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Properties and Construction of Plasmid pFW213, a Shuttle Vector with the Oral *Streptococcus* Origin of Replication▽

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Streptococcus parasanguinis **is among the most successful colonizers of the human body. Strain FW213 harbors a 7.0-kb cryptic plasmid, pFW213, with a copy number at 5 to 10 per chromosome. Sequence and functional analyses of pFW213 revealed that the open reading frame (ORF) encoding the replication protein (Rep) is essential for the replication of pFW213, and the putative plasmid addiction system (RelB and RelE) and an ORF (ORF6) with no known function are required for its stability. The minimal replicon of pFW213 contains the *rep* gene and its 5′-flanking 390-bp region. Within the minimal replicon, an A/T-rich region followed by 5 contiguous 22-bp repeats was located 5′ of the ATG of *rep*. No single-stranded replication intermediates were detected in the derivatives of pFW213, suggesting that pFW213 replicates via the theta replication mechanism. The minimal replicon was unstable in streptococcal hosts without selection, but the stability was greatly enhanced in derivatives containing the intact *relBE* genes. A *Streptococcus-Escherichia coli* shuttle vector, pCG1, was constructed with the pFW213 replicon. Plasmid pCG1 features a multiple cloning region and a spectinomycin resistance determinant that is expressed in both *Streptococcus* spp. and *E. coli*. Various streptococcal DNA fragments were cloned in pCG1, and the recombinant constructs were stably maintained in the streptococcal hosts. Since pCG1 is compatible with the most widely used streptococcal replicon, pVA380-1, pCG1 will provide a much needed tool allowing the cloning of two genes that work in concert in the same host.**

Plasmids are autonomously replicating extrachromosomal elements that generally do not carry genes essential for host cell survival. Knowledge of the basic replicon of naturally occurring plasmids is the foundation for building cloning, expression, and shuttle vectors. Most of the plasmids of Gram-negative bacterial origin replicate via theta replication, whereas most of the plasmids of Gram-positive bacterial origin replicate via rolling circle replication (RCR); exceptions exist in both cases (9). Generally, vectors derived from theta replication replicons have larger cloning size capacities and are more stably maintained than those of RCR replicons (24, 34, 40). Cloning of DNA fragments into RCR plasmids from *Staphylococcus aureus* leads to the formation of high-molecular-weight plasmid multimers, whereas DNA fragments of the same size inserted into the theta-replicating plasmid pAMβ1 remain segregation stable, indicating that the instability of the recombinant plasmids is a function of the RCR replication (18). The replication protein (Rep) of RCR plasmids possesses nicking-closing activity that recognizes and nicks a specific sequence within the double-stranded origin (*dso*). Therefore, it is hypothesized that the Reps of RCR plasmids could nick at sites with similar target sequences and, thus, generate various deletion derivatives during replication (2, 30).

The most commonly used replicons in the genetic analysis of oral streptococci are pAMβ1 and pVA380-1. Plasmid pAMβ1, which was originally isolated from *Enterococcus faecalis*, is a broad-host-range, conjugative, erythromycin (Em) resistance gene-carrying plasmid (7). The transmission of this plasmid between *E. faecalis*, *Lactococcus lactis*, and *Lactobacillus* spp. by conjugation is well documented (32, 36, 38). Plasmid pAMβ-1 replicates via the theta-type mechanism and, like most conjugative plasmids, is large (26.5 kb). Therefore, the use of pAMβ-1 as a cloning vector is limited. On the other hand, plasmid pVA380-1 is a 4.2-kb RCR plasmid isolated from *Streptococcus ferus* (29). This plasmid replicates in a broad range of Gram-positive bacterial hosts (11, 25). Similar to many RCR plasmids, pVA380-1 also contains a *mob* gene, which encodes a protein responsible for its mobilization. The mobilization of derivatives of pVA380-1 by a conjugative plasmid is an effective way to introduce foreign DNA into non-transformable streptococcal hosts (4, 22). Currently, most of the available streptococcal cloning and *Streptococcus-Escherichia coli* shuttle vectors are constructed based on the pVA380-1 replicon (11, 25, 27, 28). Given that plasmids of the same replication family are incompatible, this lack of diversity poses a limitation in genetic studies. The identification of other replicons able to function efficiently and stably in oral streptococci would allow the construction of cloning vectors and shuttle vectors that would be compatible with pVA380-1.

The human isolate *Streptococcus parasanguinis* FW213 (8) is a primary colonizer of the tooth surface and an opportunistic pathogen for subacute endocarditis (5). In an attempt to identify replicons for developing genetic tools that function in streptococci, we analyzed the cryptic plasmid pFW213 found in *S. parasanguinis* FW213 and utilized it to construct the shuttle vector described in this study.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Phenotype[a]	Description	Source or reference
Streptococcal strains			
S. parasanguinis FW213		Wild-type strain harboring pFW213	7
S. gordonii CH1		Plasmid-free, naturally competent streptococcal host	D. J. LeBlanc
S. mutans GS5		Plasmid-free, naturally competent streptococcal host	H. K. Kuramitsu
S. sanguinis SK36		Plasmid-free, naturally competent streptococcal host	T. Kitten
E. coli plasmids			
pCTL2	Km^r	pDL290 harboring an internal fragment of pFW213 (nt 2996 to 5589) at SacI and NsiI sites	This study
pCTL4	Km^r	pDL290 harboring an internal fragment of pFW213 (nt 1064 to 3621) at EcoRI site	This study
pDL290	Km^r	pSC101 replicon-based *E. coli* vector	D. J. LeBlanc
pSU21	Cm^r	p15A replicon-based *E. coli* vector	3
Streptococcal plasmids			
pCG1	Sp^r	*Streptococcus-E. coli* shuttle vector composed of p15A origin, pFW213 basic replicon, and a *spe* encoding Sp resistance in both *E. coli* and streptococci	This study
pCTL14	Sp^r	pFW213::*spe* at Klenow fragment-treated ClaI site	This study
pCTLr2	Sp^r	Derivative of pCTL14, containing pFW213 nt 3800 to 5129	This study
pCTLr3	Sp^r	Derivative of pCTL14, containing pFW213 nt 3800 to 6180	This study
pCTLr4	Sp^r	Derivative of pCTL14, containing pFW213 nt 3800 to 6897	This study
pCTLr5	Sp^r	Derivative of pCTL14, containing pFW213 nt 3800 to 500	This study
pDL276	Km^r	pVA380-1 replicon-based *Streptococcus-E. coli* shuttle vector	11
pFW213	Cryptic	Naturally occurring plasmid in *S. parasanguinis* FW213	This study
pT12	Sp^r	ExoIII deletion derivative of pCTL14, containing pFW213 nt 900 to 5700	This study
pT16R	Sp^r	ExoIII deletion derivative of pCTL14, containing pFW213 nt 3800 to 900	This study

[a] Cm, chloramphenicol; Km, kanamycin; Sp, spectinomycin; r, resistant.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *S. parasanguinis* FW213, *Streptococcus gordonii* CH1, *Streptococcus mutans* GS5, *Streptococcus sanguinis* SK36, and their derivatives were grown in Todd-Hewitt (TH; Difco) broth or on agar plates at 37°C in a 5% CO_2 atmosphere. Where indicated, Em at 5 μg ml^{-1}, kanamycin (Km) at 250 μg ml^{-1}, or spectinomycin (Sp) at 250 μg ml^{-1} was included in the growth medium (35). Recombinant *E. coli* strains were grown at 37°C with aeration in LB medium containing ampicillin (Ap) at 100 μg ml^{-1}, Em at 200 μg ml^{-1}, Km at 50 μg ml^{-1}, Sp at 50 μg ml^{-1}, or chloramphenicol (Cm) at 25 μg ml^{-1} as needed.

Standard molecular manipulations. Plasmid DNA was isolated from streptococcal strains by the method of Anderson and McKay (1). For large-scale preparation, plasmid DNA was purified further by centrifugation to equilibrium in cesium chloride-ethidium bromide (35). Plasmid DNA was introduced into *S. parasanguinis* FW213 by electroporation (6) and into *S. gordonii* CH1, *S. mutans* GS5, and *S. sanguinis* SK36 by natural transformation (23).

Total RNA was isolated from mid-exponential-phase cultures (optical density at 600 nm, ≈0.6) of *S. parasanguinis* FW213 by the method of Chen et al. (6) and further purified by using an RNeasy minikit (Qiagen). For reverse transcription-PCR (RT-PCR), the first-strand cDNA was synthesized from 10 μg of total cellular RNA with random hexamer primers. The cDNA was then amplified by PCR at high stringency (primer annealing set at 58°C) with primers specific for each open reading frame (ORF).

Restriction endonuclease and DNA-modifying enzymes were purchased from New England BioLabs (United States). *Taq* and Phusion DNA polymerase were purchased from Toyobo (Japan) and Finnzymes (Finland), respectively. Primers used in this study are listed in Table 2.

Relative plasmid copy number determination. Primers for quantitative real-time PCR (qPCR) were designed to have a predicted melting temperature of about 60°C and to generate products of approximately 100 bp in length. qPCRs were conducted using iQ SYBR green supermix (Bio-Rad). Serial dilutions (10^{-1} to 10^{-4}) of total cellular DNA (10 μg) isolated from *S. parasanguinis* (containing native pFW213) were analyzed by qPCR using primer pairs specific for the *rep* of pFW213, the chromosome-borne *secA2* (GenBank sequence accession number AY338765), *gly* (39), and *galT2* (39). The copy numbers were calculated as the mean threshold cycle (C_T) values of the amplicons of the chromosomal genes (single-copy reference) compared to the amplicon of the plasmid-borne *rep* using the formula $2^{\Delta CT}$, where ΔC_T is the difference between the threshold cycle number of the reference gene and that of the *rep* reaction.

Southern DNA-DNA hybridization and single-stranded DNA (ssDNA) detection. Total cellular DNA was isolated from recombinant *S. parasanguinis* strains according to the method of Anderson and McKay (1) as modified by LeBlanc et al. (25). Four micrograms of isolated DNA was treated with RNase A and then divided into two aliquots. One aliquot was treated with S1 nuclease, and the other was not treated. Both S1 nuclease-treated and untreated samples were separated on 0.8% Tris-acetate-EDTA (TAE) agarose gels by electrophoresis and transferred to Hybond N$^+$ membranes (Amersham) (37), with or without prior denaturation. Southern DNA-DNA hybridization was performed using a digoxigenin (DIG) DNA labeling and detection kit (Roche) according to the manufacturer's instructions.

Plasmid construction, sequencing analysis, and characterization of minimal replicon. On the basis of the restriction endonuclease site map, two internal fragments of pFW213 were subcloned onto pDL290 (D. J. LeBlanc, personal communication) at the compatible sites to generate plasmids pCTL2 and pCTL4 for sequencing. Plasmid pCTL2 contains the SacI-NsiI fragment (nucleotides [nt] 2996 to 5589) of pFW213, and pCTL4 contains the EcoRI-EcoRI fragment (nt 1064 to 3261) (Fig. 1). The sequence of the region between these two clones (nt 5589 to 1064) was completed by primer walking. Automated DNA sequencing was done by Tri Biotech, Inc. (Taiwan), and the complete sequence of pFW213 was obtained from both directions. All sequence data assembly and analysis were performed using the Vector NTI software package and The European Molecular Biology Open Software Suite (EMBOSS). Database searches were performed using BLAST, located at the NCBI website.

A DNA fragment containing the Sp resistance gene (*spe*) (26) flanked by BamHI-PvuI and ApaI-NcoI sites was generated by PCR and cloned into the Klenow fragment-treated ClaI site of plasmid pFW213 to facilitate exonuclease III (ExoIII) analysis. The resulting plasmid (pCTL14) was digested with either BamHI-PvuI or ApaI-NcoI to allow for nested deletions generated by ExoIII from opposite ends of pFW213 (20). The resulting products were self-ligated and introduced into *S. gordonii* CH1. The properties of the plasmids isolated from Sp-resistant (Sp^r) transformants were verified by plasmid isolation and restriction endonuclease digestion. To define the minimal replicon of pFW213, fragments covering different lengths of the plasmid were generated by PCR using plasmid pT16R (Table 1) as the template. PCR products were digested, self-ligated, and transformed into *S. gordonii* CH1. The presence of desired recom-

TABLE 2. Primers used in this study

Primer	Sequence[a]	Purpose
pFW213_ORF1_AS	5′-GGTTGCTCAAATCGCCTTG	Expression analysis of ORF1
pFW213_ORF1_S	5′-GCTACATCCCAACGCATG	
pFW213_ORF2_S	5′-GGCAGAATGGCCTCCCTAC	Expression analysis of ORF2
pFW213_ORF2_AS	5′-GACCGTGAGAATACGACGC	
pFW213_ORF3_S	5′-GGTGCGTTATTCGCGCCG	Expression analysis of ORF3
pFW213_ORF3_AS	5′-GCTATTCCACGCCAACTATTG	
pFW213_ORF4_S	5′-CTGGCATGACAAATATACGTC	Expression analysis of ORF4
pFW213_ORF4_AS	5′-GAGGAGCTCCATCTTCGTC	
pFW213_ORF5_S	5′-GGTGATGTCTTTACGGTTC	Expression analysis of ORF5
pFW213_ORF5_AS	5′-GGTCCAGCATCTTCAGAG	
pFW213_ORF6_S	5′ CGGGGTGAAAGTTTTGACTG	Expression analysis of ORF6
pFW213_ORF6_AS	5′-CAAAATTCCACCATCTCTTCG	
pFW213_ORF7_S	5′-TTGATACCTGAAGCACAAGATGAT	Expression analysis of ORF7
pFW213_ORF7_AS	5′-TCACTTCTCTTACCAATAGCAATGA	
pFW213_ORF8_S	5′-TTAAAACAGGAAGTCGGTGAGG	Expression analysis of ORF8
pFW213_ORF8_AS	5′-CCAAACCTTCACGCCAATTT	
pSU21AS	5′-TTTTAAGGCAGTTATTGGTGCCT	To generate psu21 internal fragment
pSU21S	5′-CATTCGCCATTCAGGCTGCG	
SpecPvuIS	5′-GGATCCAAGCTTCGATCGTTCGAA	To generate pCTLr2, pCTLr3, pCTLr4, and pCTLr5
Replicon2	5′-GATATTAGAATAGAGCGATCGAGCCATT	
Replicon3	5′-CCCCTAAAGCGATCGAAAGCTGGATGAC	
Replicon4	5′-CCGTACCGATCGCTGAAAATAGACCAG	
Replicon5	5′-GTTAGTACCTCCGATCGAAAAATTACAC	
secA2_S	5′-AAAGGTGTTGCAGAATTAGGCGGC	qPCR for *secA2*
secA2_AS	5′-TCGTCCTCTCAACTGCCAGTCAAT	
gly_S	5′-GTTGAAAGCGTGCGAACCCAGATA	qPCR for *gly*
gly_AS	5′-CTTGTGGCACCAATTCCCTCGTTT	
galT2_S	5′-GGAATACCTTCGCCCTTGTTTGGA	qPCR for *galT2*
galT2_AS	5′-AATTCCTTGCTGCACCAACTCCAC	
qPCR_S	5′-TGGTTGGCATCCGTCTATCCCTAA	qPCR for pFW213
qPCR_AS	5′-TAAGAGGAATGCTCTCATGGTGGC	

[a] Introduced restriction sequences are underlined.

binant plasmids in Sp^r transformants was confirmed as described above. All recombinant plasmids were then introduced into *S. parasanguinis* by electroporation and confirmed as described above.

Plasmid stability. Derivatives of pFW213 were tested for stability in the streptococcal host. Briefly, overnight cultures of selected clones were grown in TH broth containing Sp to ensure the presence of the respective plasmid. The culture was then diluted at 10^{-4} in fresh TH broth without antibiotics and incubated for 16 h (13 generations) for a total of 10 days (117 generations). Isolated colonies of each subculture were obtained by serial dilution and plating on TH agar, and the percentage of Sp^r isolates at each time point was determined by inoculating 300 random colonies onto TH agar plates with and without Sp. The plasmid stability was calculated as the percentage of clones in the population that maintained the test plasmid.

Incompatibility assay. The compatibility of pFW213 and pVA380-1 replicons was determined according to the method of Miki et al. (31), with minor modifications. The pVA380-1 replicon-based pDL276 (harboring *kan*) (11) was introduced into a pCTL14 (harboring *spe*)-containing *S. parasanguinis* strain, and transformants that were resistant to both Sp and Km were selected. The presence of both plasmids was confirmed as described above. Two Km^r and Sp^r double resistance colonies were suspended in 10 mM sodium phosphate buffer (pH 7), serially diluted, and then plated onto plain TH agar. After 16 h of incubation without selection (approximate 20 generations), 100 colonies were picked and patched onto TH agar plates. The patches were tested for the resistances conferred by either pDL276 (Km^r) or pCTL14 (Sp^r) by replica plating to TH agar containing Km or Sp, respectively. The process was repeated for 4 passages.

Construction of the shuttle cloning vector pCG1. A DNA fragment containing the p15A origin of replication and the β-galactosidase gene (*lacZ*) of *E. coli* was amplified from pSU21 (3) by PCR using Phusion DNA polymerase with primer pair psu21S-psu21AS. The PCR product was then cloned into pCTLr2 at the T4 polymerase-treated PvuI site. The ligation mixture was used to transform *E. coli* DH10B, and Sp^r blue colonies on LB agar with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and Sp were selected. Recombinant plasmids were verified by plasmid isolation and restriction enzyme analysis, and the correct clone was further confirmed by sequence analysis. The resulting plasmid was designated pCG1.

Nucleotide sequence accession number. The complete sequence of pFW213 has been deposited in GenBank with the accession number NC_012642.1.

RESULTS AND DISCUSSION

Identification and sequence analysis of pFW213. One plasmid, designated pFW213, was detected in *S. parasanguinis* FW213 by agarose gel electrophoresis of the gradient-purified plasmid preparation (Fig. 1A). Sequence analysis revealed circular molecules of 7,078 bp with an overall 35% G+C, which is lower than the average G+C content (approximately 41%) of the known ORFs in *S. parasanguinis* FW213. The copy number of pFW213 in *S. parasanguinis* was estimated at 5 to 10 per chromosome by real-time qPCR analysis. Annotation of the sequence identified 8 ORFs greater than 50 amino acids (aa) in length. Among the predicted ORFs, 5 were transcribed from one orientation and 3 were transcribed from the opposite orientation (Fig. 1B). Specific mRNA was readily detected in all but ORF3 by RT-PCR; only a very faint band was detected for ORF3 under the experimental conditions used (Fig. 1C). The basic properties and promoter predictions of these ORFs are listed in Table 3.

The deduced amino acid sequence of ORF1 was 94% identical to that of a hypothetical protein (SSA_2031) of *S. sanguinis* SK36. A high degree of homology (95% identity) was also observed with the C-terminal portion of the putative inorganic

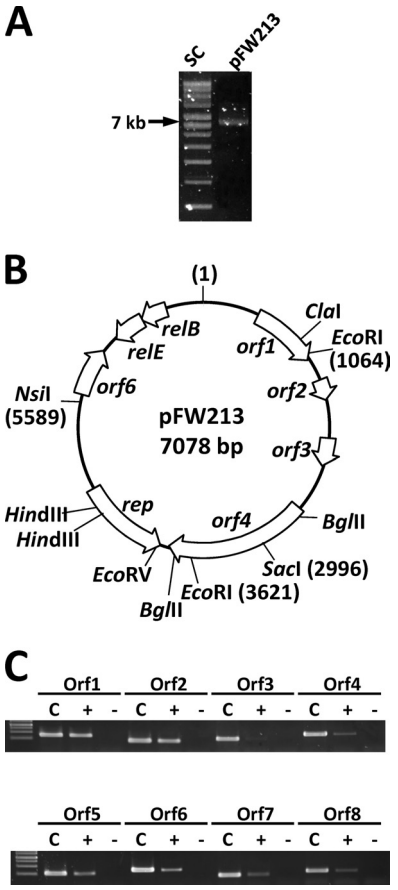

FIG. 1. Plasmid pFW213. (A) Agarose gel electrophoresis of cesium chloride gradient-purified pFW213. SC, supercoiled DNA ladder. (B) Physical and genetic map of pFW213. Relative sizes, directions of transcription, and assigned designations are indicated. The assigned first base and positions of restriction sites used in subcloning pFW213 are listed in parentheses. (C) RT-PCR analysis of ORFs in pFW213. Two percent of the total cDNA generated from RT was amplified with specific primers, and 10% of the PCR products was run on a 1.8% TAE gel. C, products generated from total cellular DNA; +, reverse transcriptase was included in the reaction mixture; −, control groups without reverse transcriptase. A 1-kb DNA ladder was used as the marker.

pyrophosphatase/exopolyphosphatase (YP_003363493) deduced from the newly sequenced *Rothia mucilaginosa* DY-18 genome. A transmembrane domain (aa 65 to 87) was suggested (http://www.cbs.dtu.dk/services/TMHMM/); however, no functional data are currently available for either protein. We also observed a putative rho-independent terminator (ΔG^o = −12.19 kcal mol^{-1}) located 19 bp 3′ of the stop codon of ORF1. Furthermore, no contiguous transcript was detected between ORF1 and ORF2 by RT-PCR, confirming that ORF1 is expressed as a single gene.

When performing BLAST analysis against the entire nonredundant GenBank database, no homology to ORF2 and ORF3 was found, albeit both ORFs contain ribosome binding sites (RBS) 5′ of the predicted translation start codon. Although the PCR product specific for ORF3 was not readily detected (Fig. 1C), we could not rule out the possibility that ORF3 was expressed at an extremely low level. On the other hand, the predicted protein encoded by ORF4 shared 35% and 31% identity with the Mob proteins of plasmid pBMYdx from *Bacillus mycoides* (10) and plasmid p9785s from *Lactobacillus johnsonii* FI9785 (21), respectively. A sequence identity of 32% was also observed with the putative Mob of pTX14-1 from *Bacillus thuringiensis* subsp. *israelensis* (GenBank sequence accession number AAB16962). These Mob proteins belong to the MOB_V family of the conjugative transfer systems (14), among which the Mob protein of streptococcal plasmid pMV158 is the best studied. When ORF4 was compared to the members of pMV158 superfamily relaxases, the consensus sequences of the highly conserved motifs I (HXXR), II (NXXL), and III (HXDEXXPHXH) (13) were also found (data not shown). Although the 5′-flanking region of ORF2 shared 54% nucleotide identity with the *oriT* of pMV158, the proposed nick site and flanking sequences (13) were not found in pFW213. With an intact *mob*, pMV158 can be mobilized by a coresident conjugative plasmid (35). Multiple attempts failed to demonstrate mobilization activity of pFW213 in the presence of the conjugative plasmid pAMβ1 (data not shown). Thus, the function of the pFW213 ORF4 remains unclear.

ORF4 and ORF5 are separated by 89 bp and transcribed convergently. An inverted repeat (ΔG^o = −10.96 kcal mol^{-1}) which may act as the transcriptional terminator for ORF4 and ORF5 was located 25-bp 3′ of ORF4. Of note, this secondary structure is flanked by 5′-GACTTG (in the ORF4 orientation)

TABLE 3. Properties of ORFs identified in pFW213

ORF	Position (nt)	Protein size (aa/kDa)	RBS[a]	Putative promoter sequence[b]	Translation start codon	Protein with greatest similarity[c]	GenBank accession no.[d]
1	502–1107	202/22.19	AGAGG (8)	TGTGTTATAAT (70)	ATG	Conserved uncharacterized protein in *S. sanguinis* SK36 (202/202)	YP_001035954
2	1336–1524	63/7.13	AGGGG (8)	TGGACG-N_{17}-TATATC (74)	ATG	No significant similarity	NA
3[e]	1871–2125	85/9.88	GGCAA (9)	TTGAGA-N_{15}-TATTTT (136)	GTG	No significant similarity	NA
4	2553–3838	432/51.5	GAGGAA (8)	TGGTCA-N_{18}-TTTAAA (118)	ATG	Mob of plasmid pBMYdx (392/426)	NP_981974
5	3928–4737	270/31.4	AAAGG (8)	TTGACT-N_{18}-TAGAAT (36)	ATG	RepB of plasmid pLME300 (242/259)	NP_783833.1
6	5615–6058	148/17.35	None	TTCACA-N_{16}-TAAACT (56)	GTG	No significant similarity	NA
7	6206–6508	101/11.6	AGAGG (4)	NA	TTG	RelE in *S. downei* F0415 (96/99)	EFQ56598.1
8	6502–6747	82/9.5	GAGG (8)	TTGAAG-N_{18}-TATAAT (26)	ATG	RelB in *S. downei* F0415 (77/79)	EFQ56528.1

[a] The distance in nucleotides from the RBS to the translation start site is listed in parentheses.
[b] The distance in nucleotides from the −10 element to the translation start site is listed in parentheses. NA, not available. N indicates G, A, T, or C.
[c] The target that the ORF shares the highest homology to. Numbers in parentheses are the length of the alignment/target size, in amino acids.
[d] GenBank sequence accession number of the locus listed under Similarity. NA, not available.
[e] No significant product was detected by RT-PCR.

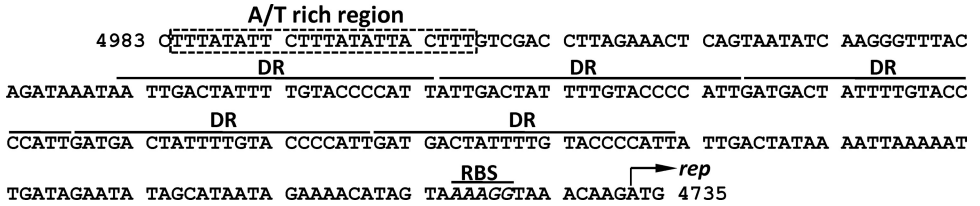

FIG. 2. Nucleotide sequence analysis of the putative *ori* of pFW213. The A/T-rich region is boxed. The DR sequence is overlined. The predicted RBS is italic and overlined, and the translation start codon of *rep* is indicated by a bent arrow.

and 5′-AATAAA (in the ORF5 orientation); thus, this shared terminator may have different strengths for ORFs 4 and 5. The highest homology (52% identity) was observed between the deduced amino acid sequence of ORF5 and the predicted theta replication protein RepB of pLME300 from *Lactobacillus fermentum* ROT1 (15). Identities of 52%, 46%, and 28% were also observed with the RepB proteins of pPLA4 (ABG23030.1) from *Lactobacillus plantarum*, of pYIT356 (YP_077168.1) from *Lactobacillus casei*, and of pKC5b (NP_862331.1) from *L. fermentum* KC5b (GenBank sequence accession numbers in parentheses). Among these three plasmids, the replication mode has been studied experimentally only in pKC5b, which replicates via the theta replication mode (34). As commonly seen in *oriV* of lactobacillus plasmids, including pLME300, pYIT356, and pKC5b, characteristics of iteron-based plasmids, such as a stretch of A/T-rich sequence and copies of about 20-bp direct-repeat (DR) sequences, are observed 5′ of the respective *rep* on each plasmid. Similarly, a 23-bp A/T-rich region and 5 copies of 22-bp DR sequences [5′-(G/A)(A/T)TGACTATTTTGTACCCCATT] were also found in the 5′-flanking region of ORF5 in pFW213 (Fig. 2).

ORF6 did not share any homology with ORFs in the database; neither could a putative RBS be found 5′ of the predicted translation start site. Thus, whether a protein is made from ORF6 is unclear. The translation start site of ORF7 is located within the 3′ end of ORF8. A contiguous transcript was identified from the midpoint of ORF7 to the end of ORF8 by RT-PCR analysis (data not shown), confirming that these ORFs are cotranscribed as an operon. A stem-loop structure ($\Delta G^{o} = -10.96$ kcal mol^{-1}) was located 38 bp 3′ of ORF7. ORF7 and ORF8 shared 59% and 47% identity with the addiction module toxin of the RelE family and the antitoxin of the RelB family, respectively, of *Streptococcus downei* F0415. Of note, homologs of this *relBE* locus have been reported in several microbes via genome projects recently. Based on these sequence homologies, ORFs 5, 7, and 8 were designated *rep*, *relE*, and *relB*.

Based on the BLAST results for pFW213 and the variations in G+C content of its ORFs (from 47.61% of ORF2 to 32.65% of ORF6), it is likely that the formation of pFW213 is the result of multiple acquisitions of DNA fragments from various microbes via horizontal gene transfer. In addition to the coding region of ORF1, the 21-bp regions 5′ to ORF1 of pFW213 and to SSA_2031 of *S. sanguinis* SK36 are identical; however, further flanking sequences in the two species are not related. Notably, we were unable to clone the NsiI-EcoRI fragment (nt 5589 to 1064) of pFW213 in *E. coli*, even on the low-copy-number cloning vector pDL290, suggesting that the expression of ORF6, *relBE*, and/or ORF1 is lethal to *E. coli*.

Identification of the minimal replicon of pFW213. The two smallest ExoIII derivatives of pFW213 that could be established in *S. gordonii* CH1 were pT12 and pT16R (Fig. 3A). The overlapping region of these two plasmids contained the entire *rep* and its 1.2-kb 5′-flanking region (Fig. 3A). To further define the minimal replicon, DNA fragments of various lengths were generated by PCR using pT16R as a template. Under selective conditions, the smallest clone, pCTLr2, harbored the

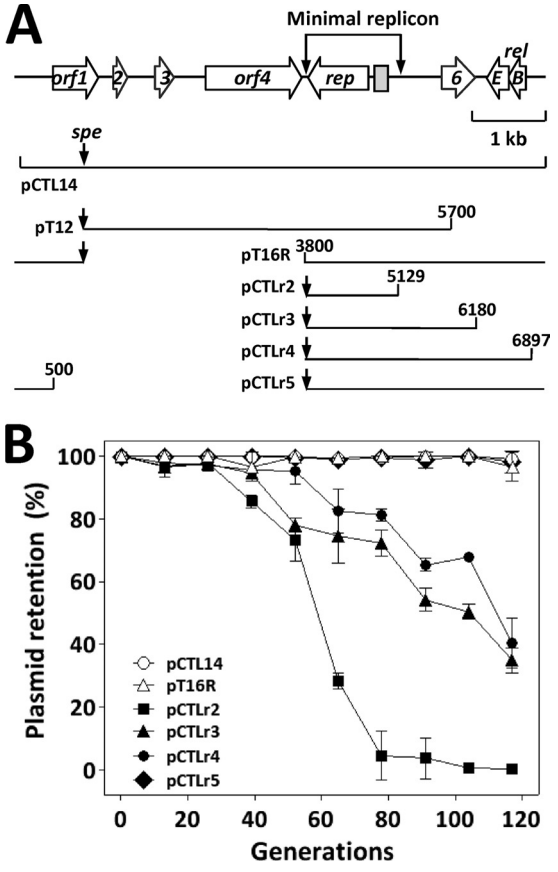

FIG. 3. The minimal replicon of pFW213. (A) Schematic diagram of pFW213. The relative location and the direction of transcription of each ORF in pFW213 are indicated by horizontal arrows. The A/T-rich sequence region and DRs are indicated by a shaded box. The smallest region that allows for replication in the streptococcal host is marked by two vertical arrows. The relative locations of the recombinant and ExoIII derivatives are listed below. The position of the *spe* in the derivatives is indicated by vertical arrows. (B) Stability of pFW213 derivatives in *S. parasanguinis* under nonselective conditions.

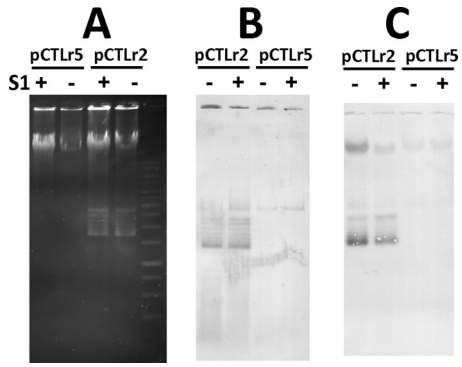

FIG. 4. Absence of detectable pFW213-specific ssDNA in total cellular DNA from FW213 strains containing pCTLr2 and pCTLr5. (A) Agarose gel electrophoresis of plasmids isolated from recombinant *S. parasanguinis* FW213. (B) Southern transfer of samples shown in panel A without denaturation of DNA. (C) Southern transfer of samples shown in panel A with denaturation of DNA. Both blots were hybridized with DIG-labeled *spe*. +, S1 nuclease treated; −, no S1 nuclease treatment prior to electrophoresis.

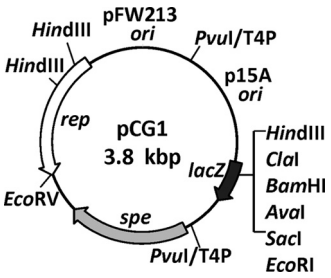

FIG. 5. Restriction endonuclease map of the *Streptococcus-E. coli* shuttle vector pCG1. The locations of the fusion sites (PvuI/T4P, T4 polymerase-treated PvuI sites) are indicated.

intact *rep*, all 5 copies of the DR sequence, and the A/T-rich region, thus defining the minimal replicon of pFW213.

Stability of pFW213 replicons and detection of ssDNA intermediates. Recombinant plasmids pCTLr2, pCTLr3, pCTLr4, and pCTLr5 were generated by PCR using pT16R as a template to characterize the effect of *relBE* on plasmid stability (Fig. 3A). As expected, the full-length pCTL14 was stable in *S. parasanguinis*, and no plasmid loss was observed after 117 generations of culturing (Fig. 3B). Both pT16R and pCTLr5 were also stably maintained over 117 generations (Fig. 3B), indicating that ORFs 1, 2, 3, and 4 are not essential for replication and stability. When the deletion was extended to nt 6897, 150 bp upstream of the translation start codon of *relB*, plasmid pCTLr4 (Fig. 3A) became unstable (Fig. 3B). After 65 generations of culturing, 18% of the colonies had lost the plasmid; at the 117th generation, the percentage of the cells that did not contain pCTLr4 was increased to 60%, indicating that the intergenic region between *relBE* and ORF1 critically affects the stability of the plasmid. The instability of the plasmid was relatively unchanged when the deletion was extended to nt 6180; plasmid pCTLr3 (Fig. 3A) was lost from 25% and 65% of the population after 65 and 117 generations of culturing, respectively (Fig. 3B). On the other hand, the stability of the replicon decreased significantly after the deletion was extended to nt 5129; plasmid pCTLr2 (Fig. 3A) was absent from 62% of the population after 65 generations of culturing and completely lost after 104 generations of culturing (Fig. 3B), demonstrating that the region upstream of *rep*, from nt 5129 to 6180, was crucial for the plasmid's stability. It is tempting to suggest that ORF6 regulates the stability of the pFW213 replicon through either the mRNA or protein generated from ORF6. Taken together, these results indicated that ORF6, the *relBE*, and the intergenic region between *relBE* and ORF1 all were important to the stability of pFW213. The toxin-antitoxin (TA) system is one of the mechanisms used by low-copy-number plasmids to maintain segregational stability (33). The inherent instability of the antitoxin (RelB) leads to activation of the toxin (RelE) in plasmid-free cells and, thus, ensures plasmid maintenance. Gotfredsen et al. (16) demonstrated that the *E. coli relBEF* operon could stabilize a mini-R1 replicon, whereas the *relBE* system of plasmid p307 is essential for the segregational stability of the plasmid (17). The RelB antitoxin is sensitive to the Lon protease in *E. coli*, and reduced segregational stability of the plasmid was observed in the Lon-deficient *E. coli* host (17). Thus, the results of the deletion analysis suggest that the *relBE* of pFW213 encodes a functional TA system that is essential for plasmid maintenance. Of note, among plasmids from Gram-positive bacteria, the RelBE system has been described in several *Lactobacillus* spp. plasmids (12, 19, 41). However, no homology of primary amino acid sequences was observed between the RelBE of pFW213 and those of *Lactobacillus* spp. plasmids.

The sequence homology between pFW213 Rep and other known RepB proteins suggests that pFW213 replicates via theta replication. To test whether pFW213 replicates via RCR, experiments were designed to determine whether ssDNA was present in streptococcal transformants containing the pFW213 derivatives pCTLr2 and pCTLr5. Plasmid pCTLr2 is the smallest replicon that replicates in both *S. parasanguinis* and *S. gordonii*, while plasmid pCTLr5 contains *rep*, *relBE*, and a partial ORF1. If pFW213 replicated via RCR, then pCTLr2 contains only *dso*, since pCTLr2 was not stably maintained in *S. parasanguinis* in the absence of selection (Fig. 3B) and, thus, accumulation of ssDNA intermediates would be apparent. However, we did not observe any S1 nuclease-sensitive targets in either strain, regardless of prior denaturation, supporting the concept that pCTLr2 does not replicate via RCR (Fig. 4). In addition, wild-type FW213 was also subject to the same analysis, and ssDNA was not detected (data not shown).

Incompatibility. The presence of pCTL14 and pDL276 in *S. parasanguinis* in the absence of selection was monitored to determine the compatibility of pFW213 and the pVA380-1 replicon. Both plasmids were stably maintained over 80 generations, indicating that these two replicons belong to different Inc groups.

Properties of the shuttle cloning vector pCG1. The smallest pFW213 derivative, pCTLr2, was chosen as the basis for the construction of pCG1 (Fig. 5). The construction is detailed in Materials and Methods. Plasmid pCG1 was maintained stably in *E. coli* DH10B over 100 generations, as determined by the lack of alterations in the restriction enzyme patterns of isolated plasmids. Attempts to construct fusions with a pUC origin of replication failed, indicating that pCTLr2 cannot be stably maintained at high copy numbers in *E. coli.*

TABLE 4. Transformation of streptococcal hosts with pCG1

Species and strain	Transformation frequency (CFU μg DNA^{-1})[a]	
	pCG1/*S. gordonii*	pCG1/*E. coli*
S. gordonii CH1	446 ± 114	193 ± 46
S. mutans GS5	416 ± 55	129 ± 26
S. sanguinis SK36	8 ± 2	53 ± 14

[a] Plasmid pCG1 isolated from recombinant *S. gordonii* (pCG1/*S. gordonii*) or *E. coli* (pCG1/*E. coli*) was used. The numbers are the means and standard deviations of the results of three independent experiments.

The transformation efficiency of pCG1 in naturally competent, plasmid-free *S. gordonii* CH1 and *S. mutans* GS5 is approximately 400 colonies per μg of DNA isolated from a streptococcal host. When plasmid DNA was isolated from an *E. coli* host, a significant reduction ($P \leq 0.05$, Student's t test) in transformation efficiency was observed (Table 4). The transformation efficiency in *S. sanguinis* SK36 was lower than that in *S. gordonii* and *S. mutans*, especially when plasmid DNA was isolated from recombinant *S. gordonii*. Restriction analysis of plasmids isolated from all streptococcal hosts confirmed that pCG1 was introduced and maintained successfully without any structural rearrangement. Furthermore, various streptococcal chromosomal fragments of 1 to 4 kb containing sequences covering an intact ORF and the promoter region were cloned into pCG1 in *E. coli* and then successfully transferred to streptococcal hosts by natural transformation or electroporation. Again, the recombinant plasmids were stably maintained in both hosts.

To further confirm the compatibility of pCG1 and pVA380-1 replicon-based vectors in streptococcal hosts, we compared the transformation efficiency of pCG1 in native *S. gordonii* CH1, *S. mutans* GS5, and *S. sanguinis* SK36 with that of the same streptococcal hosts harboring pDL276. In agreement with the observation that pCTL14 and pDL276 can coexist in *S. parasanguinis*, a comparable transformation efficiency was detected with pCG1 in the same streptococcal host regardless of the presence of pDL276, confirming that the pFW213 and pVA380-1 replicons are compatible with each other.

Summary. A theta-replicating *Streptococcus-E. coli* shuttle vector, pCG1, that allows for blue-white (X-Gal) selection in the *E. coli* host and is compatible with pVA380-1-based streptococcal vectors was constructed. Plasmid pCG1 contains the *E. coli* p15A origin of replication, the replication region of pFW213, a spectinomycin resistance gene expressed constitutively in both streptococcal and *E. coli* host strains, and the multiple cloning region of plasmid pSU21. This shuttle vector will facilitate experiments that require two compatible streptococcal plasmids in the same host.

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