *et al.*, 2001, 2002). PCR products were analysed by 2.0% agarose gel electrophoresis and sized by comparison with a 100-base-pair (bp) DNA ladder (Takara Biomed., Otsu, Japan).

Cytotoxic assay

Cytotoxic assay of *A. actinomycetemcomitans* was determined as previously described (Brogan *et al.*, 1994). Briefly, HL60 cells were obtained from Riken BioResource Center (Ibaraki, Japan) and grown in RPMI-1640 medium (Wako Co., Japan) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and gentamicin (10 μg ml^{-1}) at 37°C in 5% CO_2. For killing assays, 50 μl HL60 cells (4×10^6 cells ml^{-1}) were mixed with 50 μl bacteria–target cell suspensions (1×10^9 colony-forming units ml^{-1}) and incubated at 37°C for 60 min. Ten microliters of mixture was mixed with 10 μl trypan blue, and dead vs. viable cells were identified microscopically. Blank and negative controls included HL60 cells mixed with phosphate-buffered saline.

Serological analyses

Serological analyses of various antigen and antiserum preparations were conducted employing the

double immunodiffusion agar method (Tsuzukibashi *et al.*, 2008). Rabbit antisera raised against reference strains were prepared as described previously (Hirasawa *et al.*, 1980; Takada *et al.*, 1984).

Preparation of serotype antigen

The organisms were cultured in BHIY broth for 48 h at 37°C in a 5% CO_2 atmosphere and harvested by centrifugation, washed three times with distilled water and lyophilized. Dried cells were suspended (300 mg ml^{-1}) in saline, and autoclaved twice at 120°C for 30 min. The extracts were processed using a two-column chromatography procedure reported by Shibuya *et al.* (1991). Briefly, the antigen was purified by diethylaminoethyl Sephadex A-25 (Amersham Bioscience, Piscataway, NJ) ion-exchange chromatography and then by gel filtration using a Sephacryl S-300 (1.5 × 130 cm; fractionation range of 20,000 to 400,000; Amersham Bioscience). A molecular weight estimate of the serotype-specific antigen was obtained by gel filtration with blue dextran, dextran T150, T10 (Pharmacia, Uppsala, Sweden), and glucose as standard markers. The serotype-specific polysaccharide contents of antigen preparations was determined using the double immunodiffusion agar method using antibody against the new isolate, and a phenol–sulfuric acid method with glucose as the standard (Takada *et al.*, 1984; Shibuya *et al.*, 1991; Tsuzukibashi *et al.*, 2008).

Sugar composition

The polysaccharide antigen was hydrolysed with 2 M trifluoroacetic acid for neutral sugars and 4 M HCl for amino sugars at 100°C for 6 h. The hydrolysates were evaporated to dryness and the residues were dissolved in water. Neutral sugars in the hydrolysates were analysed by HPLC (LC-9A, Shimadzu Co., Kyoto, Japan) using a TSK-gel Sugar AXG column (150 × 4.6 mm; Toso Co., Tokyo, Japan). Amino sugars were analysed using an amino acid analyser (L-8500, Hitachi Co., Tokyo, Japan).

Gas chromatography

Gas chromatography (GC) was performed with a Hewlett-Packard 5890H gas chromatograph fitted with a flame ionization detector employing a capillary column (0.25 mm by 30 m; fused silica SPB-5) and a temperature program (80–280°C at 1°C min^{-1}) for methylated alditol acetate derivatives. GC-mass spectrometry (GC-MS) was carried out on a JMS-700V (Nippon Dennshi Co., Tokyo, Japan) applying electron impact ionization with an ionization potential of 70 eV.

Nuclear magnetic resonance

The ^{1}H-nuclear magnetic resonance (NMR) and ^{13}C-NMR spectra of polysaccharides were obtained with a Varian Unity Inova 600 spectrometer equipped with standard Varian software (^{1}H at 599.7 MHz and ^{13}C at 150.8 MHz) in deuterium oxide at 45°C with [2,2,3,3-D4]sodium 3-3-(trimethlsilyl) propanoate as an internal standard.

RESULTS

Biochemical and serological characteristics of the new isolate

During the isolation of *A. actinomycetemcomitans* strains using selective medium (Tsuzukibashi *et al.*, 2008), we obtained a serologically untypeable strain, designated as NUM-Aa 4039, from a patient with chronic periodontitis. The biochemical characteristics of the isolate and other serotype strains of *A. actinomycetemcomitans* were similar. The strain fermented glucose, fructose, mannose and maltose. Acid was not produced from sucrose, lactose, cellobiose, trehalose, melibiose, arabinose or salicin, and it produced catalase. *O*-nitrophenol galactoside was not hydrolysed. There was no dependence on X and V factors. *A. actinomycetemcomitans* NUM-Aa 4039 on BHIY agar exhibited an opaque star-like inner structural colony (Fig. 1A) and made aggregative cells in broth culture (Fig. 1B). In adherence assay, the biofilm-forming ability of the isolate was the strongest among a to f serotype strains of *A. actinomycetemcomitans* (Fig. 1C). The strong biofilm formation and cell aggregation in broth culture were not reduced upon continuous passage (once a week for 18 months). However, the colonies on plates became smooth in appearance (data not shown).

In a double immunodiffusion precipitin reaction the anti-NUM-Aa 4039 serum gave a single precipitin band with the corresponding NUM-Aa 4039 antigen but did not react with antigens from the other serotypes

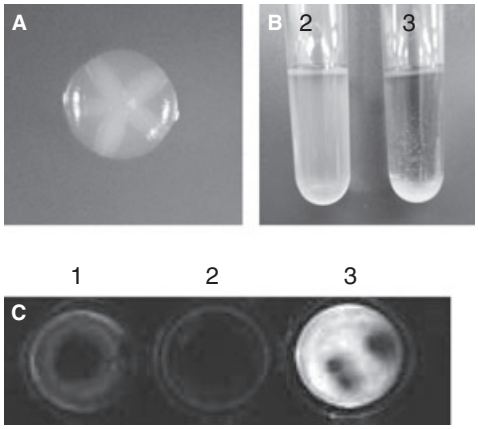

Figure 1 (A) Colony of NUM-Aa 4039 after growth on brain–heart infusion with yeast extract (BHIY) for 72 h at first isolation; (B) BHIY broth culture for 24 h; (C) qualitative adherence assay. Lane 1, ATCC43718; lane 2, ATCC33384^T; lane 3, NUM-Aa 4039.

(Fig. 2). Each serotype antibody reacted with the corresponding antigen.

Cytotoxic assay

The cytotoxic effect of *A. actinomycetemcomitans* ATCC700685-treated HL60 cells revealed >85% cell death, compared with <10% of untreated HL60 cells (Table 1). However, strains ATCC43718 and NUM-Aa 4039 did not cause significant HL60 cell death.

Polymerase chain reaction analysis

Species-specific products (557 bp) corresponding to *A. actinomycetemcomitans* were observed following PCR on genomic templates from strains ATCC33384^T and NUM-Aa 4039 (Fig. 3A). PCR using the serotype-specific (O-PS) primers was also performed. The serotype-specific PCR-amplified products of a through f produced 293, 333, 268, 411, 311, and 232-bp

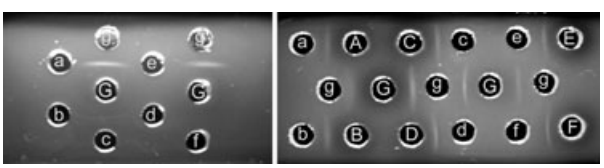

Figure 2 Double immunodiffusion analysis of autoclaved extract antigens of *Aggregatibacter actinomycetemcomitans* with serotype specific antibodies. Well: a, ATCC29523 (serotype a); b, ATCC 43718 (b); c, ATCC 33384^T (c); d, IDH781 (d); e, OMZ541 (e); f, NUM-Aa 5005 (f); g, NUM-Aa 4039; A, anti-a; B, anti-b; C, anti-c; D, anti-d; E, anti-e; F, anti-f; G, anti- NUM-Aa 4039.

Table 1 *Aggregatibacter actinomycetemcomitans*-mediated HL60 cell killing

Strain	% HL60 cell death
None	8.8 ± 1.1[1]
ATCC700685 (JP2-like)	42.7 ± 8.3
ATCC43718 (Y4)	13.3 ± 2.5
NUM-Aa-4039	12.7 ± 3.2

[1]Mean ± SD.

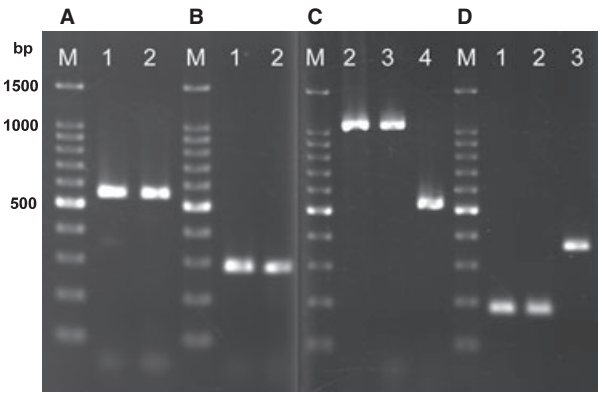

Figure 3 Polymerase chain reaction amplification of DNA from *Aggregatibacter actinomycetemcomitans* strains using primers to: (A) 16S rRNA; (B) leukotoxin A; (C) leukotoxin promoter; (D) *flp-1* start codon. Lane 1, ATCC33384^T; lane 2, NUM-Aa 4039; lane 3, ATCC43718 (Y4); lane 4, ATCC700685 (JP2-like); lane M, 100-base-pair ladder.

fragments, respectively, from each serotype strain. However, NUM-Aa 4039 was not amplified by any of these primers (data not shown). Leukotoxin A PCR products (285 bp) corresponding to central region of the *A. actinomycetemcomitans* leukotoxin gene (*lkt A*) were observed in the tested strains ATCC33384^T and NUM-Aa 4039 (Fig. 3B). However, both ATCC43718 and NUM-Aa 4039 strains gave rise to a single PCR product of 1034 bp (minimally leukotoxic), and ATCC700685, JP2-like strain gave an amplicon of 504 bp (highly leukotoxic) using primers within the leukotoxin promoter region (Fig. 3C). The PCR assay for *flp-1* upstream region showed the products of NUM-Aa 4039 and ATCC33384^T were both 179 bp and that of ATCC43718 was 363 bp (Fig. 3D).

Genetic characterization of the new isolate

The G+C content of DNA of the isolate was 41–43 mol%. The 16S rDNA sequence of strain NUM-Aa 4039 was determined for 1508 bases. Higher-level

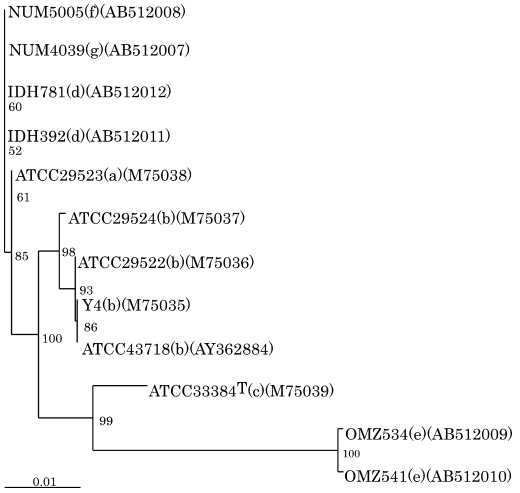

Figure 4 Phylogenetic tree derived from 16S ribosomal DNA sequences of *Aggregatibacter actinomycetemcomitans*. The tree, constructed using the neighbor-joining method, was based on comparisons of approximately 1330 nucleotides. Numbers on the tree indicate bootstrap values calculated for 1000 subsets for branch-points greater than 50%. Bar, 0.01 substitutions per nucleotide position. Serotype and accession number in parentheses.

homology was shown on comparison with *A. actinomycetemcomitans* serotype f, at 99.9%. A phylogenetic tree constructed by the neighbor-joining method is shown in Fig. 4. The new strain was located in the cluster of *A. actinomycetemcomitans*. The 16S rRNA gene sequences of strain NUM-Aa 4039 have been deposited in the DNA Database of Japan under accession number AB512007.

Characterization of the new serotype-specific antigen

The molecular weight of the column-purified surface antigen was estimated to be approximately 180,000. The yield of the purified antigen was about 24 mg from 43 g (dry weight) of whole cells. The purified antigen yielded a single precipitin band with the corresponding rabbit serotype-specific antiserum on double immunodiffusion, but did not react with antisera to the other serotypes (Fig. 2).

Structure of the new serotype-specific antigen

GC-MS analysis of the partially methylated alditol acetates obtained by the hydrolysis of methylated antigen showed the presence of 2,4-di-*O*-methyl-rhamnose and 2,3,6-tri-*O*-methyl-glucose, with a 1 : 1 M ratio.

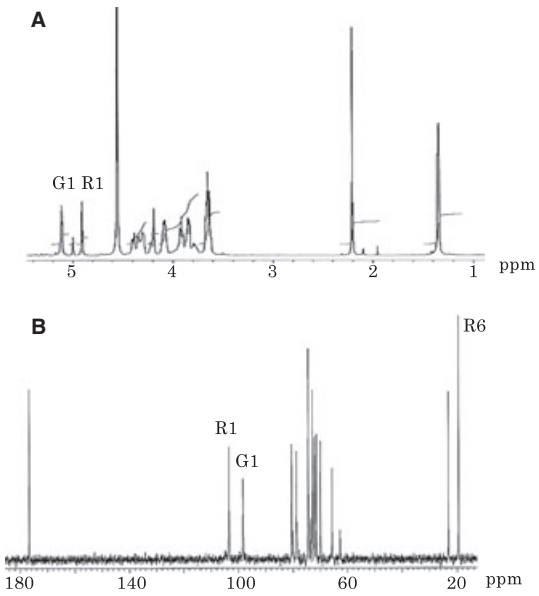

Figure 5 ^{1}H-Nuclear magnetic resonance (NMR) (A) and ^{13}C-NMR (B) spectra of serotype-specific antigen of *Aggregatibacter actinomycetemcomitans* NUM-Aa 4039 showing carbon atom resonances for glycose residues G1 and G6 [→ 4)-α-D-Glc*p*-(1 →], R1 [→ 3)-α-L-Rha*p*(1 →].

The ^{1}H-NMR (Fig. 5A) and ^{13}C-NMR (Fig. 5B) spectra of the antigen showed two anomeric proton signals at 5.08 and 4.86 p.p.m. and two anomeric carbon signals at 98.4 and 103.5 p.p.m. for glucose and rhamnose residues, respectively. Both constituent glycoses should have an α-anomeric configuration.

DISCUSSION

A. actinomycetemcomitans has been implicated as causative agent of localized aggressive periodontitis (LAP), which occurs in adolescents, resulting in marked inflammation, destruction of the periodontal ligaments and tooth loss (Slots *et al.*, 1980; Zambon, 1985).

Two of the pathogenicity factors in *A. actinomycetemcomitans* are aggregation and biofilm formation. Fresh clinical isolates of *A. actinomycetemcomitans* display a rough colony morphology phenotype with star-like centers and the cells adhere to surfaces to form extremely strong biofilms. However, most subcultured strains show smooth colony type (Tomich *et al.*, 2006). The aggregation and biofilm formation properties of NUM-Aa 4039 were strongest among the a to f serotype strains tested. Tight adherence is mediated by long fibrils of bundled pili that form on the surface of cells (Tomich *et al.*, 2006). The PCR

data showed that the sequences upstream from the *flp-1* gene in strain NUM-Aa 4039 grouped into type 2, including serotype c, d, e and f strains, whereas serotype b strains were grouped in type 1 (Kaplan *et al.*, 2002).

Production of leukotoxin, which kills host immune cells, is considered to be a key virulence factor in *A. actinomycetemcomitans*. The cytotoxic activity of NUM-Aa 4039 was similar to that of ATCC43718, and lower than that of ATCC700685. The PCR data support to these observations. NUM-Aa 4039 carried the leukotoxin A gene, whereas this strain and ATCC43718 (Y4) contained the minimally leukotoxic promoter. The highly leukotoxic JP2-like strain (ATCC700685) has a 530-bp deletion in this promoter region, resulting in enhanced production of leukotoxin (Brogan *et al.*, 1994). Kaplan *et al.* (2002) reported that all highly leukotoxic strains, except ATCC43718 (Y4) and ATCC29524, were serotype b, and that these comprised 25% of the isolates from African-American patients with LAP.

Studies on the prevalence of *A. actinomycetemcomitans* serotypes have suggested that serotypes a to c occur much more frequently among oral isolates than serotypes d and e, and that serotype b is the dominant *A. actinomycetemcomitans* serotype in subjects with LAP (Slots *et al.*, 1980; Gmür *et al.*, 1993; Asikainen *et al.*, 1995; Kaplan *et al.*, 2001). Some *A. actinomycetemcomitans* isolates do not react with any of the five (a–e) serotype-specific antisera (Paju *et al.*, 1998; Nakano *et al.*, 2007; Tsuzukibashi *et al.*, 2008). Kaplan *et al.* (2001) proposed a sixth *A. actinomycetemcomitans* serotype, designated serotype f. However, this was very close to serotype b. The serotype distribution from cardiovascular and dental plaque showed that most of the specimens could not be classified as any known serotype (a–f) (Nakano *et al.*, 2007). We have isolated a new serotype of *A. actinomycetemcomitans* from a periodontitis patient. The new serotype antigen of NUM-Aa-4039 consists of a disaccharide repeating unit composed of D-glucose and L-rhamnose, with a 1 : 1 M ratio. *A. actinomycetemcomitans* strains of all serotypes commonly produce polysaccharide antigens consisting of 6-deoxyhexoses. The deoxyhexose is 6-deoxytalose in serotypes a and c, and rhamnose in serotypes b, d, e and f strains (Shibuya *et al.*, 1991; Perry *et al.*, 1996a,b; Kaplan *et al.*, 2001). The serotype-specific antigen of NUM-Aa 4039 consists of rhamnose and glucose. The serotype d antigen consists of β-glucose residues. However, the configuration of glucose in NUM-Aa 4039 is α-anomeric.

Phylogenetic analysis showed that the 16S rDNA sequence of NUM-Aa 4039 was very close to those of serotype f and d strains. The upstream sequence of the 16S RNA gene of NUM-Aa4039 was identical with that of type 1, encompassing serotype a, d, e and f strains. Among type 1, the downstream sequences of serotypes a and e were different from those of serotypes d, f and g.

The experimental evidence leads to the characterization of the serotype antigen of *A. actinomycetemcomitans* serotype g as an unbranched high-molecular-weight polymer composed of repeating disaccharide units, with the structure: $\rightarrow 4)$-α-D-Glc*p*-$(1 \rightarrow 3)$-α-L-Rha*p*$(1 \rightarrow$. We propose that this untyped strain be assigned to a new *A. actinomycetemcomitans* serotype, designated as serotype g.

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