Plasmid Content of Some Oral Microorganisms Isolated from Subgingival Plaque

P.A. VANDENBERGH, S.A. SYED, C.F. GONZALEZ, W.J. LOESCHE, and R.H. OLSEN*

*Department of Microbiology, Medical School, and *Department of Oral Biology, Dental Research Institute, School of Dentistry, The University of Michigan, Ann Arbor, Michigan 48109*

Eighty-five strains of bacterial species selected from the predominant cultivable dental plaque flora of patients with different periodontal pathologies were examined for their plasmid content. Microorganisms studied included: Actinomyces viscosus, A. odontolyticus, Bacteroides asaccharolyticus (B. gingivalis), B. melaninogenicus *subspecies intermedius, and subspecies melaninogenicus,* Capnocytophaga ochracea (B. ochraceus), *and* Fusobacterium nucleatum. *Three* B. melaninogenicus *isolates showed plasmids of approximately 2.7-2.9 Mdalton (mega-dalton) molecular size. Restriction enzyme digests of the plasmids demonstrated dissimilar patterns when electrophoresed on agarose gels. In other microorganisms, including the Actinomyces species, plasmids were not observed.*

J Dent Res 61(3):497-501, March 1982

Introduction.

Streptococcus, Actinomyces, and Bacteroides species are prominent members of the dental plaque flora.[1-3] Some of these organisms appear to be specifically involved in certain forms of either dental caries or periodontal disease.[4] In particular, *Actinomyces viscosus* is associated with human root surface caries[5,6] and gingivitis,[7] *Bacteroides asaccharolyticus (B. gingivalis)* with periodontitis,[8] and *Capnocytophaga ochracea (B. ochraceus)* with juvenile periodontitis.[9] A strain of *A. viscosus* designated as T14 V was shown to be periodontopathic in animal models[10] and possibly to possess a 5 Mdalton plasmid.[11] A spontaneously occurring mutant of this strain known as T14 AV had reduced pathogenicity and was devoid of plasmid. A *C. ochracea* strain has been shown to have plasmids of 26 and 70 Mdaltons, the latter coding for resistance to chloramphenicol, kanamycin, tetracycline, and streptomycin.[12] These observations suggest that odontopathic members of the oral flora harbor plasmids. Enteropathogenic bacteria have been shown to possess both antibiotic and/or pathogenic determinants associated with their resident plasmids.[13] In order to determine whether a similar phenomenon occurs with the oral odontopathogens, a large number of recent isolates from clinical sites was surveyed for the presence of plasmids. In particular, strains of *A. viscosus, A. odontolyticus, A. israelii, B. melaninogenicus* subspecies intermedius and subspecies melaninogenicus, *B. asaccharolyticus, C. ochracea,* and *Fusobacterium nucleatum* were examined for the presence of indigenous plasmids using a sensitive lysis technique. Plasmids were rarely found and then only in the *B. melaninogenicus* subspecies.

Materials and methods.

Plaque collection. – Plaque was collected from diabetic children, ages six to 12 yr, and from adult patients with periodontitis. In the children, gingival-margin plaque was removed from the mesio-buccal surface of the maxillary first molar by means of an abrasive metal strip (lightening strip)[1] held in a hemostat. The strip with adherent plaque was dropped into 4 ml of a reduced transport fluid (RTF).[14] In the adults, all supragingival plaque was removed from a tooth and discarded. A separate curette was inserted to the base of the pocket, and the adherent plaque was removed and placed into the RTF.

Identification of isolates. – The plaque samples were brought into an anaerobic glove box* and dispersed for 20 s using a Kontes sonifier.† The dispersed plaque was serially diluted in RTF and plated on a variety of selective and nonselective media by means of a Spiral plater.§ After six to eight d of anaerobic incubation, colonies on an enriched Trypticase Soy Agar (ETSA),[15] which had a brown-black or red-brown pigment, were subcultured for purity and characterized as Bacteroides or Actinomyces species by the brief scheme shown in Table 1. Flat spreading colonies typical of *Capnocytophaga ochracea*[16] were found to be gram-negative, long, thin rods on microscopic examination. *A. viscosus* colonies were isolated on the selected GMC medium and shown to be gram-positive branching rods that were catalase positive.[17] Colonies typical of *F. nucleatum* and *A. israelii* were also subcultured and characterized (Table 1).

The various isolates were grown anaerobically to approximately 2 x 10^8 cells per ml in 250 ml of glucose broth for 48-72 h. This medium contained per liter: 10 g of Tryptone,‡ 5 g of yeast extract, 5 g of sodium chloride, 0.5 g of potassium nitrate, 10 g of glucose, 1 mg of hemin, 1 mg of menadione, 0.5 g of sodium carbonate, 0.2 g of dithiothreitol, and 75 ml each of mineral salt solutions 1 and 2.[14] The purity of the broth cultures of each microorganism was confirmed by microscopic examination of gram-stained smears, cultivation of the organism on ETSA agar medium, and by cultural tests.

Preparation of lysate – Method I. – Cells were harvested by centrifugation and suspended in 4 ml of 9% sucrose in 0.05 M Tris (hydroxymethyl) aminomethane (Tris), pH 8.0 (T/S buffer). Lysates were prepared by a modification of the method of Guerry *et al.*[18] Disodium ethylenediaminetetracetate (0.8 ml of 0.5 M EDTA, pH 8.0) was added to the suspension and then mixed. After the addition of 0.4 ml of lysozyme, ¶ 10 mg/ml in 0.25 M tris, pH 8.0, the suspension was placed in a 37°C water bath for five min. Sodium dodecyl sulfate (SDS), 0.8 ml of a 15% solution, was added, and the mixture was incubated in a 37°C water bath for 15-20 min with several inversions until lysed. After cellular lysis, 1.4 ml of 5 M NaCl were added to the suspension and were mixed with gentle inversion. The lysates were stored

Received for publication March 19, 1981
Accepted for publication July 16, 1981
This investigation was partially supported by the DRI Associate Program from the Office of the Vice President for Research, The University of Michigan, and NIH-NIDR Grant No. DE-02731.
Requests for reprints: Dr. P.A. Vandenbergh, Microlife Genetics, POB 2339, 1817-57th St., Sarasota, FL 33578

*Coy Laboratory Products, Ann Arbor, MI
†Kontes Biomedical Products, Vineland, NJ
§Spiral Systems, Inc., Gaithersburg, MD
‡Difco Laboratories
¶Sigma Chemical Co.

overnight at 4°C, then centrifuged at 17,000 xg (4°C, 30 min). The volume of the supernatant was measured and was decanted into chilled polypropylene centrifuge tubes; 0.313 volume of PEG 6000[#] (42% wt/vol, in 0.01 M sodium phosphate buffer, pH 7.0) was added. The tubes were refrigerated overnight, followed by centrifugation at 3000 xg (4°C, ten min). The resulting pellets were resuspended in 0.15 ml of TES buffer containing 0.005 M Tris, 0.005 M NaCl, and 5 mM Na_2 EDTA (pH 8.0).[13]

Preparation of small-volume culture lystate –Method II. – The above lysis procedure was modified for the utilization of an Eppendorf centrifuge (Model 5412). Bacteria were grown to approximately 2 x 10^8 cells per ml in a 10-ml broth culture. A 1.5-ml aliquot of this suspension was pelleted by centrifugation in 1.5 ml polypropylene tubes. The pellet was resuspended in 0.65 ml of T/S buffer, using a Pasteur pipette. After the addition of 0.13 ml EDTA and of 0.10 ml lysozyme, the suspension was mixed and was incubated for five min in a 37°C water bath. To this mixture, 0.13 ml of SDS was added, and the suspension was incubated for 15-20 min in a 37°C water bath until the cells were lysed. After cellular lysis, 0.23 ml of 5 M NaCl solution was added to the above mixture, and the tube and its contents were immersed in an ethanol-dry ice bath for two min. This immersion was followed by centrifugation for two min. The supernatant was removed and was dispensed into another cold 1.5-ml polypropylene tube. To the measured supernatant, 0.313 volume of PEG 6000 (42% wt/vol, in 0.01 M sodium phosphate buffer, pH 7.0) was added. Tubes were then incubated overnight at 4°C. Samples were then centrifuged for two min, and the precipitates were resuspended in 0.05 ml TES buffer.

Preparation of lysate – Method III. – Lysates were prepared by a modification of the procedure of Guerry *et al.*[18] All solutions utilized were as prepared in the Method I procedure. Cells were harvested, were suspended in 10 ml of T/S buffer, and then exposed to an increased amount of EDTA (2 ml). Lysozyme (1 ml) was added, followed by incubation for 60 min in a 37°C water bath. The remainder of this procedure followed that of Method I.

Cesium chloride-ethidium bromide gradients. – Cells from a 250-ml broth culture were concentrated and were lysed by either Method I or III as described above. The PEG-precipitated DNA was resuspended in 8 ml of TES, to which was added 8 g of CsCl and 0.6 ml ethidium bromide (10 mg/ml). Centrifugation was carried out at 15°C in a Spinco Type 65 fixed angle rotor for 60 h at 40,000 rpm using an L3-50 Beckman ultracentrifuge.

Restriction endonuclease digestion. – Restriction endonucleases *Eco*RI, *Bam*HI, *Bgl*I, *Bgl*II, *Hind*III, *Sal*I, *Sma*I, and *Xba*I were purchased from the Bethesda Research Laboratories, Inc. Plasmid DNA preparations (1.0 – 2.0 μg) were digested by one of the following enzyme preparations: with *Eco*RI in the presence of 100 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, and 50 mM NaCl; with *Bam*HI in the presence of 20 mM potassium phosphate (pH 7), 100 mM NaCl, 7 mM $MgCl_2$, and 2 mM 2-mercaptoethanol; with *Bgl*I in the presence of 20 mM Tris-HCl (pH 7.4), 7 mM $MgCl_2$, and 7 mM 2-mercaptoethanol; with *Hind*III in the presence of 20 mM Tris-HCl (pH 7.4), 7 mM $MgCl_2$, and 60 mM NaCl; with *Sal*I in the presence of 8 mM Tris-HCl (pH 7.6), 6 mM $MgCl_2$, 0.2 mM Na_2EDTA, and 150 mM NaCl; with *Sma*I in the presence of 15 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, and 15 mM KCl; or with *Xba*I in the presence of 6 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 6 mM $MgCl_2$. The sizes of the restriction fragments were estimated from the distribution of *Hind*III fragments of bacteriophage lambda DNA.

Agarose gel electrophoresis. – Samples of 10-30 μl of PEG-precipitated DNA were subjected to electrophoresis as previously described.[13,19]

[#]Carbowax 6000, Union Carbide, Schwarz/Mann

Results.

Fifty-nine isolates of Bacteroides species were surveyed for their plasmid content. Three strains of *B. melaninogenicus* were found to contain plasmid DNA (Table 2). No plasmids were found in the *B. asaccharolyticus* strains or other gram-negative species examined, including *F. nucleatum* or *C. ochracea.*

Plasmid DNA was not observed in the Actinomyces species, which we surveyed utilizing Method III, including the Hammond strain T14V. These results corresponded to similar studies performed by D.B. Clewell *et al.* (personal communication), using a different lysis technique specific

TABLE 1
CRITERIA USED TO CHARACTERIZE RECENT PLAQUE ISOLATES

Species	Colony	Gram Reaction	Glucose Fermentation	Indole Production	Gelatinase	Esculin hydrolysis	NO_3 Reduction	Catalase
F. nucleatum	opalescent	– R[a] fusiform	–	+	–	–	–	–
B. asaccharolyticus	black pigment	– CB[b]	–	+	+	–	–	–
B. melaninogenicus								
subspecies melaninogenicus	black pigment	– CB	+	–	+	+	–	–
subspecies intermedius	black pigment	– CB	+	+	+	–	–	–
A. odontolyticus	red-brown pigment	+ R branching	+	–	–	V	+	–
C. ochracea	spreader, yellow pigment	– fusiform	+	–	–	+	–	–
A. viscosus	growth on GMC agar	+ R branching	+	–	–	V	+	+
A. israelii	white, molar tooth	+ R branching	+	–	–	+	+	–

[a]Rods.
[b]Coccobacilli.

TABLE 2
PRESENCE OF PLASMIDS IN HUMAN PERIODONTOPATHIC BACTERIA

Organism	No. of Strains Tested	No. of Strains with Plasmid	Lytic Procedure
A. viscosus	17[a]	0	Method III
A. odontolyticus	4	0	Method III
A. israelii	1	0	Method III
B. melaninogenicus			
subspecies intermedius	44[b]	2[e]	Method I, II
subspecies melaninogenicus	4[c]	1[f]	
B. asaccharolyticus	11[d]	0	Method I, II
Capnocytophaga ochracea	4	0	Method I, II
F. nucleatum	7	0	Method I, II

[a]Four reference strains ATCC 15987, ATCC 19246, T14V, and T14AV included.
[b]Reference strain ATCC 25611 was included.
[c]Reference strain ATCC 25485 was included.
[d]Reference strain ATCC 25260 was included.
[e]Positive strain JPD was isolated from patient with juvenile periodontitis; strain DM from advanced periodontitis and Diab. J-1 from a diabetic child.
[f]Strain DM was isolated from advanced periodontitis.

for gram-positive microorganisms. These investigators independently received the Hammond strains T14V and T14AV, which had been tested for their pathogenicity in the animal model. After thorough examination, these investigators were unable to detect plasmid DNA in the *A. viscosus* strain T14V.

Lysates of the three plasmid-containing strains of *B. melaninogenicus* were analyzed for the presence of covalently closed circular (CCC) DNA by centrifugation in ethidium bromide-cesium chloride gradients. The partially purified (CCC) DNA was then dialyzed against TES buffer and subjected to electrophoresis (Fig. 1). Plasmid DNA from *E. coli* strain V 517 was co-electrophoresed with DNA from the plaque isolates as a size standard.[20] The plasmids from *B. melaninogenicus* subspecies melaninogenicus, strain DM, was designated pPV 3. This plasmid was determined to be 2.7 Mdalton in size.

The purified plasmid DNA was subjected to restriction endonuclease digestion utilizing *Eco*RI, *Bam*HI, *Bgl*II, *Hind*III, *Sal*I, *Sma*I, and *Xba*I. As depicted in Fig. 2, the plasmid pPV 1 was observed to have one *Hind*III site, while being resistant to cleavage by the remainder of the above-mentioned enzymes. The plasmid pPV 2 showed two *Hind*III sites, resulting in fragments of 1.79 Mdalton and 1.13 Mdalton, and one *Eco*RI site. The plasmid pPV 2 was observed to possess one *Bgl*I site, whereas pPV 2 was not cleaved by *Bam*HI, *Bgl*II, *Sal*I, *Sma*I, or *Xba*I, respectively (data not shown). The plasmid pPV3 was observed to have one *Hind*III site, while being resistant to cleavage by the remainder of the enzymes tested (data not shown).

Discussion.

The lytic procedures, Method I and Method II, developed in our laboratory have enabled us to accurately estimate the plasmid content of many oral bacterial isolates. These results were obtained after the examination of each isolate three times. Plasmid-containing, gram-negative bacteria were utilized as positive controls. The miniaturization of Method I allowed for a more rapid procedure to effectively evaluate large numbers of isolates for the presence of resident plasmids. The plasmid content of the Actinomyces species was surveyed by Method III, utilizing the plasmid containing *S. coelicolor* A 332 strain as a control.

This survey indicated that, of the periodontopathic species tested, *B. asaccharolyticus, C. ochracea, F. nucleatum,* and *A. viscosus* did not harbor resident plasmids.

The inability to demonstrate plasmids from *A. viscosus* strains T14V, ATCC 19246, and ATCC 15987 was unexpected, since these strains had previously been reported to contain a 5 Mdalton plasmid.[11] The reported antigenic gene product 6-deoxy-L-talose has been the subject of reports concerning the role of *A. viscosus* in periodontal disease.[10,11]

The Bacteroides species' plasmids studied demonstrated similar sizes, yet variable electrophoretic patterns upon digestion with restriction endonucleases. These results indicate uncommon evolutionary origins for these plasmids. Previously, other investigators have reported the isolation of several cryptic plasmids from *B. fragilis;*[21,22] a conjugative lincosamide-macrolide resistance plasmid in *B. fragilis* and *B. uniformis;* cryptic plasmids from *B. ochraceus;*[24] and an R plasmid from *B. ochraceus* coding for chloramphenicol, tetracycline, kanamycin, and streptomycin resistance.[12]

Similar studies completed by other investigators involved the oral microorganism *Streptococcus mutans.*[25] Based on their survey of 86 isolates, approximately 5% of all naturally occurring strains of this microorganism contained a 3.6 Mdalton cryptic plasmid. Recently, this cryptic plasmid pVA318 of *Streptococcus mutans* has been cloned into the vector pBR322 in *Escherichia coli.*[26] Cloned variants produced a pVA318-specific protein of unknown biological function.

Conclusions.

Eighty-five bacterial isolates, which included *Actinomyces viscosus, A. odontolyticus, Bacteroides asaccharolyticus, B. melaninogenicus* subspecies intermedius, and subspecies melaninogenicus, *Capnocytophaga ochracea (B. ochraceus),* and *Fusobacterium nucleatum,* were examined for their resident plasmid content. Three *B. melaninogenicus* isolates showed plasmids with molecular sizes of approximately 2.7-2.9 Mdalton.

Plasmid DNA was not detected in any of the Actinomyces species utilizing our lysis procedure. These results were similar to the observations of other investigators utilizing a different lysis procedure. Contrary to earlier reports, we were unable to detect extrachromosomal DNA

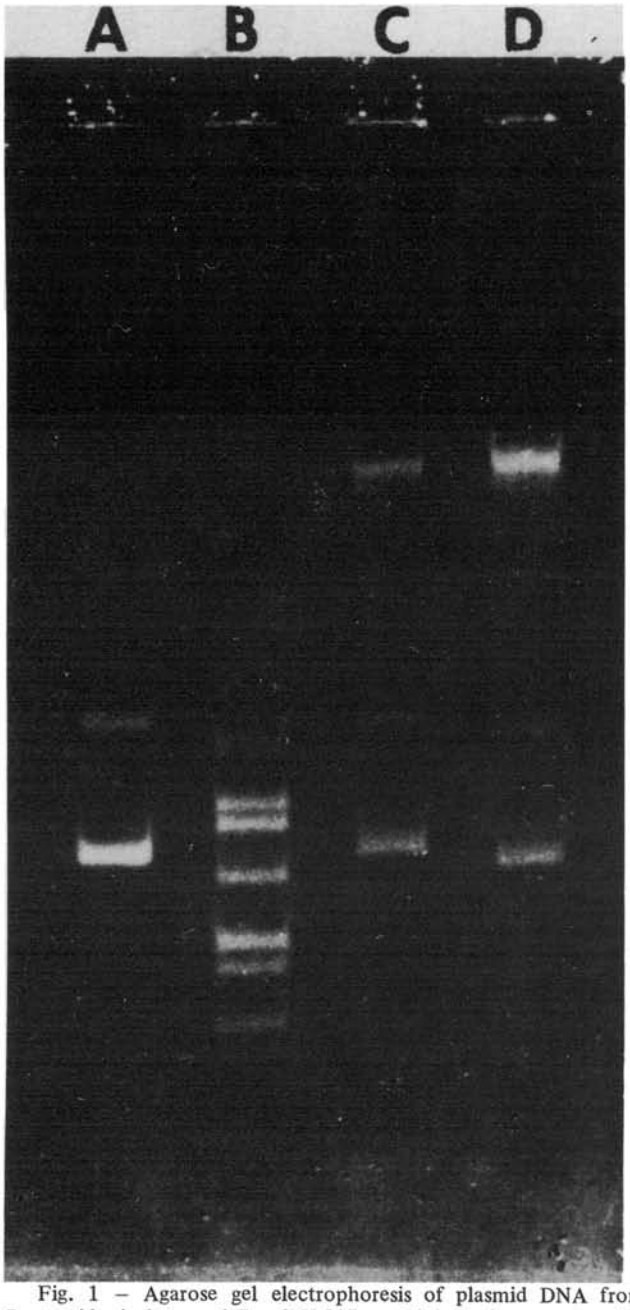

Fig. 1 – Agarose gel electrophoresis of plasmid DNA from Bacteroides isolates and *E. coli* V 517 containing reference plasmids. (A) *B. melaninogenicus* J-1 (pPV 1), CCC DNA lower band, open circular (OC) DNA middle band and fragmented chromosomal DNA upper band; (B) *E. coli* V 517, 1.4, 1.8, 2.0, 2.6, 3.4, 3.7, and 4.8 Mdalton (lower to upper bands, respectively): (C) *B. melaninogenicus* JPD (pPV 2), CCC DNA lower band, OC DNA middle band and fragmented chromosomal DNA upper band; (D) *B. melaninogenicus* DM (pPV 3), CCC DNA lower band, OC DNA middle band, and fragmented chromosomal DNA upper band.

from the *A. viscosus* strains T14V, ATCC 19246, and ATCC 15987.

Acknowledgments.

The authors wish to thank Dr. Benjamin F. Hammond who provided *A. viscosus* strains T14V and T14AV.

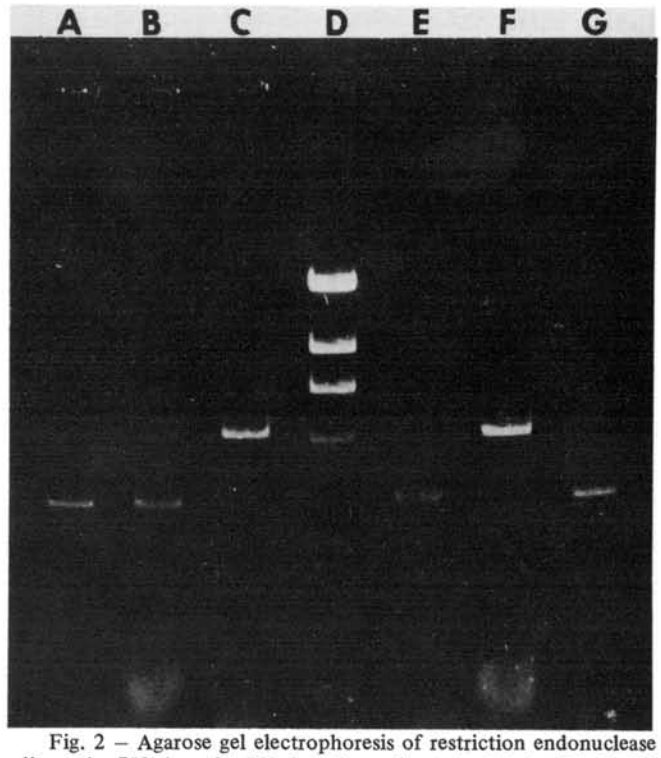

Fig. 2 – Agarose gel electrophoresis of restriction endonuclease digested pPV 1 and pPV 2. (A) undigested pPV 1; (B) *Eco*RI digested pPV 1; (C) *Hind*III digested pPV 1; (D) *Hind*III treated λ-DNA; (E) *Hind*III digested pPV 2; (F) *Eco*RI digested pPV 2; and (G) undigested pPV 2.

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