Native Plasmids of *Fusobacterium nucleatum*: Characterization and Use in Development of Genetic Systems[†]

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Received 15 July 1999/Accepted 19 November 1999

Three native plasmids of *Fusobacterium nucleatum* were characterized, including DNA sequence analysis of one plasmid, pFN1. A shuttle plasmid, pHS17, capable of transforming *Escherichia coli* and *F. nucleatum* ATCC 10953 was constructed with pFN1. pHS17 was stably maintained in the *F. nucleatum* transformants, and differences in the transformation efficiencies suggested the presence of a restriction-modification system in *F. nucleatum*.

Fusobacterium nucleatum is a gram-negative anaerobe of interest due to its central role in the ecology of dental plaque, a complex microbial biofilm that forms on teeth (6, 17), and its association with human infections (5, 12, 21, 22). A significant hindrance to the study of *F. nucleatum* is the lack of genetic and molecular systems for the construction of trait-specific isogenic mutants which are essential for delineation of gene function in the native cell background. A homologous family of native cryptic plasmids has been reported to occur in 18% of the *F. nucleatum* strains examined (20). This study was initiated to investigate the utility of native plasmids in the development of gene transfer systems for *F. nucleatum*. Data are presented on the characterization of three native *F. nucleatum* plasmids isolated in our laboratory, including determination and analysis of the complete DNA sequence of one plasmid, pFN1. Also described are the use of pFN1 in the construction of an intergeneric shuttle plasmid in *Escherichia coli*, transformation of *F. nucleatum* with the shuttle plasmid, and analysis of its stability in the *F. nucleatum* host cell background. To our knowledge, this is the first report of transformation of *F. nucleatum* by electroporation.

Isolation and characterization of *F. nucleatum* plasmids. Three native plasmids (pFN1, pFN2, and pFN3) (Table 1; Fig. 1A) were isolated from strains of *F. nucleatum* by routine techniques (Wizard Plus Minipreps [Promega, Madison, Wis.]; Midi Preps [Qiagen, Inc., Valencia, Calif.]) and visualized on ethidium-stained 0.8% agarose gels. Restriction endonuclease mapping (16) demonstrated that the plasmids varied in size and in the occurrence of several restriction endonuclease sites (Fig. 1A), suggesting that the plasmids were unrelated. However, Southern hybridization studies (15) indicated that pFN1 and pFN2 share homology with each other but not with pFN3 (Fig. 1B and C). Nitrocellulose blots of plasmid and chromosomal DNA preparations from the plasmid-containing host strains were probed with pFN1 and pFN3 DNA. The pFN1 probe hybridized to pFN1 and pFN2 DNA but not pFN3 DNA (Fig. 1B), whereas the pFN3 probe hybridized only to pFN3 DNA (Fig. 1C). No hybridization to chromosomal DNA from any of the host strains was evident (data not shown). The strain harboring pFN3, ATCC 10953, was previously reported to lack

plasmid DNA (20). Due to this discrepancy, we obtained a new culture from the American Type Culture Collection (Rockville, Md.) and confirmed the presence of pFN3 in this strain. These data reveal the existence of two nonhomologous groups of plasmids indigenous to *F. nucleatum*, the first represented by pFN1 and pFN2 and the second represented by pFN3.

Determination and analysis of pFN1 DNA sequence. Due to its small size and superior plasmid yields, pFN1 was chosen for further analysis. The DNA sequence was determined for both strands. Analysis of the compiled sequence revealed a circular structure of 5,887 bp with 23% G+C content and seven putative open reading frames (ORFs) (defined as ≥ 150 bp [Fig. 2A]). Similarity searches were performed using the National Center for Biotechnology Information BLAST server (1, 2). The sequence of pFN1 was highly homologous to the sequence of a 6,281-bp *F. nucleatum* plasmid (pAD52; GenBank accession no. AF022647). No similarity was found to any gene encoding antibiotic resistance or other selectable phenotypic marker. Antibiotic susceptibility testing indicated that the pFN1 host strain *F. nucleatum* 12230 was susceptible to penicillin G, tetracycline, chloramphenicol, clindamycin, cefoxitin, ampicillin-sulbactam, imipenem, metronidazole, and streptomycin and resistant to erythromycin at a concentration of 25 μ g/ml, as is common in other strains of *F. nucleatum* (7). These data suggested that pFN1 is a cryptic plasmid with respect to antibiotic resistance, comparable to previous findings with this group of plasmids (20).

ORF1 is related to DNA relaxase (mobilization) proteins which mediate the initiation of conjugal transfer of plasmid DNA (13). Alignment of the complete predicted amino acid sequences using Clustal W (11, 28) of ORF1 with *Staphylococcus* plasmid relaxases demonstrated 23 to 29% identity and 30 to 34% similarity. Homology to the four regions of the consensus sequence defined for relaxase proteins (13) was evident (Table 2). The putative active tyrosine was the only residue of N-terminal motif 3 of the consensus sequence identical to a residue of the pFN1 ORF1, but this motif demonstrated a weak consensus sequence in the other proteins analyzed (13). Additional studies are needed to clarify the functional properties of this putative relaxase in *F. nucleatum*.

ORF5 analyses indicated that it is related to plasmid replication proteins, including those of *Lactobacillus acidophilus* plasmid pLA103 (14), *Staphylococcus aureus* plasmid pJE1 (4), and *Pediococcus halophilus* plasmid pUCL287 (3). Alignment of the complete ORFs of homologues with pFN1 ORF5 demonstrated 10 to 19% identity and 21 to 34% similarity. The association of ORF5 with replication was strongly supported

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[†] We dedicate this work to the memory of Susan E. Valone in recognition of her support and contributions to this research.

TABLE 1. Relevant characteristics of the bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristics ^a	Source and/or reference
Strains		
<i>E. coli</i> DH5 α	Ery ^r	Life Technologies
<i>F. nucleatum</i> 12230	Cln ^s ; transtracheal isolate and source of pFN1	S. Finegold, Wadsworth Anaerobe Laboratory, West Los Angeles VA Medical Center, Los Angeles, Calif.
<i>F. nucleatum</i> 10113	Cln ^s ; clinical isolate and source of pFN2	S. Finegold
<i>F. nucleatum</i> ATCC 10953	Cln ^s ; gingival isolate and source of pFN3	American Type Culture Collection
Plasmids		
pBluescript SK(–)	Amp ^r ; 3.0-kb <i>E. coli</i> cloning vector	Stratagene Cloning Systems
pVA2198	Ery ^r ; 9.2-kb plasmid with the <i>ermF-ermAM</i> cassette	F. Macrina (8)
pFN1	5.9-kb native <i>F. nucleatum</i> plasmid	This study
pFN2	7.2-kb native <i>F. nucleatum</i> plasmid	This study
pFN3	11.1-kb native <i>F. nucleatum</i> plasmid	This study
pHS17	Amp ^r Ery ^r ; 10.0-kb plasmid consisting of pFN1, <i>ermF-ermAM</i> , and pBluescript minus its ampicillin resistance determinant	This study
pHS19	Amp ^r Ery ^r ; 4.1-kb plasmid consisting of <i>ermF-ermAM</i> and pBluescript minus its ampicillin resistance determinant	This study

^a Abbreviations: Amp, ampicillin; Ery, erythromycin; Cln, clindamycin.

by analyses of the upstream DNA sequence, which demonstrated six perfect 22-bp direct repeats (or iterons) preceded by an approximately 200-bp A-T-rich region (Fig. 2B). Multiple putative DnaA binding sites were also identified, based on matching 8 of the 9 bp comprising the DnaA binding consensus sequence (26). This organization is characteristic of the origin of replication of iteron-regulated theta-replicating plasmids (10). A general model of replication initiation involves the binding of the plasmid replication protein to the iteron sequences, resulting in structural changes (including melting of the adjacent A-T-rich region) to form an open complex. The replication protein, possibly in conjunction with the host DnaA protein, is then responsible for guiding host replication proteins into the open complex (10). It is also significant that the pFN1 replication protein homologue was related to the replication protein of pUCL287, which has been shown to utilize a theta mode of replication (3).

Transformation of *F. nucleatum* with shuttle plasmid pHS17. The shuttle plasmid pHS17 (Fig. 1) was constructed sequentially in *E. coli* DH5 α cells (Life Technologies, Gaithersburg, Md.) as follows: *AvrII*-digested pFN1 was cloned into the *XbaI* site of pBluescript; an *ermF-ermAM* cassette (8) was added by cloning into *KpnI-PstI* sites; and the pBluescript ampicillin resistance determinant was deleted by digestion with flanking *BspHI* sites. The resulting construct included both *E. coli* and *F. nucleatum* origins of replication from pBluescript and pFN1, respectively. The junctions of DNA fragments joined by cloning were confirmed by DNA sequencing, and the phenotypic properties of the construct were confirmed on selective medium.

Transformation studies were performed with plasmid DNA isolated by alkaline lysis-column purification techniques (Promega Wizard Plus Minipreps; Qiagen Midi Preps) and further purified by cesium chloride-ethidium bromide density gradient centrifugation (25). Bacterial cells were washed and resuspended in electroporation buffer (8) at a calculated optical density of 6.0, and 100- μ l aliquots were electroporated by standard techniques (27). The electroporated cells were immediately diluted in 0.9 ml of Columbia broth (BBL Microbiology Systems, Cockeysville, Md.) with MgCl₂, and the number of viable cells were determined by plating a diluted aliquot on

FIG. 1. Restriction maps and Southern blots of the *F. nucleatum* plasmids. (A) Partial restriction map of *F. nucleatum* plasmids pFN1, pFN2 and pFN3 and shuttle plasmid pHS17. Selected restriction endonuclease sites in the native plasmids are presented. Restriction endonuclease sites indicated for pHS17 relate to the plasmid construction. The pFN1 portion of pHS17 is indicated by the thick solid bar, with the position of the *repA* homologue (ORF5) and putative *ori* indicated. (B and C) Plasmids from *F. nucleatum* strains 12230 (pFN1, lanes 1), 10113 (pFN2, lanes 2), and ATCC 10953 (pFN3, lanes 3) were probed with pFN1 DNA with *EcoRI* digests (B) or pFN3 DNA with *EcoRV* digests (C). The *HincII*-digested pFN1 and *AseI*-digested pFN3 probes were ³²P labeled (specific activity of 25 × 10⁸ and 4.5 × 10⁸ dpm/μg of DNA, respectively). The positions of molecular size markers are indicated on the left, and the linear forms of pFN1, pFN2, and pFN3 are indicated on the right.

FIG. 2. Physical characteristics of pFN1 based on DNA sequence analysis. (A) ORFs, the putative origin of replication (*ori*), and selected restriction endonuclease sites are indicated. (B) Structural elements of putative origin of replication found upstream of ORF5, the *repA* homologue. The putative origin contains an A-T-rich region (crosshatched bar), six perfect 22-bp direct repeats (▶) termed iterons, and several putative DnaA-binding sites (■).

nonselective medium. The transformation mix was incubated anaerobically, followed by plating on Columbia agar (BBL Microbiology Systems) with clindamycin. Variables examined included the bacterial cell growth phases (early log, mid-log, and stationary phases), the source of pHS17 DNA (heterologous versus homologous host sources), electroporation parameters (resistance of 50 to 500 Ω ; field strength of 24 or 25 kV/cm; capacitance of 25 or 50 μ F), the concentration of $MgCl_2$ in the Columbia broth (0.5, 1.0, and 2.0 mM), and the clindamycin concentration used in the selective medium (0.2 or 0.4 μ g/ml).

Initial attempts to transform *F. nucleatum* ATCC 10953 with

FIG. 3. Plasmid DNA from *F. nucleatum* ATCC 10953 transformants consists of the shuttle plasmid, pHS17, and the native plasmid, pFN3. Plasmid preparations from *E. coli* (pHS17), *F. nucleatum* ATCC 10953 transformant strain KH21 (pHS17 and pFN3), and *F. nucleatum* ATCC 10953 (pFN3) were analyzed. The preparations were either not digested (lanes 1) or predigested with *EcoRV* (lanes 2) or *EcoRI* (lanes 3), separated on 0.8% agarose gels, stained with ethidium bromide, and visualized under UV illumination. The open circular (OC), linear (L), and covalently closed circular (CC) forms of pHS17 and pFN3 are indicated on the left and right, respectively.

pHS17 were successful under previously defined conditions (9, 24, 27). Preliminary results indicated optimal recovery of transformants with a field strength of 25 kV/cm, a capacitance of 25 μ F, and resistance ranging from 200 to 400 Ω . Analysis of the transformants revealed the presence of pHS17 and the ATCC 10953 native plasmid pFN3. The two plasmids were easily distinguished, based on their sizes and restriction endonuclease digestion patterns (Fig. 3). Electroporation controls included nonelectroporated cells with or without the addition of DNA as well as electroporated cells without DNA added, and all yielded negative results. Electroporation with pHS19 also yielded negative results, suggesting that pFN1 is essential for replication in *F. nucleatum*. The single parameter found to have a major effect on transformation efficiency was the pHS17 DNA source. Transformation efficiency using 1 μ g of plasmid DNA ranged from 1.6×10^2 to 2×10^2 transformants per μ g of DNA from the homologous *F. nucleatum* host, as compared to no transformants with DNA from the heterologous *E. coli* host (Table 3). Transformation efficiency was optimal at a resistance setting of 200 Ω with the homologous host DNA,

TABLE 2. Amino acid alignment of fusobacterial and staphylococcal plasmid relaxase proteins within the relaxase consensus sequence motifs

Plasmid and protein	Amino acid alignment in motif:			
	3	z	2a	2
Consensus ^a	-----n- <u>Y</u> ---	----HuUU-Sf--ge	u-ua--uH-d-	--pHuHuuu--u-----
Alternate		w t	u	
pFN1 ORF1 ^b	LYNILK-YV- 41 : *	GRQYRHHIQSFKPGE 20 *****:*	FDFVISTHIDK : * : * * :	GHIHNHIIINTVNIDTG * * * * * : * : * *
pS194 R1x	SRA-IN-YA- 33	VQA-HTVIQSFKPGE 20	YQVAVYTHTDK	DHYHNHIIINSVNLETG
pC223 Orf1	SRA-IN-YA- 33	NEG-HVVIQSFKPNE 20	HQVQVYTHNDT	DHVHNHIVINSIDLETG
pC221 OrfA	SRA-IN-YA- 33	IQA-HTVIQSFKPGE 20	HWVAVYTHTDK	DHYHNHIVINSVDLETG
pSK639 ORF337	SRA-IN-YA- 32	GNQGHVIIQSFKPGE 20	HQVAVYTHNDT	DHVHNHIVINSIDLETG
pIP1630 MobA2	SRA-IN-YA- 32	GNQGHVIIQSFKPGE 20	HQVAVYTHNDT	DHVHNHIVINSIDLETG
pIP1629 MobA1	SRA-IN-YA- 32	GNQGHVIIQSFKPGE 20	HQVAVYTHADT	DHVHNHIVMNSIDLETG

^a Consensus sequence abbreviations: uppercase letters, conserved; lowercase letters, present in 50% of sites; U or u, bulky hydrophobic residues (I, L, V, M, F, Y, and W); -, no consensus at this site; Y, putative active tyrosine residue.

^b Asterisks (*) above the pFN1 amino acid residues indicate identity with the consensus sequence. Asterisks below the pFN1 amino acid residues indicate identity with all the staphylococcal sequences, while a colon (:) indicates identity with most, or similarity with all, of the staphylococcal sequences. Numbers between motifs indicate numbers of intervening residues.

TABLE 3. Transformation efficiency in *F. nucleatum* ATCC 10953 with DNA isolated from homologous versus heterologous strains^a

Resistance (Ω) ^b	Transformation efficiency		
	Plasmid DNA from heterologous strain (pHS17)		Plasmid DNA (1 μ g) from homologous strain (pHS17 + pFN3)
	1 μ g	5 μ g	
400	0	1.2×10^1	1.6×10^2
300	0	0	1.9×10^2
200	0	1	2.0×10^2

^a Transformation efficiency was calculated as the number of transformants per milliliter per microgram of DNA (27). The average number of viable cells remaining after electroporation at 400, 300, and 200 Ω was 0.39×10^8 , 1.1×10^8 , and 2.1×10^8 , respectively. Plasmid DNA was isolated from a heterologous host (*E. coli*; pHS17) or from a homologous host (*F. nucleatum* KH21; pHS17 and pFN3). The quantitation of plasmid DNA is based on total DNA in the preparation.

^b See text for additional electroporation parameters. Outgrowth period was approximately 12 h.

although pronounced differences were not evident over the range examined. Transformation with 5 μ g of *E. coli* pHS17 DNA was demonstrated; however, the efficiency was still less than that observed with 1 μ g of homologous DNA. The 100-fold or greater increase in transformation efficiency with homologous DNA suggests the presence of a functional restriction-modification system in *F. nucleatum* ATCC 10953. Restriction-modification systems have been found in *F. nucleatum* (18, 19). Growth phase also influenced the transformation efficiency, but to a lesser extent than the DNA source. Increased transformation efficiencies were routinely obtained with early-log-phase cells. For example, in one experiment using early-log-phase, mid-log-phase, and stationary-phase recipient cells and an outgrowth period of 5 h (approximately two generations), the transformation efficiencies were 7.2×10^3 , 4.8×10^3 , and 5.0×10^3 , respectively. No significant differences were observed with variations in the concentration of MgCl₂ in the outgrowth broth or with 0.2 versus 0.4 μ g of clindamycin per ml in the selective agar medium.

Stability of shuttle plasmid in *F. nucleatum* transformants. The structural stability of pHS17 in representative transformants was evaluated by restriction endonuclease mapping, PCR amplification of pHS17-specific DNA regions, and Southern analysis of the transformant DNA with pFN1 and pHS17 DNA probes (data not shown). In all of the analyses done, no evidence of DNA rearrangement or deletion was detected. The segregational stability of pHS17 was examined in the transformant strain KH21, maintained in liquid cultures without antibiotic selection (23). After 100 generations, the percentage loss of plasmid per generation was 0.02, with an average of 98% of the viable cells demonstrating the clindamycin resistance phenotype. The shuttle plasmid was present in all colonies subcultured at baseline and after 100 generations, with no evidence of DNA rearrangement or deletion. Thus, pHS17 was found to be both structurally and segregationally stable in the *F. nucleatum* host cell background. Interestingly, both pHS17 and pFN3 were stably maintained in the transformants, indicating that these two plasmids are compatible and that pFN3 may be useful in developing plasmid vectors for use in conjunction with pFN1-derived plasmids.

The data presented here document key findings that provide a foundation for developing *F. nucleatum* genetic systems. These findings include the following: the occurrence of two distinct groups of plasmids native to *F. nucleatum*, one of which

appears to be a theta-replicating iteron-regulated plasmid; the ability of the *ermF-ermAM* cassette to confer clindamycin resistance in *F. nucleatum*; the ability of *F. nucleatum* to be transformed by electroporation using the pFN1-derived shuttle plasmid; and the stability of the shuttle plasmid in *F. nucleatum*. Additional studies are in progress to examine the copy number of the native plasmids and to confirm the mode of replication of the pFN1 group of plasmids. Further, we are actively pursuing strategies to identify additional selectable markers and further optimize the structural features of the shuttle plasmid for use in molecular manipulations of fusobacteria.

Nucleotide sequence accession numbers. The nucleotide sequences of pFN1 and the *ermF-ermAM* cassette (8) have been deposited in the GenBank database under accession no. AF159249 and AF219231, respectively.

Grants from the UCLA School of Dentistry Opportunity Fund and the UCLA Academic Senate and PHS grant DE12639 to S.K.H. supported this work.

The authors thank E. Lee for technical assistance, V. L. Miller and D. A. Haake for helpful discussions and review of this material, and S. Hunt Gerardo for editorial review.

REFERENCES

- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403–410.
- Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389–3402.
- Benachour, A., J. Frere, and G. Novel. 1995. pUCL287 plasmid from *Tetragenococcus halophilus* (*Pediococcus halophilus*) ATCC 33315 represents a new theta-type replicon family of lactic acid bacteria. *FEMS Microbiol. Lett.* **128**:167–176.
- Berg, T., N. Firth, S. Apisiridej, A. Hettiaratchi, A. Leelaporn, and R. A. Skurray. 1998. Complete nucleotide sequence of pSK41: evolution of staphylococcal conjugative multiresistance plasmids. *J. Bacteriol.* **180**:4350–4359.
- Bolstad, A. I., H. B. Jensen, and V. Bakken. 1996. Taxonomy, biology, and periodontal aspects of *Fusobacterium nucleatum*. *Clin. Microbiol. Rev.* **9**:55–71.
- Bradshaw, D. J., P. D. Marsh, G. K. Watson, and C. Allison. 1998. Role of *Fusobacterium nucleatum* and coaggregation in anaerobe survival in planktonic and biofilm oral microbial communities during aeration. *Infect. Immun.* **66**:4729–4732.
- Brazier, J. S., D. M. Citron, and E. J. C. Goldstein. 1991. A selective medium for *Fusobacterium* spp. *J. Appl. Bacteriol.* **71**:343–346.
- Fletcher, H. M., H. A. Schenkein, R. M. Morgan, K. A. Bailey, C. R. Berry, and F. L. Macrina. 1995. Virulence of a *Porphyromonas gingivalis* W83 mutant defective in the *prtH* gene. *Infect. Immun.* **63**:1521–1528.
- Fletcher, M. 1986. Measurement of glucose utilization by *Pseudomonas fluorescens* that are free-living and that are attached to surfaces. *Appl. Environ. Microbiol.* **52**:672–676.
- Helinski, D. R., A. E. Toukdarian, and R. P. Novick. 1996. Replication control and other stable maintenance mechanisms of plasmids, p. 2295–2324. In F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 2. ASM Press, Washington, D.C.
- Higgins, D. G. 1994. CLUSTAL V: multiple alignment of DNA and protein sequences. *Methods Mol. Biol.* **25**:307–318.
- Holst, E., A. R. Goffeng, and B. Andersch. 1994. Bacterial vaginosis and vaginal microorganisms in idiopathic premature labor and association with pregnancy outcome. *J. Clin. Microbiol.* **32**:176–186.
- Ilyina, T. V., and E. V. Koonin. 1992. Conserved sequence motifs in the initiator proteins for rolling circle DNA replication encoded by diverse replicons from eubacteria, eukaryotes and archaeobacteria. *Nucleic Acids Res.* **20**:3279–3285.
- Kanatani, K., T. Tahara, M. Oshimura, K. Sano, and C. Umezawa. 1995. Identification of the replication region of *Lactobacillus acidophilus* plasmid pLA103. *FEMS Microbiol. Lett.* **133**:127–130.
- Kinder, S. A., J. L. Badger, G. O. Bryant, J. C. Pepe, and V. L. Miller. 1993. Cloning of the *YenI* restriction endonuclease and methyltransferase from *Yersinia enterocolitica* serotype O8 and construction of a R–M+ mutant. *Gene* **136**:271–275.
- Kinder Haake, S., and X. Wang. 1997. Cloning and expression of *fomA*, the major outer membrane protein gene from *Fusobacterium nucleatum* T18. *Arch. Oral Biol.* **42**:19–24.

17. Kolenbrander, P. E., and J. London. 1993. Adhere today, here tomorrow: oral bacterial adherence. *J. Bacteriol.* **175**:3247–3252.
18. Leung, D. W., A. C. P. Lui, H. Merilees, B. C. McBride, and M. Smith. 1979. A restriction enzyme from *Fusobacterium nucleatum* 4H which recognizes GCNGC. *Nucleic Acids Res.* **6**:17–25.
19. Lui, A. C. P., B. C. McBride, G. F. Vovis, and M. Smith. 1979. Site specific endonuclease from *Fusobacterium nucleatum*. *Nucleic Acids Res.* **6**:1–15.
20. McKay, T. L., J. Ko, Y. Bilalis, and J. M. DiRienzo. 1995. Mobile genetic elements of *Fusobacterium nucleatum*. *Plasmid* **33**:15–20.
21. Mikamo, H., K. Kawazoe, Y. Sato, and T. Tamaya. 1999. Elastase activity of anaerobes isolated from amniotic fluid with preterm premature rupture of membranes. *Am. J. Obstet. Gynecol.* **180**:378–380.
22. Moore, W. E. C., and L. V. H. Moore. 1994. The bacteria of periodontal diseases. *Periodontol.* **2000** **5**:66–77.
23. Roberts, R. C., and D. R. Helinski. 1992. Definition of the minimal plasmid stabilization system from the broad-host-range plasmid RK2. *J. Bacteriol.* **174**:8119–8132.
24. Rosey, E. L., M. J. Kennedy, D. K. Petrella, R. G. Ulrich, and R. J. Yancey, Jr. 1995. Inactivation of *Serpulina hyodysenteriae* *flaA1* and *flaB1* periplasmic flagellar genes by electroporation-mediated allelic exchange. *J. Bacteriol.* **177**:5959–5970.
25. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
26. Schaefer, C., and W. Messer. 1991. DnaA protein/DNA interaction. Modulation of the recognition sequence. *Mol. Gen. Genet.* **226**:34–40.
27. Sreenivasan, P. K., D. J. LeBlanc, L. N. Lee, and P. Fives-Taylor. 1991. Transformation of *Actinobacillus actinomycetemcomitans* by electroporation, utilizing constructed shuttle plasmids. *Infect. Immun.* **59**:4621–4627.
28. Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**:4673–4680.