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ISOLATION AND CHARACTERIZATION OF SMALL NATURALLY OCCURRING PLASMIDS FROM *Desulfovibrio* spp.

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By

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ISOLATION AND CHARACTERIZATION OF SMALL NATURALLY OCCURRING PLASMIDS FROM *Desulfovibrio* spp.

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY

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Abstract

Molecular techniques applied to microbial taxonomy were used for the identification of *Desulfovibrio* SR-1, which was isolated from sediments of a uranium contaminated subsurface aquifer. The complementary phenotypic characterization included the morphology and the pyruvate fermentation. The genotypic characterization included phylogenetic analyses (16S rRNA gene, 16S-23S ITS and *dsrAB* gene), DNA-DNA hybridization, G+C mol% content, and DNA fingerprinting (PFGE, ribotyping and BOX-PCR). Discrimination at the subspecies level was facilitated by the consistent phenotype differences and the DNA typing methods targeting whole genomes and gene clusters. Our data indicate that *Desulfovibrio* SR-1 represents a novel subspecies of *D. africanus*.

To establish a vector system that facilitates genetic manipulation in *Desulfovibrio* species, we have screened naturally occurring sulfate-reducing bacteria for small endogenous plasmids. A low-copy number plasmid pDaf was isolated from *Desulfovibrio* SR-1. Sequence analysis of this 8568 bp plasmid revealed a G+C content of 47.2% and nine open reading frames encoding polypeptides greater than 100 amino acids. The molecular and functional characterization of pDaf has revealed that this plasmid contains enough genetic information to be mobilized, to display a low copy number, and to be maintained stably under non-selective pressure. The results of this study provide potential avenues for genetic tool development.

Chapter 1

Genetic Characterization of *Desulfovibrio* SR-1

Abstract

Molecular techniques applied to microbial taxonomy were used for the discrimination between *Desulfovibrio* SR-1 and *Desulfovibrio africanus*. *Desulfovibrio* SR-1 has a single polar flagellum and ferments pyruvate. *D. africanus* has lophotrichus flagella and pyruvate does not support its growth in sulfate-free media. The 16S rRNA gene and the ITS sequence analyses showed the same phylogenetic affiliation for both strains. The DNA sequences of the ITS region of *Desulfovibrio* SR-1 and *D. africanus* contain two transfer RNA genes ($tRNA^{Ile}$, $tRNA^{Ala)}$, and they are 300 and 299 bases in length, respectively. The analysis of *dsrAB* sequences showed a better discrimination power than the 16S rRNA sequences in defining their phylogenetic affiliation. The 72% DNA-DNA similarity value did not conclusively resolve whether these strains should be considered as members of one species. The 62.4 mol% G+C content of *Desulfovirbio* SR-1 differs from the 63.1 mol% G+C of *D. africanus*. Significant differences in the genomic fingerprints (16S rDNA, PmeI-PFGE and BOX-PCR) between both strains were observed. These results suggest that these strains have different genomic organization. The presence of plasmid DNA was only detected in *Desulfovibrio* SR-1. On the basis of distinct phenotypic and genotypic characteristics, strain *Desulfovibrio* SR-1 represents a novel subspecies of *D. africanus*.

Introduction

Sulfate-reducing bacteria represent a class of anaerobic microorganisms that are primarily isolated from environmental sources, such as soil, water, and sediments. *Desulfovibrio* is one of the first genera described and the most thoroughly studied genus among the sulfate-reducing bacteria (Postgate and Campbell, 1966).

Desulfovibrio species are of a great environmental interest as they are involved in the reduction and subsequent immobilization of toxic metals and metalloids such as chromium (Goulhen et al., 2006), uranium (Payne et al., 2002) and arsenate (Macy et al., 2000). However, they have also been regarded as the principal mercury methylators in sediments (Jay et al., 2002; Ekstrom et al., 2003).

Desulfovibrio SR-1 has been characterized only partially since its isolation from uranium contaminated subsurface sediments (Castañeda, 2001). Based on the partial 16S rRNA gene sequence, *Desulfovibrio* SR-1 is closely related to *D. africanus*, but some phenotypic differences were detected between these strains (Castañeda, 2001). Comparative sequence analysis of the 16S rRNA gene is generally used to determine the phylogenetic position of novel isolates. However, the resolution of 16S rRNA gene sequence analysis between closely related species is generally low, and there is no threshold value of 16S rRNA gene sequence identity for species recognition (Coenye et al., 2005).

Sequence polymorphisms and length variations of the16S-23S ITS sequences have been used to discriminate several bacterial species and subspecies (Conrads et al., 2002; Conrads et al., 2005; Guasp et al., 2000). The ITS sequence analyses have been used to characterize *Desulfovibrio* species rather than discriminate closely related strains.

In *Desulfovibrio* species, the 16S-23S ITS spacer regions can have one or two transfer RNA genes and range in size from 274 to 529 nucleotides (Loubinoux et al., 2002).

Phylogenies from protein coding genes provide additional insights into the relationships within groups sharing similar functional properties. The *dsrAB* gene encodes a key enzyme that catalyzes the reduction of sulfite to sulfide. This gene is highly conserved across sulfate-reducing bacteria, and it is considered as a potential molecular marker for phylogenetic affiliation in these organisms (Wagner et al., 1998; Dar et al., 2007).

DNA-DNA hybridization experiments have been performed to determine relatedness between bacteria, and it is one of the three criteria used in defining prokaryotic species (Wayne et al., 1987; Gevers et al., 2005; Roselló-Mora, 2006). The mol% G+C content of DNA is considered part of the standard description of bacterial species, and it is optional for a type strain of a new species in an established genus (Stackebrandt et al., 2002).

DNA typing methods that target whole genomes are recommended to determine genomic relatedness (Van Belkum et al., 2001; Stackebrandt et al., 2002). Pulsed field gel electrophoresis (PFGE) has been useful in discriminating *Desulfurella acetivorans* DSM 5264^T from *D. multipotents* DSM 8415^T (Pradella et al., 1998) and *Bacillus anthracis* from its closest relatives *B. cereus* and *B. thuringiensis* (Zhong et al., 2007). PFGE has been primarily used to determine genome size of several *Desulfovibrio* species (Devereux et al., 1997; Walker et al., 2006).

Ribotyping has been used to discriminate bacterial strains based on the location of restriction enzyme recognition sites within the 16S rRNA genes and around the ribosome

RNA operons (*rrn*). Specific ribotype profiles have been shown in *Burkholderia* species such as *B. xenovorans*, *B. sacchari*, *B. phenazinium*, *B. hospita*, *B. fungorum*, *B. caledonica*, and *B. glathei* (Goris et al., 2004). *Bifidobacterium infantis* could be differentiated from *B. longum* based on the size of their ribosomal bands despite the same number of bands (Mangin et al., 1994; Klein, 2007). The ribotyping method has also been used to determine the 16S rRNA gene copy number in *Campylobacter helveticus* (Linton et al., 1994).

The repetitive DNA sequences are commonly present in multiple copies in the genomes of most gram-negative bacteria (Healy, 2005). PCR of these repetitive elements has been used to reveal the genetic diversity of closely related bacterial strains (Koeuth et al., 1995; Rademaker et al., 2000; Tacão et al., 2005). The repetitive PCR with REP and ERIC primers has been recommended for identification, discrimination and analysis of strain relatedness within *Desulfovibrio* species (Dzierżewicz et al., 2003).

We hypothesized that the taxonomic status of *Desulfovibrio* SR-1 will be elucidated by consistent phenotype differences, phylogenetic analysis, DNA-DNA hybridization, G+C mol% content, and DNA typing methods targeting whole genomes and gene clusters.

To test this hypothesis, a phenotypic and genotypic comparison between *Desulfovibrio* SR-1 and the type strain *D. africanus* DSM 2603^{T}was performed. The complementary phenotypic characterization included morphology and pyruvate fermentation. The genotypic characterization included phylogenetic analyses (16S rRNA gene, 16S-23S ITS and *dsrAB* gene), DNA-DNA hybridization, G+C mol% content, and genomic fingerprints (PFGE, ribotyping, BOX-PCR).

Materials and Methods

Desulfovibrio strains

Desulfovibrio SR-1 was isolated from a shallow uranium contaminated subsurface aquifer in Shiprock-New Mexico (Castañeda, 2001). The most relevant chemical properties of the groundwater were: 36.4 mM sulfate, 2.7 µM U (VI), and absence of nitrate. The selected sediment to isolate *Desulfovibrio* spp. contained 4.27 x 10^4 SRB/g.

The type strain *Desulfovibrio africanus* DSM 2603^T (Campbell et al., 1966) was used to determine the phenotypic and genetic differences between both strains.

Complementary Phenotypic Characterization

Electron-microscope analysis

Desulfovibrio SR-1 and *D. africanus* DSM 2603^T strains were grown in lactate sulfate medium (Castañeda, 2001) at 37°C for 12 hours. Cells from 1-ml culture were washed gently with 3mM HEPES (pH 7.0) and resuspended in 100 µl of the same buffer. Samples were prepared according to standard protocols for the negative stain with uranyl acetate. Electron micrographs were obtained using the transmission electron microscope JEOL 2000FX.

Pyruvate fermentation

10-ml exponential phase culture of *Desulfovibrio* SR-1 grown in lactate sulfate medium was pelleted by centrifugation and rinsed with sulfate-free pyruvate medium (Castañeda, 2001). Washed cells were inoculated into 100 ml of 10 mM pyruvate medium and incubated at 37°C. After 2 week incubation, the concentration of acetate was quantified by Gas Chromotography (GC) using a Varian 3400 GC equipped with a

column 80/120 carbopack B.DA*/4% carbowax 20M. Nitrogen carrier gas flow was 24 ml/min. The sample was diluted in nanopure water and acidified with oxalic acid.

Genetic Characterization

Culture conditions for molecular analysis and chromosomal DNA Isolation

Desulfovibrio SR-1 and *Desulfovibrio africanus* DSM 2603^T were cultured in lactate sulfate medium at 37°C. Genomic DNA was isolated from 10-ml exponentially grown cultures using the Easy-DNA Kit (Invitrogen).

Amplification of 16S rRNA gene and 16S-23S rRNA internal transcribed spacer region.

The 16S rRNA genes from *Desulfovibrio* SR-1 and *D. africanus* DSM 2603^T were amplified using the consensus primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1496R (5'-TACCTTGTTACGACTT-3') (Kane et al., 1993). DNA templates were amplified in a total reaction of 50 µl containing 500 ng of chromosomal DNA, 400 µM dNTPs, 400 nM each primer, 2.5 mM $MgCl_2$ and 2.5 U of Platinum Taq DNA polymerase (Invitrogen). An initial denaturation step of 94°C for 5 min was followed by 30 cycles of 94°C per 45 s, 55°C per 45 s and 72°C per 1 min 40 s; and a final extension of 15 min at 72°C. The PCR products were then cloned into the TOPO-TA cloning vector according to the manufacturer's instructions (Invitrogen). Twenty randomly selected clones were screened by KpnI-restriction digestion of the purified plasmids, and two similar clones were then sequenced using the vector primers.

The16S-23S rRNA internal transcribed spacer region (ITS) from *Desulfovibrio* SR-1 and *D. africanus* DSM 2603^T were amplified using primers complementary to

conserved regions in the 23S rRNA gene (García-Martínez et al., 1999) and in the 16S rRNA gene of *Desulfovibrio* species (Devereux et al., 1992). Sequences of the forward (683-702 position in *Desulfovibrio* SR-1) and reverse (38-21 *E. coli* numbering) primers were: F-16S (5'-TGTAGGAGTGAAATCCGTAG-3') and R-23S (5'-TGCCAAGGCATCCACCGT-3'). DNA templates were amplified in a total reaction of 50 µl containing 500 ng of chromosomal DNA, 400 µM dNTPs, 400 nM each primer, 2.5 mM $MgCl_2$ and 2.5 U of Platinum Taq DNA polymerase (Invitrogen). An initial denaturation step of 94°C for 5 min was followed by 30 cycles of 94°C per 45 s, 55°C per 45 s and 72°C per 1 min 20 s; and a final extension of 15 min at 72°C. The PCR products were then cloned into the TOPO-TA cloning vector as described for the 16S rRNA gene. Twenty randomly selected clones were screened by SphI-restriction digestion analysis of the purified plasmids, and two similar clones from each strain were then sequenced using the vector primers.

Sequence reactions were performed on double-stranded DNA-templates with the primers M13-F (5'-GTAAAACGACGGCCAG-3') and M13-R (5'-CAGGAAACAGCTATGAC-3') using the BigDye Terminator Cycle sequencing kit. Sequences were obtained with a 3730 DNA analyzer (Applied Biosystems).

The 16S rDNA sequences were assembled using the program "bl2seq" (Tatusova and Madden, 1999) and blasted to GenBank using the blastn algorithm (Altschul et al., 1990). Complete 16S rDNA sequences from other *Desulfovibrio* species were obtained from the GenBank database. CLUSTAL_X (Thompson et al., 1997) was used to align all 16S rDNA sequences. The sequence data of 1521 aligned bases, between *Escherichia coli* positions 30 and 1541, were used for phylogenetic analysis. The neighbour-joining

phylogenetic analysis (Saitou and Nei, 1987) with bootstrap values (1000 replicates) was carried out using PAUP 4.0* software package (Swofford, 1998). A distance matrix was computed using PHYLIP Maximum Parsimony and Jukes-Cantor correction (Thompson et al., 1994).

Similarity to ITS sequences from other *Desulfovibrio* species was also determined using the blastn algorithm (Altschul et al., 1990). The structure of ITS regions was analyzed using the IWoCS software at http://egg.umh.es/iwocs/ (D'Auria et al., 2006). The presence of transfer RNA genes with predicted secondary structures was detected using the program tRNAscan-SE v.1.21 at http://lowelab.cse.ucsc.edu/tRNAscan-SE/ (Lowe and Eddy, 1997). Complete ITS sequences from other *Desulfovibrio* species were obtained from the GenBank database. All ITS sequences were then aligned using the MUSCLE algorithm at http://www.drive5.com/muscle/ (Edgar, 2004) and imported into the ARB Software package at http://www.arb-home.de (Ludwig et al., 2004). The sequence alignment was then checked for misalignment using the ARB integrated aligner. A filter was created using the Maximum Frequency method, which takes only the tRNA gene sequences into account and screens out the variable regions. The phylogenetic tree for the tRNA-coding sequences was then generated using the ARB software. A distance matrix was computed using PHYLIP Maximum Parsimony and Jukes-Cantor correction (Thompson et al., 1994).

Amplification of the dissimilatory sulfite reductase gene (*dsrAB*)

The *dsrAB* gene from *Desulfovibrio* SR-1 was amplified using the consensus primers DSR1F (5'-AC[C/G]CACTGGAAGCACG-3') and DSR4R (5'-GTGTAGCAGTTACCGCA-3') (Wagner et al., 1998). DNA template was amplified in a

50-µl reaction mixture containing 500 ng of genomic DNA, 400 µM dNTPs, 400 nM each primer, 2.5 mM $MgCl_2$ and 2.5 U of platinum Taq DNA polymerase (Invitrogen). An initial denaturation step of 94°C for 5 min was followed by 30 cycles of 94°C per 45 s, 51°C per 45 s and 72°C per 2 min; and a final extension of 15 min at 72°C. The PCR products were directly cloned into the TOPO-TA cloning vector as described above. Twenty randomly selected clones were screened by BamHI-restriction digestion analysis of the purified plasmids. The *dsrAB*-fragments from two clones were sequenced on both DNA strands. DNA sequencing was performed using the M13 vector primers and two custom-designed internal primers DSR1F-2nd (5'-TTCCGACTTCTCGGTCATCG-3') and DSR4R-2nd (5'-TCCAGGATCTCGTGGTACAG-3). Sequences were also obtained with a 3730 DNA Analyzer .The *dsrAB* nucleotide sequences were assembled using the program "bl2seq" (Tatusova and Madden, 1999). Similarity to *dsrAB* sequences from cultured sulfate reducing bacteria was determined using blastn and tblastx algorithms (Altschul et al., 1990) (http://www.ncbi.nlm.nih.gov/BLAST/). The nucleotide sequences of *Desulfovibrio dsrAB* genes were aligned according to the deduced amino acid sequences. DsrAB amino acid sequences deduced from *Desulfovibrio dsrAB* gene sequences greater than 1941 nucleotides were considered for comparative analysis. The 327 amino acid positions of alpha subunit and the 216 amino acid positions of beta subunit were used for phylogeny inference (Wagner et al., 2005). The consensus phylogenetic tree based on the FITCH distance method was generated using ARB software (Ludwig et al., 2004) at http://www.arb-home.de . A distance matrix was generated using PHYLIP and Jones-Taylor-Thorton models.

DNA-DNA hybridization and DNA base composition (G+C mol% content)

Spectroscopic DNA-DNA hybridization between *Desulfovibrio* SR-1 and *D. africanus* DSM 2603^T was performed at the DSMZ (Deutche Sammlung von Mikroorganismen und Zellkulturen) Braunschweig, Germany. DNA was purified by chromatography on hydroxyapatite (Cashion et al., 1977), and the DNA-DNA hybridization was carried out at 67°C (De Ley et al., 1970; Huβ et al., 1983). The DNA-DNA relatedness percentage is reported as a mean value based on duplicate measurements.

The DNA base composition of *D. africanus* SR-1 was determined by reverse phase HPLC using non-methylated Lambda-DNA as a calibration reference (Mesbah et al., 1989). DNA from three species with complete genome sequence was also used as reference (*Bacillus subtilis* DSM 402, *Xanthomonas campestris* pv. *campestris* DSM 3586, and *Streptomyces violaceoruber* DSM 40783). The G+C content determination was also performed at the DSMZ.

Pulsed field gel electrophoresis (PFGE)

3-ml exponential phase cultures of both *Desulfovibrio* strains were washed three times with phosphate buffered saline (PBS). The cell pellets were then resuspended in 500 µl of PBS, mixed gently with 500 µl of 2% molten agarose, and poured into plug molds. The embedded bacteria were lysed with the lysozyme (1%) and proteinase K (1%) buffers at 37°C for 30 minutes, and 55°C for 12 hours, respectively. Plugs were then washed five times with 1X TBE buffer, twice with acetone, and three times with 1X TBE buffer (Bautsch, 1992). Slices of plugs (2mm wide) were digested with 20U of PmeI in a 200 µl restriction mixture for 16 hours at 37°C. The plugs were then loaded onto a 1%

agarose gel in 0.5 X TBE buffer. PFGE was performed on a CHEF-DF III system (Bio-Rad Laboratories, 1992). Running conditions included the following: 0.5X TBE running buffer, 14°C running temperature, 6.0 V cm^{-1} for 24 hours, and a switching time linearly ramped from 60-120 s.

Ribotyping and Southern blot analysis

The DNA hybridization signals for the 16S rRNA genes in *Desulfovibrio* SR-1, and *D. africanus* DSM 2603^T genomes were detected by Southern blot hybridization. The primers F-16S-Hyb (5'-GGTGTAGGAGTGAAATCCGTAG-3) and R-16S-Hyb (5'-AGTTTCAGCCTTGCGACCGTAC-3') were designed to amplify a 227-bp labeled probe using the PCR DIG Probe Synthesis Kit (Roche). Based on restriction site computer analysis of their 16S rDNA sequences, genomic DNA was digested with restriction enzymes that cut this gene once (SspI, StuI, kpnI, EcoRI, SphI, MluI, SacII) and twice (SmaI, XmaI). The probe sequence does not contain recognition sites for these restriction enzymes. Genomic DNA was also digested with enzymes that might have recognition sites around the rRNA operons (BamHI, ClaI, HindIII, NruI, PstI, ScaI, BmgBI, MboI, PvuII). Approximately 5 μg of digested genomic DNA was separated by agarose gel electrophoresis on 0.8% agarose gels in 1X Tris-borate-EDTA buffer (pH 7.9) at 90 volts for 4 hours. Gels were blotted by downward capillary transfer (Chomczynski, 1992) on positively charged nylon membranes (Nytran SuperCharge, 0.45 um pore-size). DNA was covalently bonded to the nylon membrane using UV cross-linking at 1250 (x100 J/cm^2) for 1 minute on each side. The DNA-DNA hybridization and the immunological detection of hybridization signals were performed according to the protocol supplied by the manufacturer (Roche). The reproducibility of the 16S rDNA

fingerprints was assessed by a similar approach using new genomic DNA samples, which were digested with the restriction enzymes BmgBI, HindIII, KpnI, PstI, SphI and XmaI.

Repetitive PCR genomic fingerprinting

BOX-PCR fingerprints of *Desulfovibrio* SR-1 and *D. africanus* DSM 2603^T were obtained with the BOX A1R primer (5'-CTACGGCAAGGCGACGCTGACG-3') (Versalovic et al., 1994). All PCR reactions were performed in 50 µl reaction mixtures containing 4 mM $MgCl_2$, 200 µM dNTPs, 1 µM BOX A1R primer, 2.5U of Platinum Taq DNA polymerase, and 400 ng of genomic DNA. Thermal cycling parameters included an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 94°C per 1 min, 50°C per 1 min, 72°C per 8 min; and a final extension of 8 min at 72°C. A 10-µl aliquot of amplified PCR products was separated by gel electrophoresis on 0.8% agarose gels in 1X Tris-borate-EDTA buffer (pH 7.9) at 80 volts for 2 hours. To examine the stability of the BOX-PCR fingerprints of *Desulfovibrio* SR-1 after several subcultures (Kang and Dunne, 2003), genomic DNA was extracted from the original culture and after several subcultures over a 5-year period. The reproducibility of BOX-PCR fingerprint patterns was verified in two independent PCR reactions.

Plasmid screening

Cultures from exponential and stationary phase were screened for the presence of small native plasmids. Different culture volumes ranging from 5 to 30 ml were assayed for the plasmid detection. Cultures were centrifuged at 8000 rpm, at 4°C for 8 minutes. Plasmid purification from the cell-pellets was performed by alkaline lysis using the QIAGEN plasmid mini prep protocol, but the bacterial lysate was precipitated with isopropanol and 70% ethanol. Plasmid DNA was resuspended in 20 µl of distilled water

and analyzed by gel electrophoresis on 0.6% agarose gels in 1X Tris-borate-EDTA buffer (pH 7.9) at 90 volts for 2 hours.

Results

Complementary Phenotypic Characterization

Electron-microscope analysis

Electron-microscope analysis of negatively stained cells of *Desulfovibrio* SR-1 showed the presence of a single polar flagellum (Fig 1.1). In contrast, polar lophotrichous flagella were observed in *D. africanus* DSM 2603^{T} (Fig 1.2).

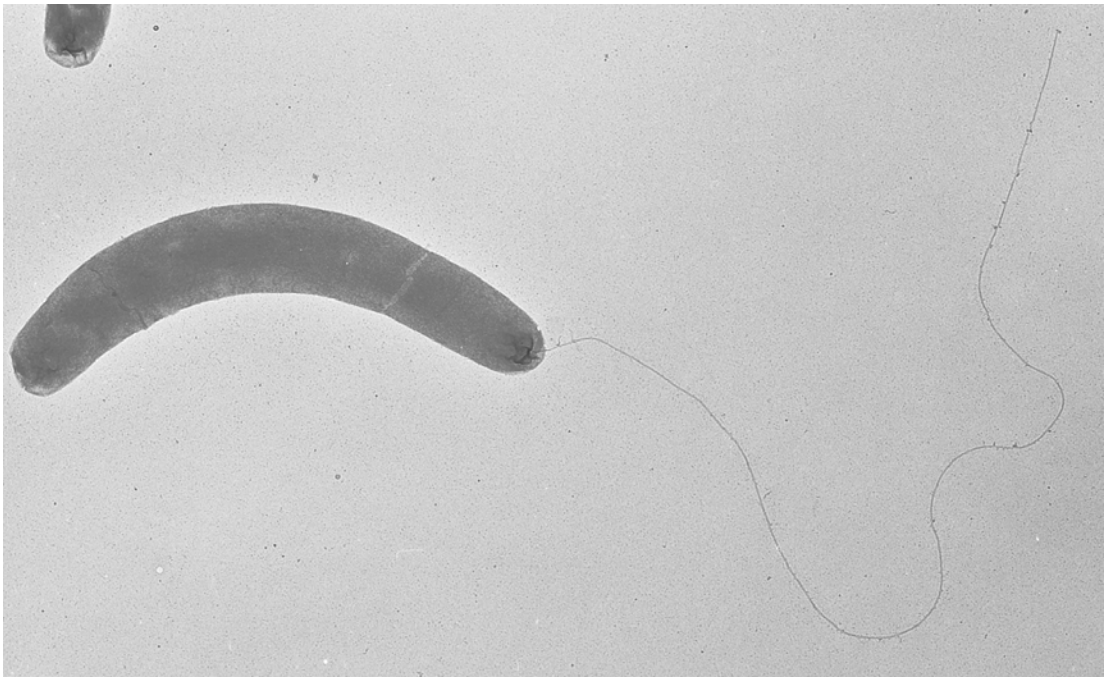

Fig 1.1. Transmission electron micrograph of negatively stained cells of *Desulfovibrio* SR-1.

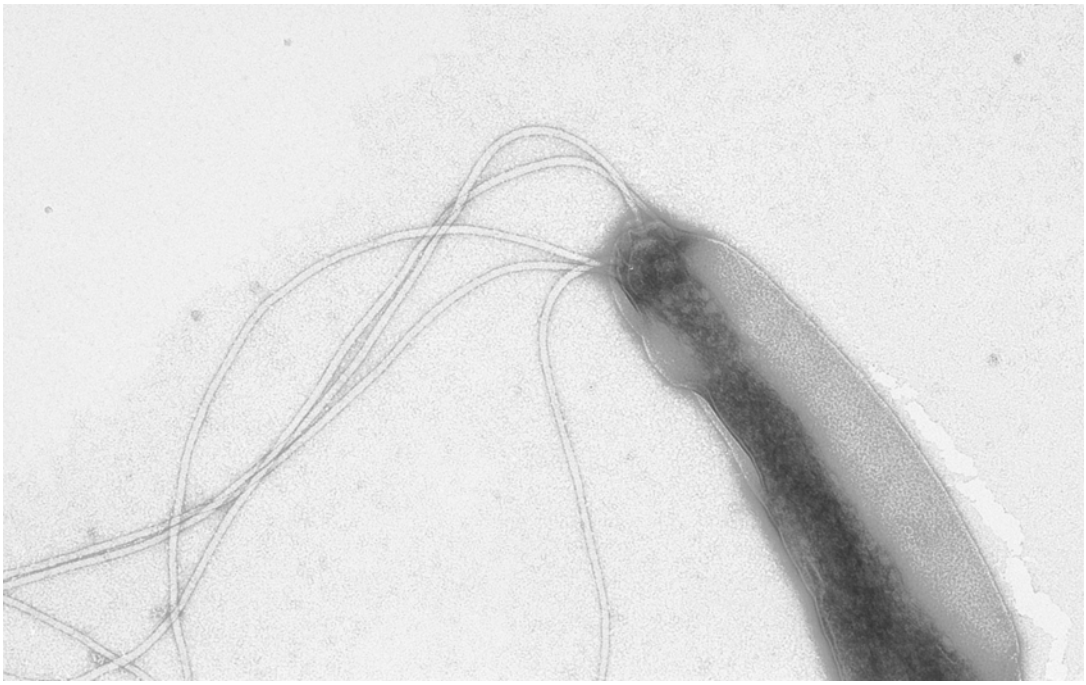

Fig 1.2. Transmission electron micrograph of negatively stained cells of *D. africanus* DSM 2603^{T}.

Pyruvate fermentation

Desulfovibrio SR-1 fermented pyruvate to acetate, giving rise to 6 mM acetate as determined by gas chromatography. *D. africanus* DSM2603^{T} did not grow in sulfate-free pyruvate medium.

Genetic Characterization

Amplification of 16S rRNA gene and 16S-23S rRNA internal transcribed spacer region.

Since complete 16S rDNA sequences allow a reliable phylogenetic comparison, we sequenced 1880 nucleotides of the rRNA operon of *Desulfovibrio* SR-1, which

included the full-length 16S rRNA gene and ITS region, and the 5' 23S rRNA gene. We also sequenced 800 nucleotides of the rRNA operon of *D. africanus* DSM 2603^T, from position1059 of the16S rDNA sequence (X99236). The complete sequence of the16S rRNA gene of *Desulfovibrio* SR-1 comprised of 1542 nucleotides corresponded to *E. coli* 16S rDNA sequence positions 9 to 1541 (Brosius, 1978). The similarity of 16S rDNA sequences between SR-1 and DSM 2603^T was 99.93% (Table 1.1). The comparative analysis of 1521 16S-rDNA nucleotide positions revealed that *Desulfovibrio* SR-1 is closely related to *D. africanus* DSM 2603^T (Fig 1.3).

Table 1.1. 16S rDNA similarity values for *Desulfovibrio* SR-1 and related *Desulfovibrio* species.

	% 16S rRNA sequence similarity							
	Desulfovibrio SR-1	*D. africanus* DSM 2603^T	*D. vulgaris* DP4	*D. burkinensis* DSM 6830^T	*D. magneticus* DSM 13731^T	*D. desulfuricans* Essex 6^T	*D. desulfuricans* F28	*D. desulfuricans* MB
Desulfovibrio africanus DSM 2603^T	99.93							
D. vulgaris DP4 (CP000527)	88.01	87.93						
D. burkinensis DSM 6830^T (AF053752)	89.18	89.11	87.12					
D. magneticus DSM 13731^T (D43944)	88.71	88.64	86.86	98.94				
D. desulfuricans Essex6^T ATCC 29577 (AF192153)	87.97	87.9	90.55	87.74	87.97			
D. desulfuricans F28 (DQ092636)	87.9	87.82	90.47	87.66	87.89	99.93		
D. desulfuricans MB ATCC2774 (AF192154)	88.05	87.97	90.32	86.88	87.03	97.18	97.12	
Eschericha coli (V00348)	75.67	75.58	75.01	73.63	73.52	75.73	75.73	75.82

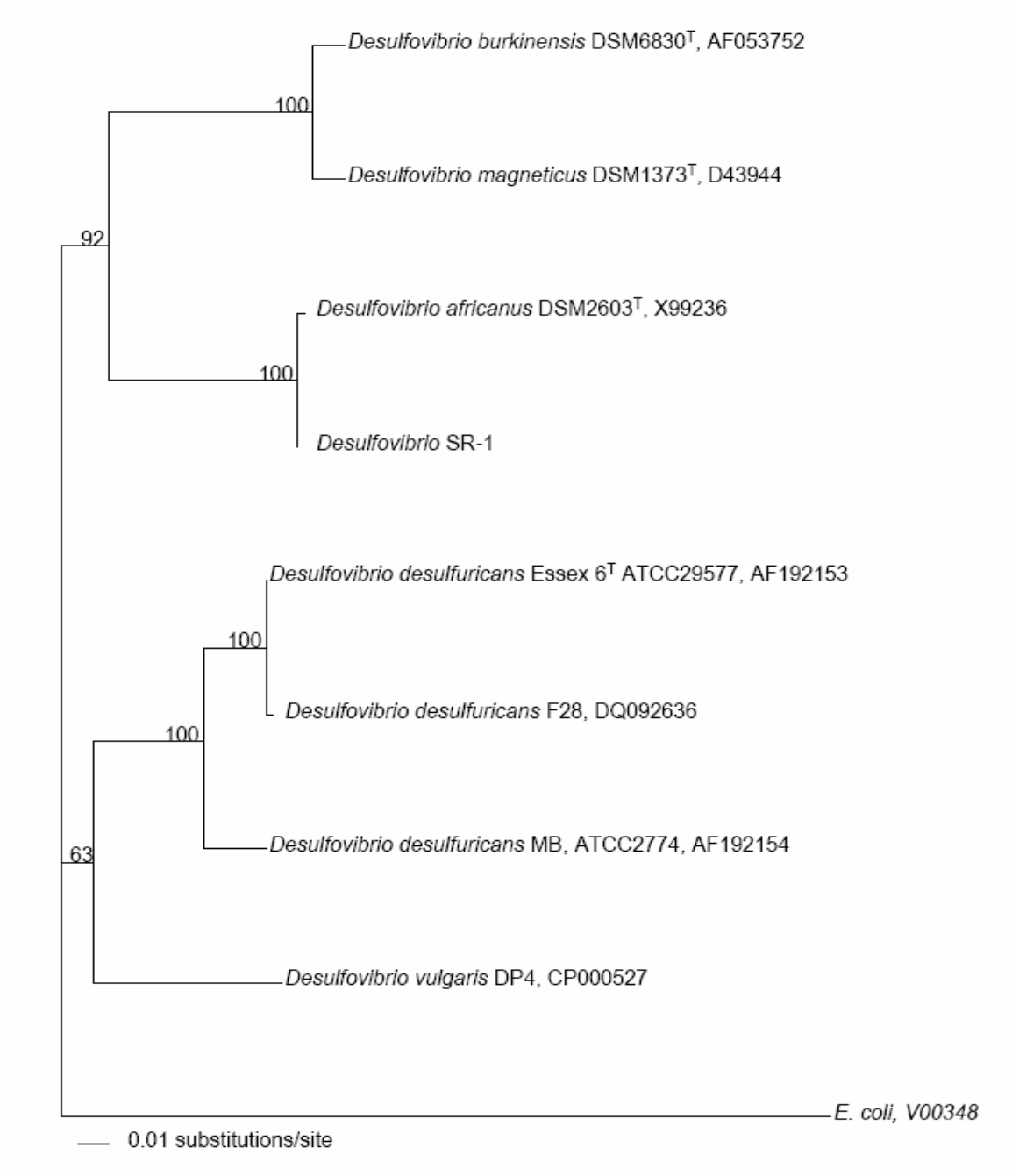

Fig 1.3. Phylogenetic tree of *Desulfovibrio* SR-1 and phylogenetically related species based on 1521 nucleotide positions of 16S rDNA sequences. The dendrogram was generated by the neighbor-joining algorithm. The scale bar indicates 1 nucleotide difference per 100 nucleotide positions.

The DNA sequences of the ITS region of *Desulfovibrio* SR-1 and *D. africanus* DSM 2603^T were 300 and 299 bases in length, respectively, with a 99% identity. Two transfer RNA genes (tRNA^{Ile}, tRNA^{Ala}) with predicted secondary structures were detected in the ITS sequences of both strains. The similarity of the tRNA gene sequences between SR-1 and DSM 2603^T was 99.38% (Table 1.2). The comparative analysis of the ITS sequences revealed that *Desulfovibrio* SR-1 is closely related to *D. africanus* DSM 2603^T (Fig 1.4).

Table 1.2. 16S-23S ITS similarity values for *Desulfovibrio* SR-1 and related *Desulfovibrio* species.

	% ITS nucleotide similarity							
	Desulfovibrio SR-1	*D. africanus* DSM 2603^T	*D. desulfuricans* G20	*D. vulgaris* DP4	*D. vulgaris* Hildenborough	*D. desulfuricans* Essex 6^T	*D. fairfieldensis*	*D. desulfuricans* MB
Desulfovibrio africanus DSM 2603^T	99.38							
D. desulfuricans G20 CP000112	92.44	92.44						
D. vulgaris DP4 CP000527	89.78	89.78	88.92					
D. vulgaris Hildenborough AE017285	89.78	89.78	88.92	100				
D. desulfuricans Essex 6^T ATCC 29577	83.61	84.26	78.37	78.14	77.99			
D. fairfieldensis ATCC700045	79.4	80.07	80.88	82.78	82.67	68.1		
D. desulfuricans MB ATCC 27774	84.26	84.92	87.11	87.56	87.47	87.52	86.07	
Escherichia coli D12649	73.84	74.52	72.49	70.87	70.67	67.98	65.72	77.54

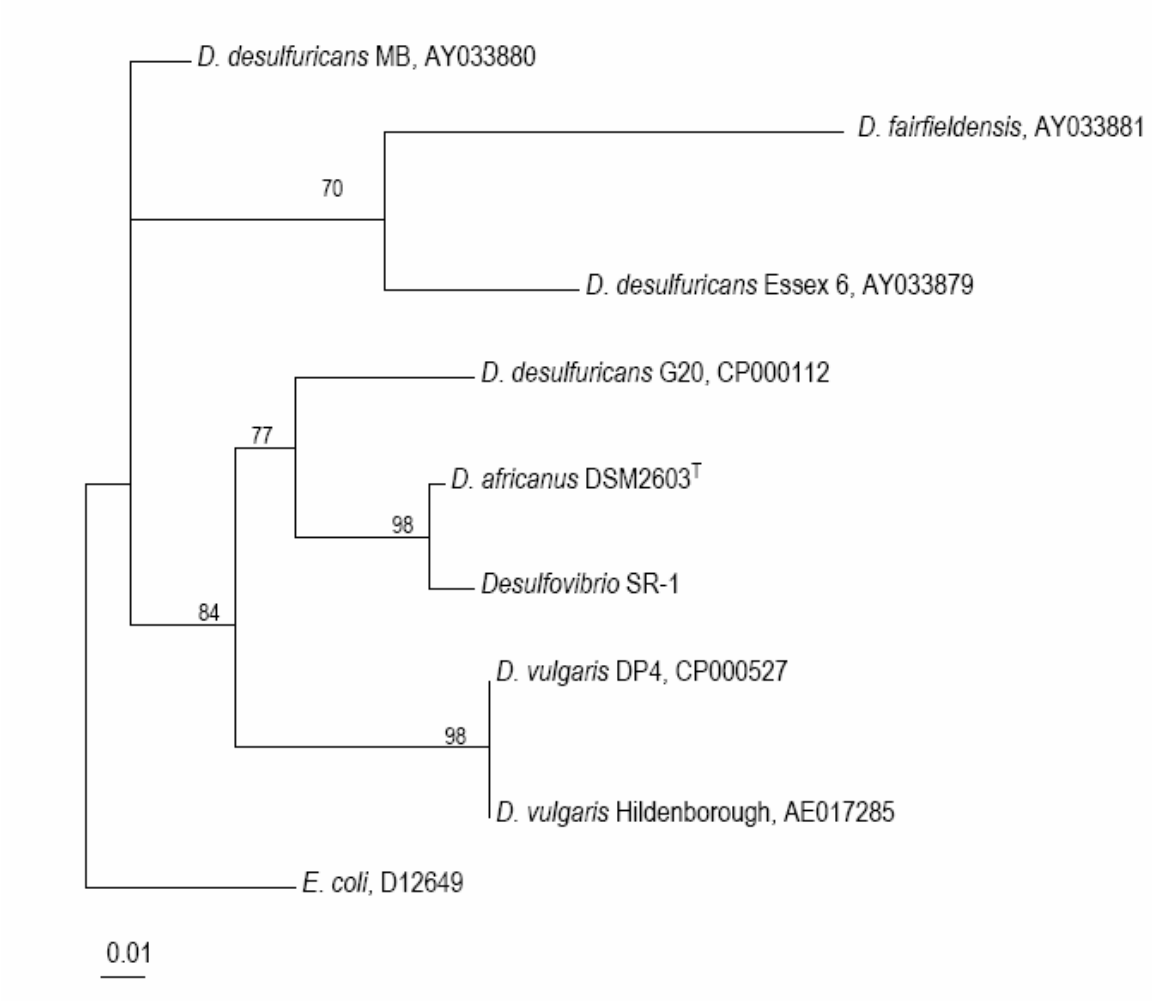

Fig1.4. Phylogenetic tree of *Desulfovibrio* SR-1 and phylogenetically related species based on the alignable stretches of ITS sequences. The dendrogram was generated by the neighbor-joining algorithm. The scale bar indicates 1 nucleotide difference per 100 nucleotide positions.

Amplification of the dissimilatory sulfite reductase gene (*dsrAB*)

The *dsrAB* gene sequences (1983 nucleotides) of *Desulfovibrio* SR-1 and *D. africanus* DSM 2603^T (AF271772) were 96% similar. The similarity of the DsrAB amino

acid sequences between SR-1 and DSM 2603^T was 94.66% (Table 1.3). The comparative analysis of the DsrAB amino acid sequences revealed that *Desulfovibrio* SR-1 is phylogenetically related to *D. africanus* DSM 2603^T (Fig 1.5).

Table 1.3. DsrAB similarity values for *Desulfovibrio* SR-1 and related *Desulfovibrio* species.

	% amino acid sequence similarity to:											
	Desulfovibrio SR-1	*D. africanus* DSM2603^T	*D. burkinensis*	*D. carbinolicus*	*D. fructosivorans*	*D. desulfuricans Essex6*	*D. simplex*	*D. piger*	*D. oxamicus*	*D. termitidis*	*D. vulgaris Hildenbourogh*	*D. vulgaris DP4*
D. africanus DSM2603^T	94.66											
D. burkinensis	55.81	51.32										
D. carbinolicus	53.83	49.74	98.46									
D. fructosivorans	54.92	49.66	87.92	86.62								
D. desulfuricans Essex6	55.78	52.74	52.10	50.87	53.93							
D. simplex	55.31	51.13	55.07	53.28	55.95	94.73						
D. piger	52.71	50.54	57.59	56.28	58.17	87.80	88.24					
D. oxamicus	59.32	57.51	60.08	59.42	59.23	72.47	72.49	67.50				
D. termitidis	60.28	56.35	59.74	59.08	60.94	73.84	73.19	72.29	96.47			
D. vulgaris Hildenborough	65.28	63.56	64.17	63.62	61.94	72.34	70.70	71.91	86.37	84.53		
D. vulgaris DP4	65.28	63.56	64.17	63.62	61.94	72.34	70.70	71.91	86.37	84.53	100	
Desulfomonile tiedjei	45.92	42.13	40.30	38.94	40.83	36.98	40.02	40.17	46.92	48.46	51.94	51.94

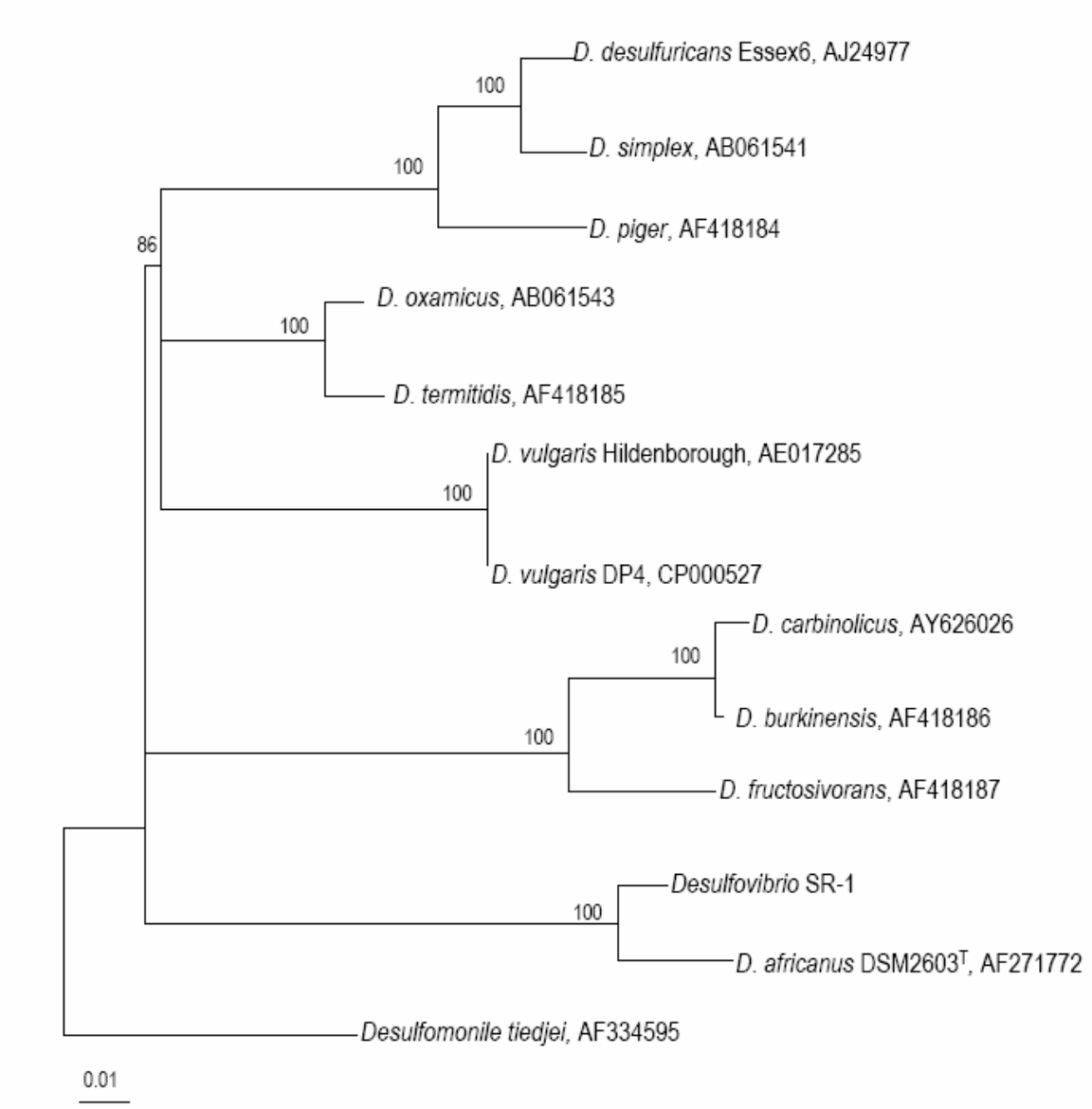

Fig 1.5. Phylogenetic tree of DsrAB amino acid sequences deduced from *Desulfovibrio* sequences greater than 1941 bases showing the affiliation of *Desulfovibrio* SR-1. The bar scale indicates 10% estimated sequence divergence.

DNA-DNA hybridization and DNA base composition (G+C mol% content)

The DNA-DNA hybridization values between *Desulfovibrio* SR-1 and *D. africanus* DSM 2603^T were 72.1% and 71.9%. The DNA-DNA similarity of SR-1 with

DSM 2603^{T} was the mean value 72%. The DNA base composition of *Desulfovibrio* SR-1 was 62.4 mol% G+C.

Pulsed field gel electrophoresis (PFGE)

PFGE patterns of both bacterial chromosomes revealed the presence of three PmeI-fragments in *Desulfovibrio* SR-1 and six PmeI-fragments in *D. africanus* (Fig 1.6).

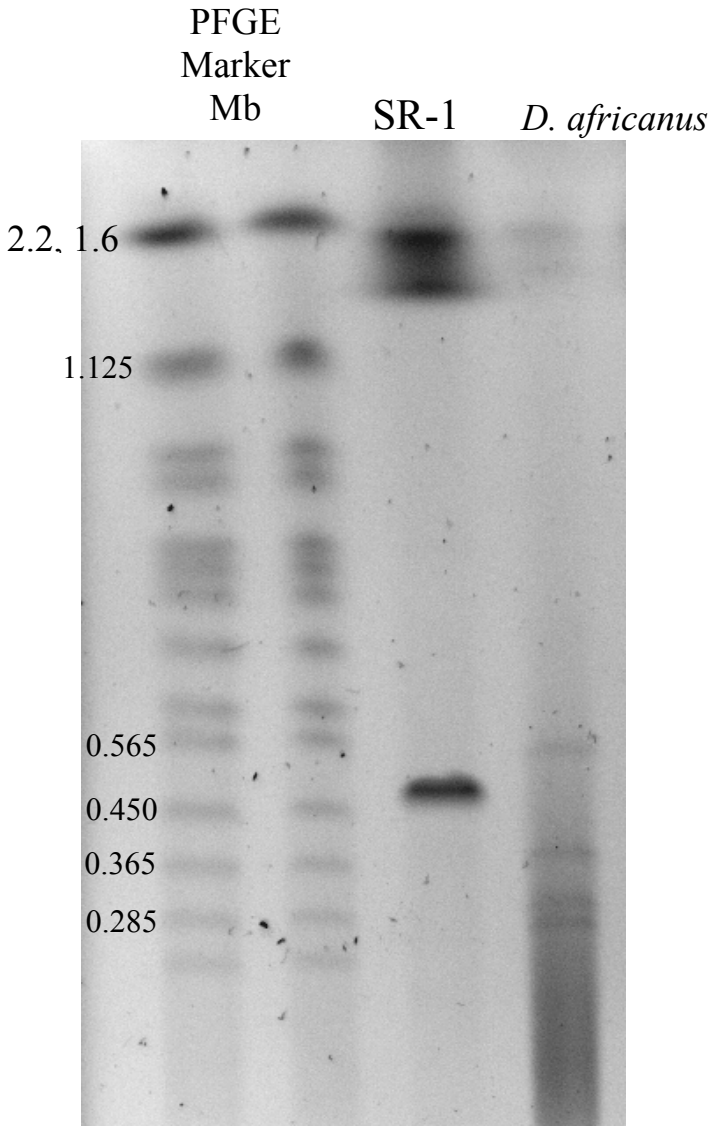

Fig 1.6. PFGE fingerprint patterns of PmeI restriction digests of *Desulfovibrio* SR-1 and *D. africanus* DSM 2603^{T}. Lanes 1, 2: *Saccharomyces cerevisiae* size marker. Lane 3: *Desulfovibrio* SR-1. Lane 4: *D. africanus* DSM 2603^{T}.

Ribotyping and Southern blot analysis

Similar 16S rDNA fingerprints were observed with the restriction enzymes EcoRI, KpnI and StuI (Fig 1.7); BamHI and NruI (Fig 1.8); MboI, PvuII and SacII (Fig 1.9). Distinct 16S rDNA fingerprints were observed with the restriction enzymes SphI and SspI (Fig 1.7); ClaI, HindIII, PstI and ScaI (Fig 1.8); BmgBI, MluI and SmaI (Fig 1.9); and XmaI (Fig 1.10). The reproducibility of the fingerprint patterns was confirmed by the identical 16S rDNA fingerprints per restriction enzyme when they were assayed on new genomic DNA samples (Fig 1.10). In general, *Desulfovibrio* SR-1 could be distinguished from *D. africanus* based on variation of restriction sites within (SspI, SphI, SmaI, XmaI) and around (BmgBI, ClaI, HindIII, MluI, PstI) the 16S rRNA gene.

The number of SspI ribosomal fragments could represent the 16S rRNA gene copy number in *Desulfovibrio* SR-1 (Fig 1.7).

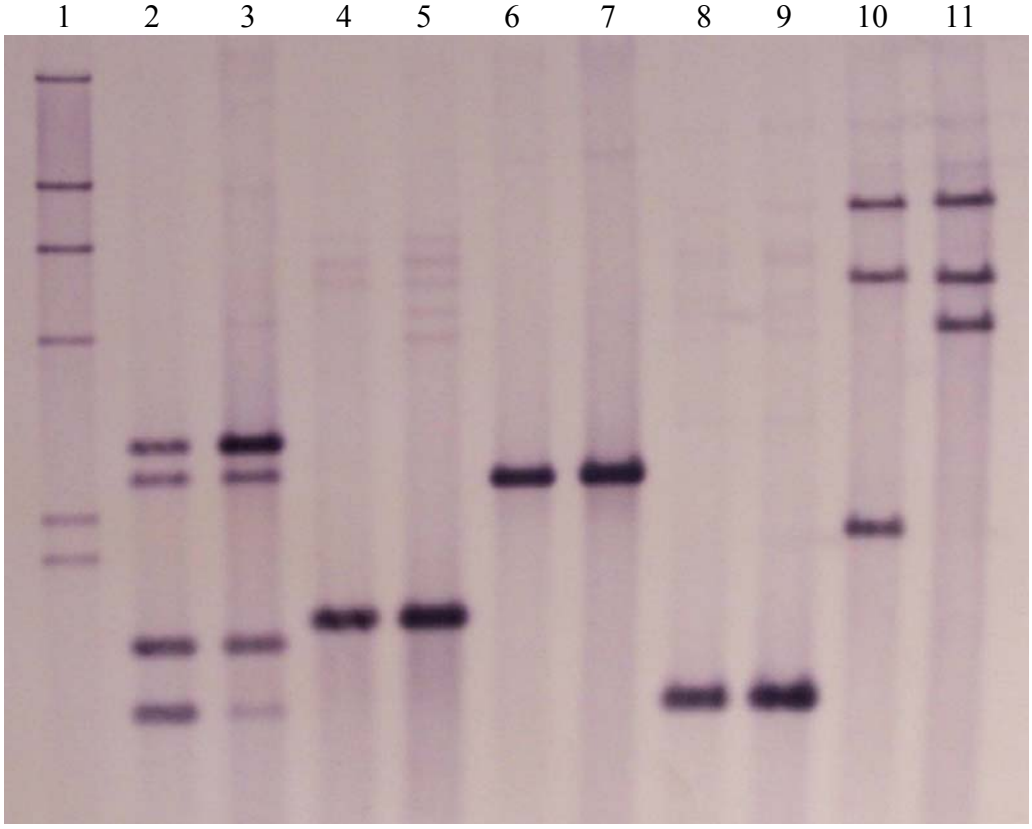

Fig 1.7. 16S rDNA fingerprints of *Desulfovibrio* SR-1 and *D. africanus* DSM 2603^T using enzymes that cut the 16S RNA gene once. *Desulfovibrio* SR-1 (lanes 2, 4, 6, 8, 10). *D. africanus* DSM 2603^T (lanes 3, 5, 7, 9, 11).Genomic DNA digested with SspI (lanes 2, 3), StuI (lanes 4, 5), KpnI (lanes 6, 7), EcoRI (lanes 8, 9) and SphI (lanes 10, 11). DNA molecular weight marker II (lane 1).

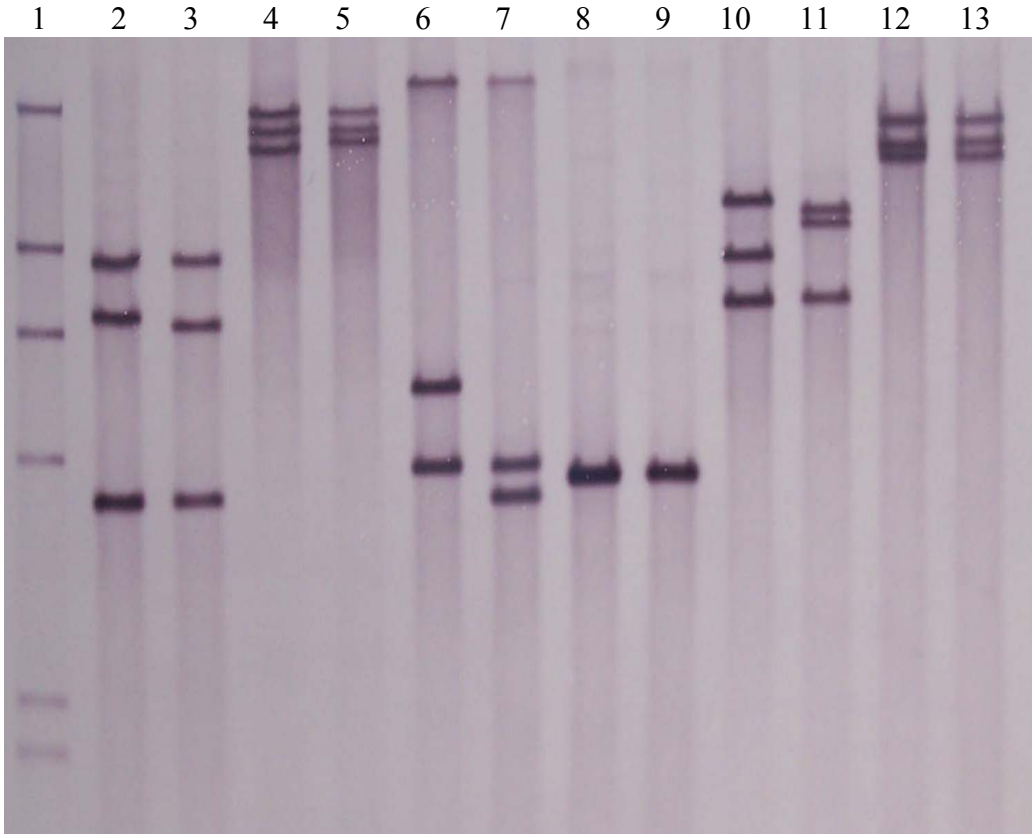

Fig 1.8. 16S rDNA fingerprints of *Desulfovibrio* SR-1 and *D. africanus* DSM 2603^T using enzymes with recognition sites around the ribosome operon. *Desulfovibrio* SR-1 (lanes 2, 4, 6, 8, 10, 12). *D. africanus* DSM 2603^T (lanes 3, 5, 7, 9, 11, 13). Genomic DNA digested with BamHI (lanes 2, 3), ClaI (lanes 4, 5), HindIII (lanes 6, 7), NruI (lanes 8, 9), PstI (lanes 10, 11) and ScaI (lanes 12, 13).DNA molecular weight marker II (lane 1).

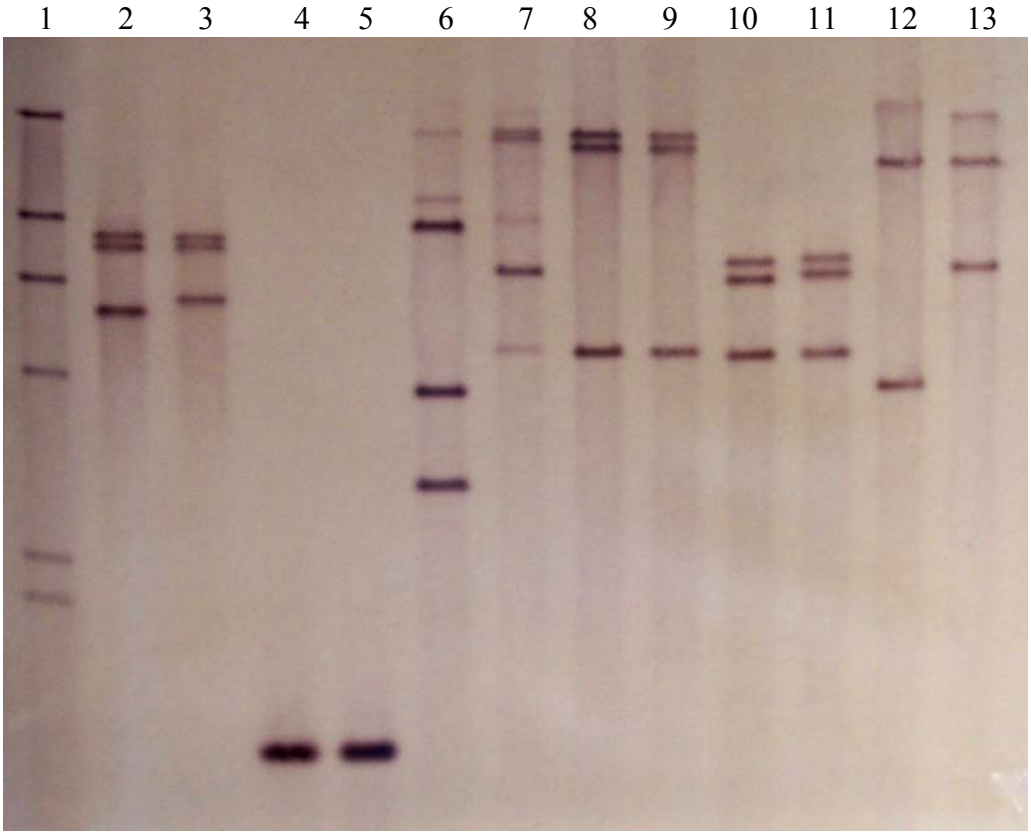

Fig 1.9. 16S rDNA fingerprints of *Desulfovibrio* SR-1 and *D. africanus* DSM 2603^T using enzymes with recognition sites within and around the ribosome operon. *Desulfovibrio* SR-1 (lanes 2, 4, 6, 8, 10, 12). *D. africanus* DSM 2603^T (lanes 3, 5, 7, 9, 11, 13). Genomic DNA digested with BmgBI (lanes 2, 3), MboI (lanes 4, 5), MluI (lanes 6, 7), PvuII(lanes 8, 9), SacII (lanes 10, 11) and SmaI (lanes 12, 13). DNA molecular weight marker II (lane 1).

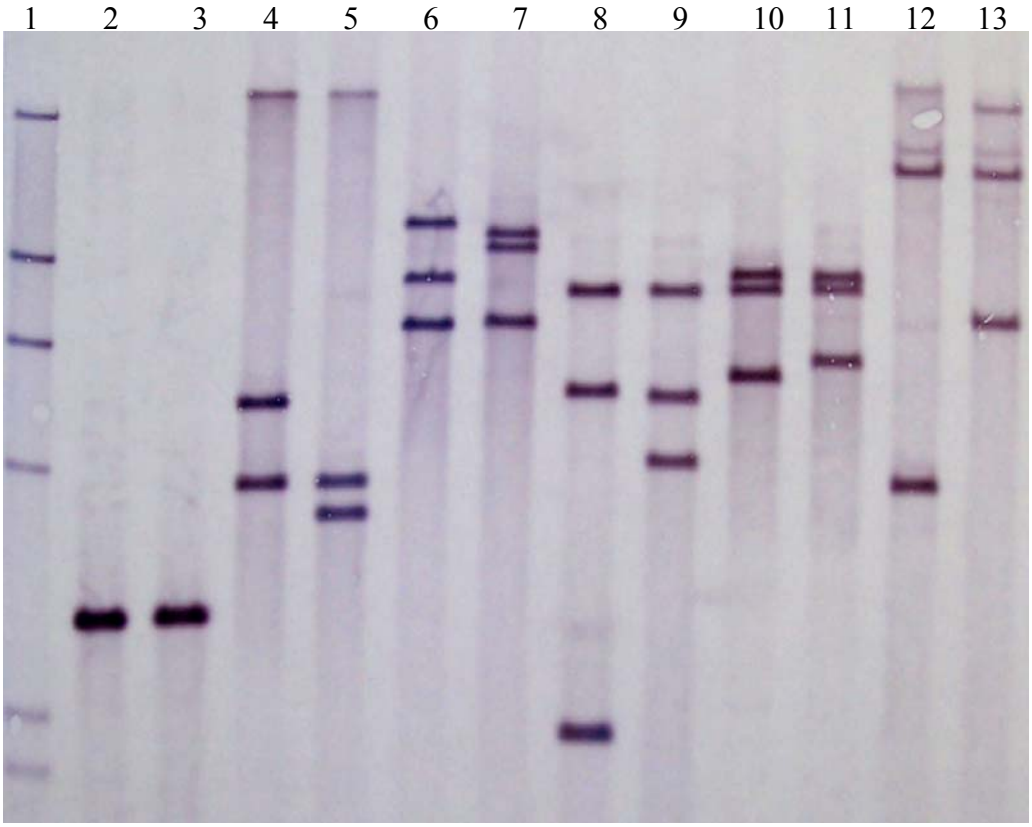

Fig 1.10. Distinct 16S rDNA fingerprints of *Desulfovibrio* SR-1 and *D. africanus* DSM 2603^T. *Desulfovibrio* SR-1 (lanes 2, 4, 6, 8, 10, 12). *D. africanus* DSM 2603^T (lanes 3, 5, 7, 9, 11, 13). Genomic DNA digested with KpnI (lanes 2, 3), HindIII (lanes 4, 5), PstI (lanes 6, 7), SphI(lanes 8, 9), BmgBI (lanes 10, 11) and XmaI (lanes 12, 13). DNA molecular weight marker II (lane 1).

Repetitive PCR genomic fingerprinting

Identical BOX-PCR fingerprint patterns were resolved on the two independent gels of *Desulfovibrio* SR-1 as well as on the gels of *D. africanus* DSM 2603^T, but significant differences in the fingerprint patterns between both strains were observed (Fig 1.11).

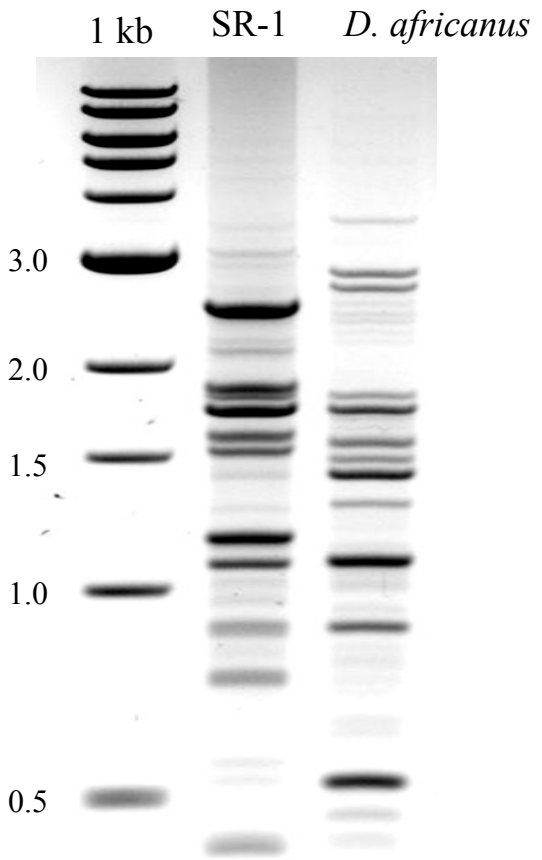

Fig 1.11. BOX-PCR fingerprint patterns of *Desulfovibrio* SR-1 and *D. africanus* DSM2603^T. *Desulfovibrio* SR-1 (lane 2). *D. africanus* DSM 2603^T (lane 3). 1kb DNA ladder (lane 1).

Plasmid screening

A small plasmid was only purified from either exponential or stationary phase cultures of *Desulfovibrio* SR-1 (Fig 12). In addition, the minimum volume that allowed the plasmid detection was 10 ml-cultures regardless of phase culture.

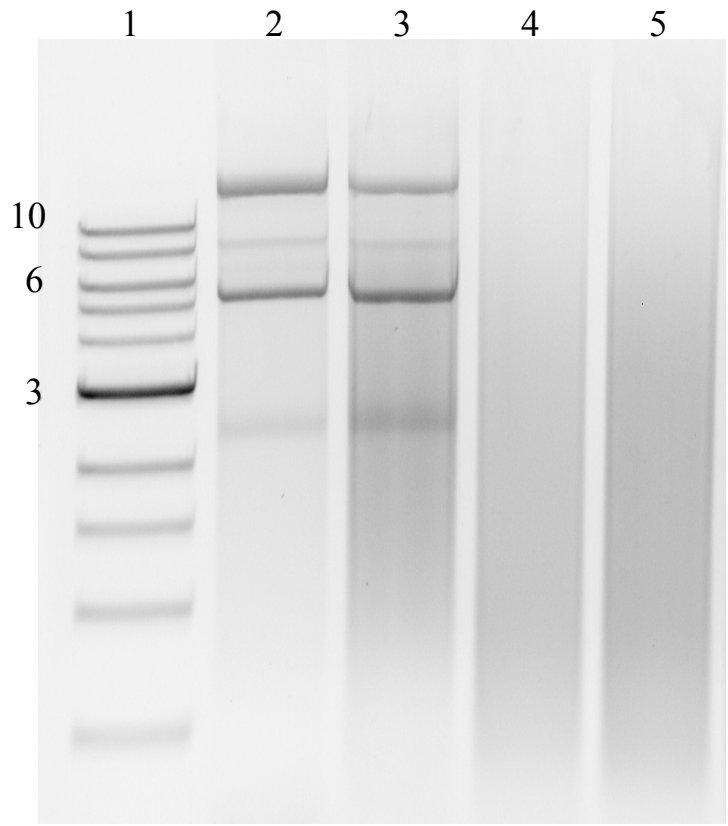

Fig 1.12. Plasmid from exponential and stationary phase cultures of *Desulfovibrio* SR-1. Exponential phase (lane 2). Stationary phase (lane 3). No plasmid from *D. africanus* DSM 2603^{T} cultures: Exponential (lane 4) and stationary (lanes 5). 1 kb DNA ladder (lane 1).

Discussion

Most *Desulfovibrio* species are characterized by a single polar flagellum or polar bundle of flagella. The presence of flagella constitutes the basis for classification within the genus *Desulfovibrio* (Postgate and Campbell, 1966). The electron-microscope analysis of negatively stained cells showed that the flagellation type of *Desulfovibrio* SR-

1 differs from *D. africanus* DSM 2603 ^{T}. A single polar flagellum was observed in *Desulfovibrio* SR-1 cells and lophotrichous flagella in *D. africanus* cells. The lophotrichous polar flagellation has been described as a morphological characteristic of *D. africanus* (Postgate and Campbell, 1966), which was independently isolated from two different sites in Africa. Both the seawater and the freshwater strains were identical in morphology, physiology and DNA base composition regardless of its isolation source (Campbell et al., 1966). Considering the number of flagella as a relevant morphological characteristic in *Desulfovibrio* species, *Desulfovibrio* SR-1 and *D. africanus* may be considered as different strains.

The growth of *Desulfovibrio* species with pyruvate and malate as carbon sources is considered taxonomically significant for species affiliation (Postgate and Campbell, 1966). Additionally, it has been reported that pyruvate does not support growth of the type strain *D. africanus* DSM 2603^{T} in the absence of sulfate (Campbell, 1966; Jones 1971). Previous results have shown that *Desulfovibrio* SR-1 differs from *D. africanus* in that it oxidizes formate but not malate and ferments pyruvate (Castañeda, 20001). In the current study, the pyruvate fermentation was determined by optical cell density and acetate detection. Thus, the growth of *Desulfovibrio* SR-1 in sulfate-free pyruvate medium coupled with acetate production confirmed the fermentative metabolism of pyruvate. No difference in the pyruvate fermentation has been observed between *Desulfovibrio* subspecies such as *D. desulfuricans* subsp *desulfuricans* Essex 6 and MB (Loubinoux et al., 2002), *D. vulgaris* subsp. *vulgaris* str. Hildenborough and *D. vulgaris* subsp. *vulgaris* str. DP4 (Walker et al., 2006). It is important to note that phenotype plays

an important role in the decision about cut off points of genomic data for species delineation (Stackebrandt et al., 2002).

The complete16S rRNA gene sequence analysis revealed that *Desulfovibrio* SR-1 was closely related to *D. africanus* with a sequence similarity of 99.93%, which is above the 97% threshold value generally used to delineate prokaryotic species (Wayne et al., 1987). However, the discriminatory power of 16S rDNA sequences is limited when closely related species and even different species within the same genus are inspected (Stackebrandt and Goebel, 1994). For example, strains with 99.9% 16S rRNA gene sequence similarity that belong to different species include *Sphingosinicella microcystinivorans* and *S. xenopeptidilytic* (Geueke et al., 2007); *Bacillus pumilus* and *B. safensis* (Satomi et al., 2006). In the *Desulfovibrio* genus, *D. alaskensis* (Feio et al., 2004) and *D. desulfuricans* G20 (http://www.jgi.doe.gov/JGI_microbial/html/) are considered as different species despite the 99% 16S rDNA sequence similarity.

The 16S-23S ITS regions from *Desulfovibrio* SR-1 and *D. africanus* were sequenced, because several studies have shown that this region provides high resolution in elucidating phylogenetic relationships among closely related species. The presence or absence of transfer RNA genes in the ITS region were determined, since they are characteristic features of certain groups of prokaryotes (García-Martínez et al., 1999). In addition, the predicted secondary structure of these tRNA genes provides an evidence of their functional role in RNA processing (Apirion and Miczak, 1993). *Desulfovibrio* species have one type of 16S-23S ITS region containing either $tRNA^{Ile}$ or $tRNA^{Ile}$ and $tRNA^{Ala}$ genes (Loubinoux et al., 2002). Whole-genome sequences of *D. vulgaris* subsp. *vulgaris* str. Hildenborough (NC_002937) and *D. vulgaris* subsp. *vulgaris* str. DP4

(NC_008751) have revealed one type of 16S-23S ITS region containing the $tRNA^{Ile}$ and $tRNA^{Ala}$ genes. It is known that the transfer RNA genes are highly conserved DNA segments of the ITS regions found in all strains of a single species, but rarely beyond the genus or family level (García-Martínez et al., 1999). Thus, the comparative 16S-23S ITS sequence analysis between *Desulfovibrio* SR-1 and *D. africanus* DSM 2603^T revealed that the ITS regions were conserved in size and type of transfer RNA genes. Moreover, the 99.38% similarity of the tRNA gene sequences supported the ITS phylogeny of *Desulfovibrio* SR-1 and *D. africanus*, where they were found as closely related strains. In contrast, the closely related *D. vulgaris* subsp. *vulgaris* strains showed the same ITS phylogenetic affiliation due to their identical ITS sequences. Thus, the ITS region is likely not a useful marker to differentiate closely related *Desulfovibrio* strains as it has been found to be in cyanobacteria, where even differences in ITS length among closely related strains were observed (Rocap et al., 2002).

By phylogenetic analysis of DsrAB amino acid sequences, *Desulfovibrio* SR-1 was related to *D. africanus* with a 94.66% sequence similarity. A similar phylogenetic affiliation has been reported for *D. aerotolerans* and *D. burkinensis* with a 96.8% DsrAB amino acid sequence similarity (Mogensen et al., 2005). However, there is no threshold value of *dsrAB* gene sequence divergence for species affiliation. The same phylogenetic affiliation has been reported for closely related *D. vulgaris* strains when partial *dsrAB* sequences were analyzed (Wagner et al., 1998). The complete genome sequences of *D. vulgaris* subsp. *vulgaris* str. Hildenborough and *D. vulgaris* subsp. *vulgaris* str. DP4 have confirmed that they have identical *dsrAB* sequences. Our results indicate that the *dsrAB* gene may offer an advantage over the 16S rRNA gene and 16S-23S ITS region in

defining the sequence divergence between *Desulfovibrio* SR-1 and *D. africanus* DSM 2603^{T}. In general, when comparing the topologies generated by the 16S rRNA gene, 16S-23S ITS region and partial *dsrAB* gene sequences, the phylogenetic relationship between *Desulfovibrio* SR-1 and *D. africanus* appears to be congruent.

The 16S rRNA gene sequences often lack resolution when compared with the DNA-DNA hybridization method. Whereas isolates that have less than 97% rRNA gene sequence similarity usually share <70 % DNA-DNA hybridization and belong to different species, isolates that have ≥ 97% identity might or might not meet the 70% DNA-DNA hybridization criterion for inclusion in the same species (Gevers et al., 2005). Some examples where *Desulfovibrio* strains with a high 16S rRNA gene sequence identity (>98.7) showed low DNA-DNA hybridization values include *D. alaskensis* and *D. vietnamensis* (Feio et al., 2004), and *D. magneticus* and *D. burkinensis* (Sakaguchi et al., 2002). Consequently, due to the high 16S rRNA gene similarity of 99.93% between *Desulfovibrio* SR-1 and *D. africanus*, the DNA-DNA hybridization was necessary to clarify the species status of *Desulfovibrio* SR-1.

The DNA-DNA hybridization value of 72% between *Desulfovibrio* SR-1 and *D. africanus* is close to the borderline applied to the bacterial species (Wayne et al., 1987). DNA-DNA hybridization values do not represent actual sequence identity or gene content differences since DNA heteroduplexes will only form between strands that show at least 80% sequence complementarity (Roselló-Mora, 2006). Moreover, the 70% DNA relatedness values should be considered as indicative rather than absolute (Roselló-Mora and Amann, 2001). The phenotypic consistency among strains should be the deciding factor when attempting to delineate a species (Stackebrandt et al., 2002). Thus, the 72%

DNA-DNA hybridization is a critical value that can not resolve whether *Desulfovibrio* SR-1 should be regarded as *D. africanus*. A 90% DNA-DNA hybridization value has been reported when closely related *Desulfovibrio vulgaris* strains were inspected (Brandis and Thauer, 1981). On the basis of some phenotypic differences, *Pseudomonas aureofaciens* has been reclassified as a subspecies of *P. chlororaphis* despite a 73% DNA-DNA similarity and almost identical 16S rDNA sequences (Peix et al., 2007). Furthermore, in contrast to most other prokaryotic taxa, where the threshold value for species affiliation is 70%, species of the *Pasteurellaceae* have mostly been defined by showing DNA-DNA hybridization values of 80-85% (Christensen et al., 2005).

The 62.4 mol% G+C obtained for *Desulfovibrio* SR-1 differs from the value of 63.1 mol% reported for the type strain *D. africanus* DSM 2603^T (Skyring and Jones, 1972). Determination of the genomic G+C content lacks resolution for determining taxonomic relationships between strains, but the G+C mol% does not vary more than 5 mol% among strains of a given species (Roselló-Mora and Amann, 2001). The difference of 7.1 mol% between the G+C content of *D. alaskensis* (64.1 mol%) and *D. desulfuricans* G20 (57 mol%) indicates that they are different species despite the 99% 16S rDNA sequence similarity. The complete genome sequences of *D. vulgaris* subsp. *vulgaris* str. Hildenborough (NC_002937) and *D. vulgaris* subsp. *vulgaris* str. DP4 (NC_008751) have revealed the same G+C content of 63 mol%, but their genomes differ in size (by 0.1 Mb) and in number of chromosomal genes (by 442 genes). The genome reduction and gene loss in *D. vulgaris* str. DP4 was previously determined by PFGE and comparative whole genome microarray analyses (Walker et al., 2006). Thus, these results confirm that the G+C content of a genome is not significantly affected by chromosomal

rearrangements or gene loss (Coenye et al., 2005). The lower G+C mol% content of *Desulfovibrio* SR-1 compared to that of *D. africanus* can not explain whether a genome reduction or gene loss has occurred in the former strain. Therefore the DNA mol% G+C determination for *Desulfovibrio* SR-1 constitutes only complementary information for the description of this new isolate.

DNA-based typing methods that target the whole genome have also been recommended for the determination of isolate relatedness (Stackebrandt et al., 2002). PFGE has been useful in discriminating species with a high degree of morphological and biochemical similarities (Pradella et al., 1998; Zhong et al., 2007). The separation of macrorestriction fragments by PFGE generates DNA banding patterns that are used to evaluate interspecies and intraspecies genetic variation (Van Belkum et al., 2001). The proposed system to determine strain relatedness considers that bacterial isolates with the same PFGE pattern are the same strain, whereas bacterial isolates differing by one to three bands are closely related strains (Tenover et al., 1995). Genomic DNA of *Desulfovibrio* SR-1 and *D. africanus* DSM 2603^T was digested with PmeI (5' GTTTAAAC 3') because its recognition site is expected to occur rarely on these high G+C genomes. In general, the distinct PFGE patterns generated by this restriction enzyme indicate the presence of more PmeI restriction cleavage sites on the genome of *D. africanus* than on the *Desulfovibrio* SR-1 genome. Considering that the PFGE patterns of *Desulfovibrio* SR-1 and *D. africanus* differ by three bands, they may be closely related strains. In contrast, *D. vulgaris* Hildenborough could be differentiated from *D. vulgaris* DP4 based on the size of their I-CeuI fragments despite the same number of fragments in the PFGE patterns (Walker et al., 2006). Since I-CeuI cuts the 23S rRNA gene, the

distinct banding patterns of these closely related strains were shown to be a consequence of chromosomal rearrangements (Walker et al., 2006).

In general, the number of 16S-hybridization bands visualized depends on the restriction enzyme selected for analysis and on the degree of polymorphism that exists within and around the ribosomal operons (Pukall, 2006). Despite the same number of bands, the different 16S ribosomal patterns generated by the same restriction enzyme indicate different location of the restriction cleavage sites on the genome of both *Desulfovibrio* SR-1 and *D. africanus*. Similar ribosomal patterns only indicate that the restriction enzymes have generated the same length of restriction fragments, but the restriction cleavage sites might not be in the same location on both genomes. Thus, the comparison of 16S rDNA fingerprints between *Desulfovibrio* SR-1 and *D. africanus* suggests that their chromosomes have a different organization. Considering that SspI cuts the 16S rRNA gene, the four SspI-ribosomal fragments may represent the number of ribosomal operons in *Desulfovibrio* SR-1. Complete genome sequences of *Desulfovibrio* species have revealed the presence of four to five copies of ribosomal operons per chromosome.

The repetitive PCR fingerprinting is a DNA-based typing method recommended to discriminate at the subspecies level (Stackebrandt et al., 2002). The rep-PCR has shown to be a valuable tool for classifying and typing a variety of Gram-negative bacteria (Versalovic et al., 1994; Healy et al., 2005; Tacão et al., 2005). Three categories of conserved repetitive sequences are used for bacterial typing: the enterobacterial repetitive intergenic consensus sequence (ERIC), the repetitive extragenic palindrome sequence (REP) and the BOX element (Versalovic et al., 1991). It is important to note that

fingerprinting with amplified repetitive elements has correlated well with DNA-DNA hybridization, and it has been suitable for delineating species (Rademaker et al., 2000). The only research related to repetitive element fingerprinting in *Desulfovibrio* species has looked exclusively at ERIC- and REP-PCR fingerprints, which were useful for differentiating between soil and intestinal strains of *D. desulfuricans* (Dzierżewicz et al., 2003). In the present study the BOX-PCR method was able to discriminate *Desulfovibrio* SR-1 from *D. africanus* based on their specific BOX-PCR fingerprints which were very stable over several subcultures.

The absence of plasmids in *D. africanus* has been previously reported (Postgate, 1984) and confirmed in the present study. The small plasmid isolated from *Desulfovibrio* SR-1 can be considered as a native plasmid since bacterial plasmids are components of the genomes of naturally occurring bacteria.

The ad hoc committee on reconciliation of approaches to bacterial systematics has proposed that subspecies designations can be used for genetically close organisms that can be differentiated by some phenotypic properties (Wayne et al., 1987). In fact, data from a combination of phenotypic and genetic approaches indicate that *Desulfovibrio* SR-1 may be considered as a distinct *D. africanus* subspecies.

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Chapter 2

Characterization of pDaf, a small native plasmid from *Desulfovibrio* SR-1

Abstract

A self-replicating plasmid pDaf was isolated from *Desulfovibrio* SR-1. Sequence analysis of this 8568 bp plasmid revealed a G+C content of 47.2% and nine open reading frames (Orfs) encoding polypeptides greater than 100 amino acids. We compared these Orfs to known nucleotide and peptide sequences available through GenBank. The Orf 1 was identified as an initiator of plasmid replication (Rep3), the Orf 2 as a mobilization gene (*mobA*), and the Orf 5 as a partitioning gene (*parA*). After insertion of an antibiotic resistance marker, pDaf could replicate in *D. africanus* and *P. aeruginosa* PA14. A series of *ColE1*-based plasmid deletion constructs were tested to determine the functional role or each Orf in the plasmid biology. The replicon is 1123-bp long and contains a *rep* gene with four 22-bp iterons. The mobilization operon is composed of three overlapped genes (*mobC*, *mob* and *mobA*) with an upstream *oriT* of 145-bp long. The partitioning operon is composed of two adjacent genes (*parA* and *parB*) with a downstream partitioning-like site containing inverted repeats. The functional analyses revealed that 53.6% of the plasmid contains genes associated with replication (Orf 1), mobilization (Orf 6, 7 and 2), and partitioning (Orf 5 and 9). pDaf is a low copy number plasmid with a high efficiency of transformation, it is mobilizable by the IncP RK2 plasmid at high transfer frequency, and the partitioning system confers high plasmid stability under non-selective pressure. A small cloning vector pDaf-MCS was constructed, with the effort to evaluate its usefulness as a genetic tool in *D. africanus*.

Introduction

Sulfate-reducing bacteria play important roles in geochemical cycles, corrosion processes, and immobilization of toxic metals (Postgate, 1984). Environmental isolates of sulfate-reducing bacteria consist mainly of *Desulfovibrio* species for which genetic tools remain to be developed. They have served as model organisms in seminal studies of mercury methylation pathways (Ekstrom et al., 2003), biofilm formation and uranium immobilization (Beyenal et al., 2004; Clark et al., 2007) as well as gene regulation under different stress conditions (Fournier et al., 2006; Zhang et al., 2006; Stoylar et al., 2007). Moreover, the genome sequences of *D. vulgaris* str. Hildenborough, *D. vulgaris* str. DP4 and *D. desulfuricans* G20 are currently available, microarrays for these organisms have been developed (Scholten et al., 2007) and proteomic studies have been undertaken (Nie et al., 2006; Caffrey et al., 2007; Luo et al., 2007b).

Plasmids have been found in all bacterial communities studied to date, including both soil and marine, and clinical environments. They have contributed to bacterial evolution and adaptation by mediating the horizontal exchange of genetic material between different bacterial species and even between bacteria and eukaryotic cells (Heinemann and Sprague, 1989; Frost et al., 2005; Sørensen et al., 2005; Johnsen and Kroer, 2007). It is well known that plasmids carry various traits that are essential for adaptation to variations in habitat conditions, but many cryptic plasmids provide no obvious benefit to their bacterial hosts (Sørensen et al., 2005).

Previous studies have reported the presence of megaplasmids in *D. gigas*, *D. vulgaris* and *D. desulfuricans* (Postgate et al., 1984). The complete sequences of megaplasmids from *D. vulgaris* have revealed important plasmid-encoded functions such

as nitrogen fixation and a type-III secretion system (Heildelberg et al. 2004). The isolation of small plasmids from *Desulfovibrio* species is limited to the pBG1 plasmid native to *D. desulfuricans* G100A (Wall et al., 1993). Since small plasmids play key roles in biological research as molecular biological vectors, the pBG1 plasmid was used to construct several *E. coli- Desulfovibrio* vectors (Rousset et al., 1998). It is important to note that derivatives of broad host range vectors have been mostly used for gene expression in some *Desulfovibrio* species (Voordouw et al., 1990; Blanchard et al., 1993; Tan et al. 1994).

Over the past 10 years, genetic and molecular studies of *Desulfovibrio* species have been well documented. In particular, the recent development of a random mutagenesis system in *D. desulfuricans* G20 has identified new roles of specific genes and the roles of genes of unknown function in sediment fitness (Luo et al., 2007c). *Desulfovibrio* mutants for specific genes have been generated by insertional inactivation (Rapp-Giles et al., 2000) and gene replacement techniques (Pohorelic et al., 2002; Rodrigues et al., 2006), but functional complementation in the corresponding *Desulfovibrio* mutants has not been reported. The *D. desulfuricans* G20 *arsC* gene was cloned into pSC27 (pBG1-based plasmid) to complement the mutant phenotype by restoring its arsenate resistance (Li and Krumholz, 2007). Each of these fields has enhanced the understanding of *Desulfovibrio* genetics, but researchers still lack plasmid vectors to add detailed genetic analyses to the already advanced biochemical characterization of various *Desulfovibrio* gene products. To study the regulation of gene expression and interactions of cellular components within *Desulfovibrio* species, genetic tools need to be developed.

Since replication initiation of most plasmids is dependent on host-encoded enzymes that limit their host range, we hypothesized that naturally occurring plasmids native to *Desulfovibrio* species are better genetic tools for gene functional analyses in these organisms.

The main objective of this research entails the molecular and functional characterization of a small plasmid native to *Desulfovibrio* SR-1 in order to determine the structural genes to be used in constructing cloning vectors, with the effort to evaluate their usefulness in genetic studies of these organisms.

Materials and Methods

Bacterial strains, plasmids, and culture conditions

The relevant characteristics of the bacterial strains and plasmids are listed in Table 2.1. *Escherichia coli*, *Shewanella oneidensis* MR-1 Ap^r, *Pseudomonas aeruginosa* PA14 and *Agrobacterium tumefaciens* GV3101 were grown in Luria-Bertani (LB) broth and LB agar. *Desulfovibrio* species were grown in lactate sulfate (LS) medium (Castañeda, 2001). The minimum inhibitory concentration (MIC) of kanamycin for *D. africanus* $DSM2603^T$ in LS broth was determined to be 200 µg/ml. *S. oneidensis* MR-1 Ap^r derivative was generated by successive transfers in 50µg-increase ampicillin concentrations. The antibiotics used for selection include ampicillin (Ap), chloramphenicol (Cm), kanamycin (km) and neomycin (Neo). The antibiotic concentrations were: 50 µg/ml km (*E. coli*, *S. oneidensis* MR-1 and *A. tumefaciens* GV3101), 175 µg/ml km in LS agar (*Desulfovibrio* spp.), 150 µg/ml Neo (*P.*

aeruginosa), 100 µg/ml Ap (*E. coli*), 250 µg/ml Ap (*S. oneidensis* MR-1), and 30 µg/ml Cm for *E. coli* HB101 (pRK600). Most cultures were incubated at 37°C. *S. oneidensis* MR-1 and *A. tumefaciens* were incubated at 30°C.

Table 2.1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristics	Source or reference
Escherichia coli K12 GM2163	F⁻ *ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 rpsL136 (*Str^R*) dam13::Tn9 (*Cm^R*) xylA-5 mtl-1 thi-1 mcrB1 hsdR2* (strain derived from GM2159 *recF143*)	Palmer and Marinus, 1994
E. coli K12 JM109	F′ *traD36 proA⁺B⁺ lacI^q Δ(lacZ)M15/ Δ(lac-proAB) glnV44 e14⁻ gyrA96 recA1 relA1 endA1 thi hsdR17*	Yanisch-Perron, 1985
E. coli K12 DH5α	F⁻8Φ80*dlacZΔM*15 Δ(*lacZYA-argF U169 deoR supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan, 1983
pGEM®-7Zf(+)	*lac*Zα,T7 and SP6 vector primers, *bla* (Ap^r)	Promega
pUC19	*lac*Zα, *ColE1-ori* (high copy number), *bla* (Ap^r), unstable origin of replication in shuttle vectors	Yanisch-Perron, 1985
pRK600	*ColE1-ori* RK2-Mob^+ RK2-Tra^+ *cat* (Cm^r)	Figurski, 1979
pBBR1MCS-2	*rep* (medium copy number) km^r , stable replicon in shuttle vectors	Kovach et al., 1995
Shewanella oneidensis MR-1 Ap^r	Ap resistant derivative from Type strain ATCC 70050	This study
Pseudomonas aeruginosa PA-14	Wild type	Rahme et al., 1995
Agrobacterium tumefaciens GV3101	Rif^r no replication of *ColE1*-based plasmids, cured Ti plasmid	Hellens et al., 2000
Desulfovibrio SR-1	Wild type strain, single flagellum, harboring a small plasmid	This study
D. africanus $DSM2603^T$	Type strain, lophotrichous , plasmid-free	F. Morel
D. desulfuricans G20	*D. desulfuricans* G100A, resistance to nalidixic acid, cured pBG1 plasmid	J. Wall
D. vulgaris Hildenborough	Type strain, megaplasmid-free (megaplasmid is lost spontaneously)	NCIMB 8303
D. desulfuricans Essex 6	Type strain, plasmid-free	DSM642

Isolation and purification of pDaf plasmid

Desulfovibrio SR-1 exponential phase cultures were used to isolate pDaf plasmid by the alkaline lysis procedure (Sambrook et al., 1989). The QIAGen Maxiprep kit was used for large-scale plasmid purification from the cell-pellets, and the supercoiled plasmid DNA was further gel purified from 0.6% agarose gels. The QIAGen Mini prep kit was used for small-scale plasmid purification, but the bacterial lysate was directly precipitated with isopropanol and 70% ethanol.

Cloning of *Desulfovibrio* plasmid (pDaf)

The single and double restriction digestions of the supercoiled plasmid DNA were assayed with the enzymes BamHI, ClaI, EcoRI and EcoRI-ClaI, respectively. The estimated DNA fragment sizes were: 9-kb (BamHI); 3- and 5-kb (EcoRI); 1-, 2-, 2.5- and 3-kb (EcoRI-ClaI). The gel purified fragments were independently cloned into the pGEM-7zf (+) vector. The recombinant plasmids were then electroporated into *E. coli* JM109 competent cells according to standard protocols (Ausubel, 1989). Transformants were selected on LB-Ap agar containing 0.1 mM IPTG and 40µg/ml X-Gal. White colonies were screened for the presence of inserts by restriction digestion analysis of purified recombinant plasmids. On the basis of the cloned fragment size, recombinant plasmids were designated as Bam9, Eco3, Eco5, EcoCla1, EcoCla2, EcoCla2.5, and EcoCla3.

Plasmid DNA sequencing and analysis

The above recombinant plasmids were used as DNA templates to determine the pDaf plasmid sequence. The vector primers SP6 (5'-TATTTAGGTGACACTATAG-3') and T7 (5'-TAATACGACTCACTATAGGG-3') were initially used for sequencing all

the cloned DNA fragments. Seventeen sequencing primers were successively designed from the new nucleotide sequences (Table 2.2). DNA sequencing by primer walking was performed on both DNA strands of the 9 kb (Bam9) and 5 kb (Eco5) fragments. Sequencing reactions were carried out with the BigDye Terminator Cycle sequencing kit using a 3730 DNA analyzer from Applied Biosystems, at the Oklahoma Medical Research Foundation (Oklahoma City, OK, USA).

Table 2.2. Oligonucleotides for the primer extension sequencing.

Primer[a]	strand	5'→ 3' oligonucleotide sequence	Binding site[b]
EcoCla-1	-	GGTCACGTGTTCAAGCCGTC	152-133
Bam-1	-	AATCATCGCCCAAGCCCATG	378-359
Bam-2	-	TTTCCACGCCAAGCCGAGCTTG	895-874
Bam-3	+	TCTCGATCTAGTCGTGAAATGG	2134-2155
Bam-4	-	ATCGAATGCGCTCATAAACG	2220-2201
Bam-5	+	CGAAGTCATATTTCGCCTTG	2955-2974
EcoCla-2	+	AGTCCTCTATGGAGCAAGAG	3521-3540
EcoCla-3	+	ACTAGGACTGGCTTTGTAGAC	4163-4183
EcoCla-4	-	TCTGTTTGCTCCATTGAATGG	4857-4837
EcoCla-5	+	AGTTATGGCAAGACGTCTCAAC	5357-5378
Eco-1	-	AGGAGACGGTCGCTATATGC	5867-5848
Eco-2	+	ACTAGGTTTGGACCTCAAATCC	6100-6121
Eco-3	+	CTAAGCATGGCAGGCCAATG	6183-6202
Bam-6	-	TGCTTCAAGAGGCTATCGAC	6783-6764
Bam-7	-	TCTAGCCAAAGCAGGAAAAGC	7347-7327
Eco-4	+	TCTCTGAAGAGGTCGATAGC	6753-6772
EcoCla-6	-	GACCTGTCTTTCTCGACATG	8182-8163

[a]Oligonucleotide name is based on the restriction fragment used as a sequence source: EcoCla (EcoRI-ClaI), Eco (EcoRI-EcoRI), Bam (BamHI).
[b] Position on assembled sequence of pDaf.

The DNA sequence was analyzed with programs available on the webpage of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The "Blast 2 sequences" program was used to assemble the plasmid sequences (Tatusova and Madden, 1997). Initial screening for open reading frames was determined by the "ORF Finder" tool. Coding sequences with associated ribosome binding sites were predicted with GeneMark (Borodovsky and McIninch, 1993). Start codon positions were confirmed by manual identification of the Shine-Dalgarno sequences (RBS) within 15 bp of a potential start site (Ma et al., 2002). All open reading frames greater than 300 nucleotides were further compared to the GenBank protein database using the blastp algorithm (Altschul et al., 1997). The online bioinformatics tool BPROM from Softberry was used to detect bacterial promoters. BPROM is a bacterial sigma 70 promoter recognition program with about 80% accuracy and specificity. The intergenic distance and the conservation of genes pairs in *D. vulgaris* megaplasmid genome were used for predicting operons. Direct repeats were searched by the tandem repeats finder (Benson, 1999). Inverted repeats were searched by the inverted repeats finder REPuter (Kurtz et al., 2001). NEB cutter v 2.0 program (http://tools.neb.com/NEBcutter) was used to search possible targets of DNA methylation (Hénaut et al., 1996) and restriction recognition sequences (Vincze et al., 2003). The GeneTool 1.0 software was used for the detection of prokaryotic transcription factor motifs and the G+C density analysis.

Relative-copy number determination

Plasmid copy number was determined in the native host by the relative standard curve method using the real-time PCR detection system (Scheurer et al., 2007). The *rep*

gene from pDaf was used as a target gene and the single copy gene *Da FdIII* (Busch et al., 2000) as a chromosomal copy number reference to which the plasmid copy number was compared. Total DNA was extracted from 10-ml of exponentially grown *Desulfovibrio* SR-1 cells. Cultures were pelleted and resuspended in 1 ml of sterile distilled water three times. The last cell pellet was resuspended in 100 µl of distilled water, boiled for 5 minutes and pelleted by centrifugation. The supernatant was treated with RNase at 40 µg/ml final concentration. Two-fold serial dilutions of total DNA were used as the DNA template for the real time PCR amplification.

The relative standard curves for target and reference genes were performed simultaneously. Specific primers were designed to amplify an equivalent 143-bp amplicon from both the plasmid (Daf-REP) and the reference (FdIII) genes. Daf-REP amplicon was amplified with primers F-AF (5'–GGATTAAGTCCTCGTCAAAGG- 3') and R-AF (5' –CGAAGCTCTAGCCGATCAAC- 3'). FdIII amplicon was amplified with primers F-FdIII (5'–ACCATCGACACCGACAAGTG- 3') and R-FdIII (5'-TCGCAAACTTCGACGCAAGAC- 3'). The real-time PCR amplification was assessed with the SYBR Green PCR Master Mix designed to use with the ABI Prism 7000 Sequence Detection System (SDS; Applied Biosystems). The 25-µl reaction mixtures contained 12.5 µl of master mix, 300 nM primers, and 5 µl of total DNA at different concentrations (two-fold increase from 0.5 to 240 ng). The 25-µl reaction mixtures were carried out in triplicate. The thermal cycling conditions were: (i) 50°C for 2 min, (ii) 95°C for 10 min, and (iii) 40 cycles (95°C per 15 s, 60°C per 1 min); followed by (iv) the dissociation curve of 60-95°C for 20 min.

To generate the standard curves at least 5 different concentrations per standard were measured, and the amount of unknown total DNA fell within the range tested. The slope and the intercept of standard curve lines based on the threshold cycle number (Ct) were generated by the ABI Prism 7000 SDS software. These parameters were then used to determine the relative plasmid copy number for a given Ct value (Table A1). The real-time PCR amplification efficiencies were estimated by the equation $E = 10^{[-1/\text{-slope}]}$ (Pfaffl, 2001). Specificity of real-time PCR products was documented with gel electrophoresis.

The plasmid copy number was also assessed by Southern blot hybridization using the DIG system from Roche. The pDaf plasmid and genomic DNA from *Desulfovibrio* SR-1 were used as DNA templates for the amplification of the plasmid and reference probes, respectively. The 143-bp DIG-labeled DNA probes were generated by the PCR labeling technique (PCR DIG Probe Synthesis Kit) using the above real-time PCR primers. Approximately 5 μg of BamHI- digested chromosomal DNA was separated by agarose gel electrophoresis on a 0.8% agarose gel in 1X Tris-borate-EDTA buffer (pH 7.9) at 90 volts for 4 hours. Gels were blotted by downward capillary transfer (Chomczynski, 1992) on positively charged nylon membranes (Nytran SuperCharge, 0.45 um pore-size). DNA was covalently bonded to the nylon membrane using UV cross-linking at 1250 (x100 J/cm^2) for 1 minute on each side. Two-probe DNA-DNA hybridization with one pDaf-derived probe and one chromosome-derived probe was performed at 51°C for 16 hours. The immunological detection of hybridization signals was performed according to the protocol supplied by the manufacturer (Roche) using the DIG Nucleic Acid detection kit. The hybridization bands obtained with both probes were scanned, and the intensity profiles of each band were generated by digital image analysis

with the program “ImageJ”. The pDaf copy number was calculated by comparing the intensity of chromosomal and plasmid bands. In order to validate this method, hybridization of two-fold increase KpnI digested pDaf DNA was performed concurrently. The two-fold increase DNA concentrations ranged from125 pg to 2000 pg.

Determination of pDaf host range

Insertion of a selectable kanamycin resistance marker

To insert an antibiotic marker gene into the KpnI and SpeI recognition site of pDaf, the amplification of the kanamycin gene from pBBR1MCS-2 was performed with primers F-km1, R-km1, F-km1 and R-km2 (Table 2.3).

The PCR amplification was performed using the Expand Long Template PCR system (Roche). The 50 µl reaction contained 200 ng of plasmid DNA (pBBR1MCS-2), 500 µM dNTPs, 300 nM forward and reverse primers, 2.75 mM $MgCl_2$ and 3.75 U of Expand Long template DNA polymerase mix. Reaction conditions were: (i) 5 min at 94°C; (ii) 30 cycles (45 s at 94°C, 45 s at 55°C, 1 min 10 s at 68°C); and (iii) a final extension of 10 min at 68°C. The PCR products were then purified using the QIAquick mini-elute PCR purification kit according to the manufacturer’s instructions. The purified PCR products were digested with the corresponding restriction enzymes and dephosphorylated according to manufacturer’s recommendations. The kanamycin PCR products were independently cloned into the KpnI and SpeI recognition sites of pDaf using standard cloning procedures (Sambrook et al., 1989). The ligation mixtures were then electroporated into *E. coli* GM2163 and JM109 competent cells (Table 2.1) with

transformation efficiencies of ~ 3 x 10^6 CFU/ μg of DNA. LB-km agar was used for the selection of probable pDaf transformants.

Table 2.3. Oligonucleotide sequences used in the construction of pDaf derivatives.

Name	5'→ 3' oligonucleotide sequence
F-km1	GGGGTACCTTACATGGCGATAGCTAGACTG
R-km1	GGGGTACCTCGCTTGGTCGGTCATTTCGAAC
F-km2	GACTAGTCTTACATGGCGATAGCTAGACTG
R-km2	GACTAGTTCGCTTGGTCGGTCATTTCGAAC
F-km	CTTACATGGCGATAGCTAGACTG
F-ori1	GGGGTACCATCCACAGAATCAGGGGATAACG
R-tag1	GTCTAGCTATCGCCATGTAAGTTTCGTTCCACTGAGCGTCAGAC
F-ori2	GACTAGT ATCCACAGAATCAGGGGATAACG
F-rep	GGGGTACCCTGCTGGACAAGCTGATGGACAG
R-tag2	GTCTAGCTATCGCCATGTAAGATAGTCTGGAACAGCGCACTTAC
F-mob1	GGGGTACCGTTTGCCTGATCTTCTGCTTTGC
F-mob2	GGGGTACCTGTTTCTTGGAAGTGTGAGGACC
R-par1	GGGGTACCAGTCAAAGCGAGACTTGGCCATG
R-par2	GGGGTACCGCTGAGTTAGGCCATAACTAGC
R-MCS	GGGGTACC**TCTAGAGATATCGTCGAC**TCGCTTGGTCGGTCATTTCGAAC

Restriction recognition sequences: KpnI (underlined) and SpeI (double underlined); overlap sequence (dot line) and MCS sequence (boldface).

Desulfovibrio-E. coli vector construction

The 1748-bp fragment containing both the antibiotic gene and the *ColE1* origin of replication was amplified by the overlap extension PCR technique (Ho et al., 1989; Horton et al., 1989). The fragment to be cloned into the KpnI site of pDaf was amplified with primers listed in Table 2.3. The *km* gene from pBBR1MCS-2 and the pUC19-*ColE1* origin of replication were amplified in two independent PCR reactions using the Expand Long Template PCR system from Roche. The 1017-bp fragment containing the *km* gene was amplified with primers F-km and R-km1. The 731-bp fragment containing the *ColE1* origin of replication was amplified with primers F-ori1 and R-tag1. The individual 50-µl PCR reactions contained 100 ng of plasmid DNA, 500 µM dNTPs, 300 nM forward and reverse primers, 2.75 mM $MgCl_2$ and 3.75 U of Expand Long template DNA polymerase mix. The amplification of both PCR products was performed under the same conditions: (i) 5 min at 94°C; (ii) 30 cycles (45 s at 94°C, 45 s at 53°C, 1 min 10 s at 68°C); and (iii) a final extension of 10 min at 68°C. The purified PCR products were then used as DNA templates for the overlap PCR with primers F-ori1 and R-km1 to generate a full-length 1748 bp fragment containing the *ColE1* origin of replication and the kanamycin gene (*ColE1-km*). The 50-µl PCR reaction containing 150 ng of each PCR product was performed under the following conditions: (i) 5 min at 94°C ; (ii)15 cycles (45 s at 94°C,45 s at 40°C, 2 min at 68°C; (iii)15 cycles (45 s at 94°C, 45 s at 45°C, 2 min at 68°C; (iv)15 cycles (45 s at 94°C, 45 s at 50°C, 2 min at 68°C); and (v) a final extension of 10 min at 68°C. The amplification of the *ColE1-km* fragment to be cloned into the SpeI restriction site of pDaf was performed with primers F-ori2, R-tag1, F-km and R-km2. The same approach was used for the amplification of a 2477-bp fragment (*rep* and

km genes from pBBR1MCS-2) with primers F-rep, R-tag-2, F-km and R-km-1 (Table 2.3), but the extension temperatures were adjusted according to the fragment lengths.

The purified PCR products were digested with the appropriate restriction enzyme, gel purified and dephosphorylated according to standard protocols. The subsequent ligations were carried out at 16°C for 16 hours. The ligation mixtures were electroporated into *E. coli* GM2163 competent cells (Ausubel, 1989). LB-km agar was used for the selection of pDaf-carrying transformants (Fig A1), which were screened by a PstI-HindIII double restriction digestion of purified plasmids.

Electroporation of pDaf into several bacterial strains

The host range of pDaf was investigated in bacterial species that belong to alpha, delta and gamma proteobacteria. The plasmid replication was tested in the following species: *Desulfovibrio desulfuricans* G20, *D. desulfuricans* Essex 6, *D. vulgaris* Hildenborough, *D. africanus, Pseudomonas aeruginosa* PA14, *Agrobacterium tumefaciens* GV3101, *Escherichia coli* and *Shewanella oneidensis* MR-1.

Only pDaf derivatives with a selectable marker inserted into a non-coding region were assayed (pDaf-Spe2 and pDaf-Spe5). Eventually, to protect pDaf from restriction modification systems during transformation, plasmid derivatives were either methylated *in vivo* by electroporating them into *E. coli* DH5α or treated with the "TypeOne Restriction Inhibitor" (Kruger et al. 1977; Walkinshaw et al., 2002). Plasmids prepared from *E. coli* GM2163 and *E. coli* DH5α were assayed simultaneously.

Desulfovibrio electrocompetent cells were prepared based on the method previously described (Castañeda, 2001), but using a 400 mM sucrose-1 mM $MgCl_2$ electroporation solution (Luo, 2007c). *Desulfovibrio* species were transformed with 1 μg

of plasmid DNA and allowed to grow in LS broth at 37°C for 16 hours. LS-km agar was used for the selection of transformants which were screened by plasmid purification and restriction analysis. Plasmid-free *D. africanus* and pDaf-carrying transformants were confirmed by Southern blot hybridization as previously described.

The pDaf derivatives isolated from *E. coli* GM2163 and *E. coli* DH5α were electroporated into *P. aeruginosa* PA14 competent cells using 300 mM sucrose electroporation solution (Smith and Iglewski, 1989). Screening of potential pDaf-carrying transformants was performed by colony PCR with primers F-ori1 and R-km1 (Table 3). Both types of pDaf derivatives (unmethylated and methylated) were also electroporated into *A. tumefaciens* GV3101 competent cells according to standard protocols (Main et al., 1995). Since pDaf derivatives contain *ColE1-ori* which is functional in *Shewanella* spp, a mixture ligation composed of the km and 4591-bp pDaf PCR products was electroporated into *S. oneidensis* MR-1 competent cells using a 10% glycerol electroporation solution (Myers and Myers, 1997). LB-agar km plates were used for the selection of probable *A. tumefaciens* and *S. oneidensis* transformants.

Functional characterization of open reading frames in plasmid biology

Construction of plasmid deletion derivatives

Several deletion derivatives were constructed to study the role of each open reading frame in the biology of pDaf plasmid (Fig 2.1, Table 2.4). The naturally occurring restriction sequences on pDaf were used to remove specific fragments from the recombinant plasmid pDaf-T1, which was digested with the restriction enzymes BmgBI, HindIII, SspI, PciI and PsiI. The subsequent self-ligations of the gel purified plasmids

lacking the specific restriction fragment were electroporated into *E. coli* GM2163. LB-km agar was used for the selection of pDaf-carrying transformants. The fragment deletion was then confirmed by restriction analysis of purified plasmids.

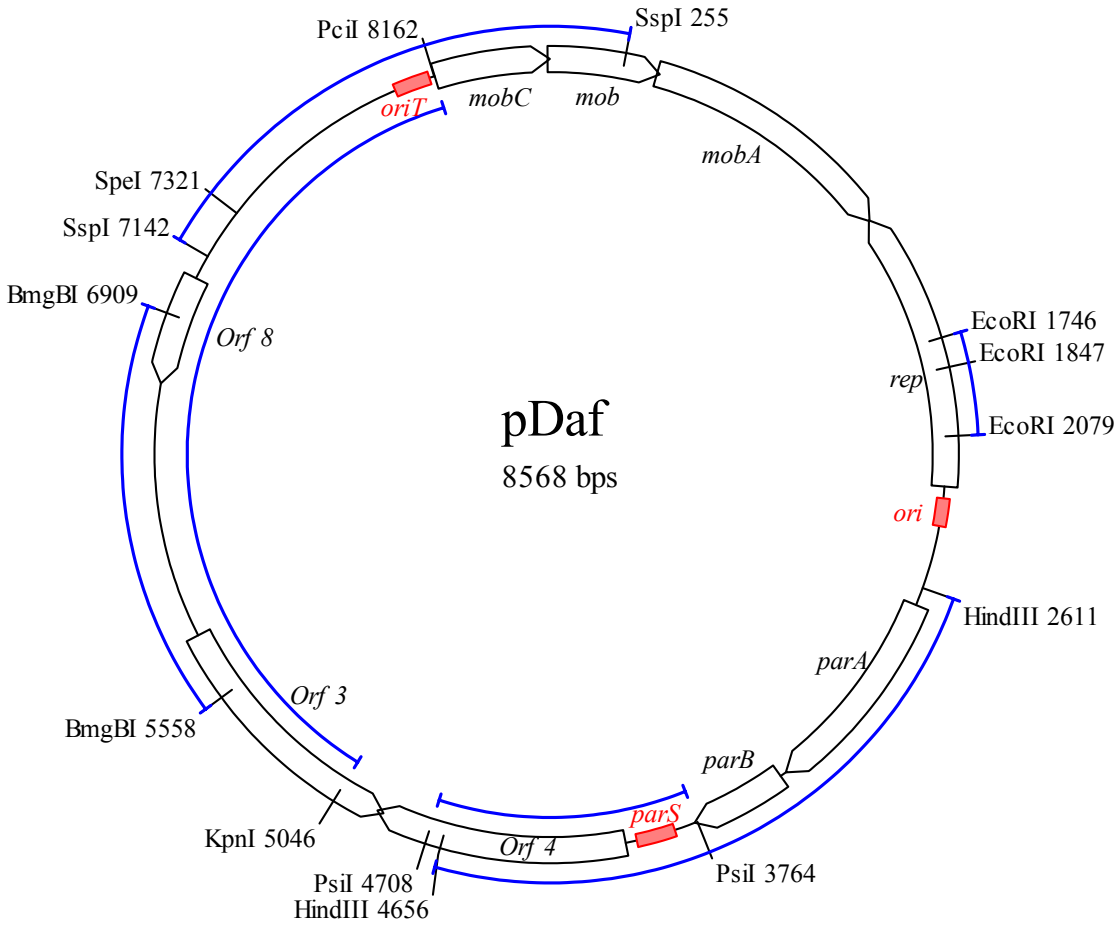


Fig 2.1. The naturally occurring recognition sites on pDaf used for the construction of a series of plasmid deletion derivatives.

Table 2.4. Deletion derivatives used in functional characterization of each open reading frame in plasmid biology.

Derivative	Description
pDaf-ΔH	Deletion of a 2045-bp HindIII fragment removes Orf 5 (*parA*), Orf 9 and partitioning-like site from *par* operon, and Orf 4 (encodes a hypothetical protein). (2611-4656)*
pDaf-ΔS	Deletion of a 1681-bp SspI fragment removes origin of transfer, Orf 6 (*mobC*) and Orf 7 (encodes a protein with no similarity) from the putative *mob* operon. (7141-255)*
pDaf-ΔB	Deletion of a 1351-bp BmgBI fragment truncates Orf 8 (encodes an unknown function protein) and removes most of Orf 3 (encodes a hypothetical protein). (5558-6909)*
pDaf-ΔP	Deletion of a 944-bp PsiI fragment truncates Orf 9 (putative *parB*) and removes Orf 4 (encodes a hypothetical protein). (3764-4708)*
pDaf-ΔPP	pDaf-ΔP derivative. Additional deletion of a 3112-bp fragment removes Orf 3, Orf 8 and the ribosomal binding site for Orf 6 (*mobC*). (5046-8162)*
pDaf-ΔE	Deletion of a 332-bp EcoRI fragment from Orf1 (plasmid replication). (1746-1847-2079)*
pDaf-ΔparS	Amplified pDaf fragment containing origin of transfer, Orf 6, Orf 7, Orf 2, Orf1, Orf 5, and Orf 9, but lacking a partitioning-like site. (7820-4042)*

*Nucleotide position on *Desulfovibrio* plasmid.

Molecular and functional characterization of the plasmid structural genes

Construction of pDaf derivatives by PCR

To better define the plasmid structural genes, additional pDaf derivatives were constructed. The full-length pDaf fragments containing the *mob*, *rep* and *par* genes were amplified from native pDaf as a DNA template using the Expand Long Template PCR system with the primers listed in Table 2.3. The PCR products were independently ligated to the *ColE1-km* fragment and the mixture ligations were electroporated into *E. coli* GM2163. LB-km agar was used for the selection of pDaf-carrying transformants.

Two different pDaf-fragments were amplified to determine whether the upstream region of *mobC* (Orf 6) and the downstream region of Orf 9 were involved in plasmid mobilization and stabilization, respectively. The amplification of a 4690-bp fragment (7679 to 3790 nucleotide positions on pDaf) was carried out with primers F-mob1 and R-par2. The amplification of a 4591-bp fragment (8020-4042 nucleotide positions on pDaf) was carried out with primers F-mob2 and R-par1. The PCR products were independently ligated to *ColE1-km* fragments to generate the corresponding recombinant plasmids pDaf-ΔparS and pDaf-mob-par. The 4591-bp PCR product was also ligated to a 2477 bp *rep-km* fragment to generate pDaf-BBR2. Plasmids were screened by PstI-HindIII double restriction digestions. To confirm that the pDaf plasmid sequence was not modified during the PCR amplification, these recombinant plasmids were analyzed by restriction digestion with enzymes that have recognition sequences on the pDaf-fragment. The selected enzymes were: BamHI, BsaAI, ClaI, EcoRI, HindIII, PciI, PsiI and SspI.

Plasmid derivatives which were selected for functional characterization of plasmid-associated functions are listed in Table 2.5.

Table 2.5. *Desulfovibrio* plasmid derivatives used for functional characterization.

Derivatives	Relevant characteristics
pDaf-T1	*km-ColE1-ori* inserted into kpnI site in a coding region. *km* and *rep* in frame
pDaf-T4	Same as pDaf-T1but *km* and *rep* not in frame
pDaf-Spe2	*km-ColE1-ori* inserted into SpeI site in a non-coding region. *km* and *rep* genes are not in frame. km^r
pDaf-Spe5	Same as pDaf-Spe2, but *km* and *rep* are in frame. km^r
pDaf-BBR1	km-pBBR1-rep inserted into kpnI site in a coding region. km^r
pDaf-ΔH	Mob^+ Rep^+ Par^- Δ(*parA-parS*) km^r
pDaf-ΔS	Mob^- Δ(*mobC*-orf 7) Rep+ Par+ km^r *ColE1-ori*
pDaf-ΔB	Mob+ Rep+ Par+ Δ(orf3-orf8) km^r *ColE1-ori*
pDaf-ΔP	Mob + Rep+ Par^- km^r *ColE1-ori*
pDaf-ΔPP	Mob^- Par^- Δ(*parB-parS*) km^r *ColE1-ori*
pDaf-ΔE	Rep^- Mob+ Par+ km^r *ColE1-ori*
pDaf-ΔparS	synthetic derivative Mob^+ Rep^+ Par^- (Δ*parS*) km^r *ColE1-ori*
pDaf-mob-par	synthetic derivative Mob^+ Rep^+ Par^+ km^r *ColE1-ori*
pDaf-km^R	synthetic derivative Mob^+ Rep^+ Par^+ km^r
pDaf-BBR2	synthetic derivative, Mob^+ Rep^+ Par^+ km^r pBBR1-rep
pDaf-MCS	cloning vector , Mob^+ Rep^+ Par^+ (MCS: XbaI, EcoRV, SalI) km^r *ColE1-ori*

Superscript "—" or "+" indicates phenotype.

Replication

The functionality of pDaf replication module was assayed in *D. africanus* with pDaf derivatives isolated from *E. coli* GM2163 (Table 2.5). The pDaf-ΔE derivative was used to confirm the role of Orf 1(*rep*) in plasmid replication. The pDaf-ΔH derivative

was used to localized the replication origin region and confirm that replication functions independently from the partitioning system. To confirm whether pDaf is a self-replicating plasmid, *D. africanus* was transformed with the ligation mixture composed of the km PCR product and the pDaf-fragment excised from pDaf-mob-par. Four-ligation mixtures containing 200 ng of plasmid DNA per reaction were concentrated using the QIAquick mini-elute purification and then electroporated into *D. africanus* competent cells. Transformants were selected on LS-km agar. The pDaf-carrying transformants were screened by plasmid isolation. The recombinant plasmid pDaf-km^r was screened by PstI-HindIII and ClaI-restriction digestion analysis. Colony-forming units (CFU) were scored to estimate the transformation efficiency.

Mobilization

Conjugation experiments were performed to determine whether pDaf is a self-transmissible or a non-self-transmissible plasmid. Plasmid derivatives assayed in conjugation experiments include: pDaf-Spe2, pDaf-mob-par and the deletion derivatives pDaf-ΔB, pDaf-ΔS, pDaf-ΔPP, and pDaf-ΔparS (Table 2.5).

Plasmid transfer between pDaf-carrying *E. coli* (donor) and *S. oneidensis* MR-1 Ap^r (recipient) was performed by biparental and triparental mating methods. The biparental mating involved donor (D) and recipient (R). The triparental mating involved pDaf-carrying *E. coli* (D), pRK600 as a helper plasmid (H) to provide the transfer function in *trans*, and *S. oneidensis* MR-1 Ap^r (R). The donor-recipient ratio in biparental conjugation was 1:3 (D: R), and the donor-helper-recipient ratio in triparental mating was 1:1:3 (D: H: R). Conjugation was performed at 30°C for 18 hours. Transconjugants were plated out on selective (km/Ap) and non-selective (Ap) media. *S. oneidensis* km^r

transconjugants were screened by plasmid isolation and restriction analysis. The plasmid transfer from *E. coli* to *S. oneidensis* MR-1 was confirmed by the absence of cleavage when purified plasmids were digested with ClaI (Mayer et al., 1981). Conjugation frequency is reported as the number of transconjugants per donor (CFU on selective medium divided by CFU on non-selective medium).

Partitioning (Plasmid Stability)

The functionality of the pDaf partitioning module was assessed by plasmid stability experiments in *E. coli, S. oneidensis* MR-1 and *D. africanus*. Plasmid stability in *E. coli* GM2603 was performed with the following derivatives which are listed in Table 2.5: pDaf-ΔH (deleted *par* operon), pDaf-ΔP (truncated *parB*), pDaf-ΔparS (lacks partitioning-like site), pDaf-T1 (intact *par* operon), and pDaf-BBR2 (intact *par* operon). Stability assays were performed by growing plasmid-carrying *E. coli* cells without selection in 5 ml of LB at 37°C over 100 generations, with consecutive subcultures to fresh LB at 20-generation intervals. Samples taken at each interval were diluted 10^{-6} fold, plated onto LB agar plates in the absence of selection, and incubated at 37°C overnight. At least 100 colonies were replica-plated on a selective medium (LB agar km plates). Plasmid stability was observed as the percentage of colonies that retained the antibiotic resistance encoded by the plasmid. A similar approach was used for plasmid stability experiments in *S. oneidensis* MR-1Ap^r with derivatives pDaf –T1 and pDaf-BBR2. The replica-plate on selective medium was performed at two time points (beginning and end of the experiment).

Plasmid stability in *D. africanus* was assessed with the following derivatives: pDaf-MCS, pDaf-ΔH, pDaf-ΔP and pDaf-ΔS. Stability assays were performed by

growing plasmid-carrying *D. africanus* cells without selection in 10 ml of LS broth at 37°C over 120 generations, with consecutive subcultures to fresh LS broth at 5-day intervals (~ 20 generations). Samples taken at each interval were screened by plasmid purification or PCR amplification of the *km-ori* fragment. Plasmid stability was observed as maintenance of the plasmid, checking every 5 days over a 2 month period.

Construction of pDaf-MCS cloning vector

The pDaf cloning vector was constructed by a combination of standard- and overlap extension-PCR techniques using the Expand Long Template PCR system as previously described with the primers listed in Table 2.3. The extension temperatures were adjusted according to fragment length. Multiple cloning site (MCS) contains recognition sequences for XbaI, EcoRV and SalI enzymes. The 1771 bp *ori-km* fragment with a flanking MCS was amplified by the overlap extension PCR technique with primers F-ori1, R-tag1, F-km and R-MCS. The PCR product was then digested with kpnI restriction enzyme and dephosporylated according to manufacturer's recommendations.

The 4591-bp pDaf fragment was excised from pDaf-mob-par by KpnI restriction digestion and ligated to MCS-PCR product using standard cloning methods (Sambrock et al., 1989). The ligation mixture was then electroporated into *E. coli* GM2163 competent cells, and the transformants were selected on LB-km agar. The recombinant plasmid pDaf-MCS was screened by XbaI, SalI and EcoRV digestions in addition to the enzymes which cut pDaf plasmid.

Results

Plasmid sequence analysis

The circular plasmid is composed of 8568 bp, and the overall G+C content is 47.2%, which is lower than the G+C content determined for the host strain *Desulfovibrio* SR-1 (62.4 mol%). Although the overall sequence of pDaf shows a low G+C content, the GC density analysis for pDaf (Fig 2.2) reveals some regions with exceptionally high G+C contents of 61% (373-1281 nucleotide positions on pDaf sequence) and 59% (8155-8568 nucleotide positions on pDaf sequence).

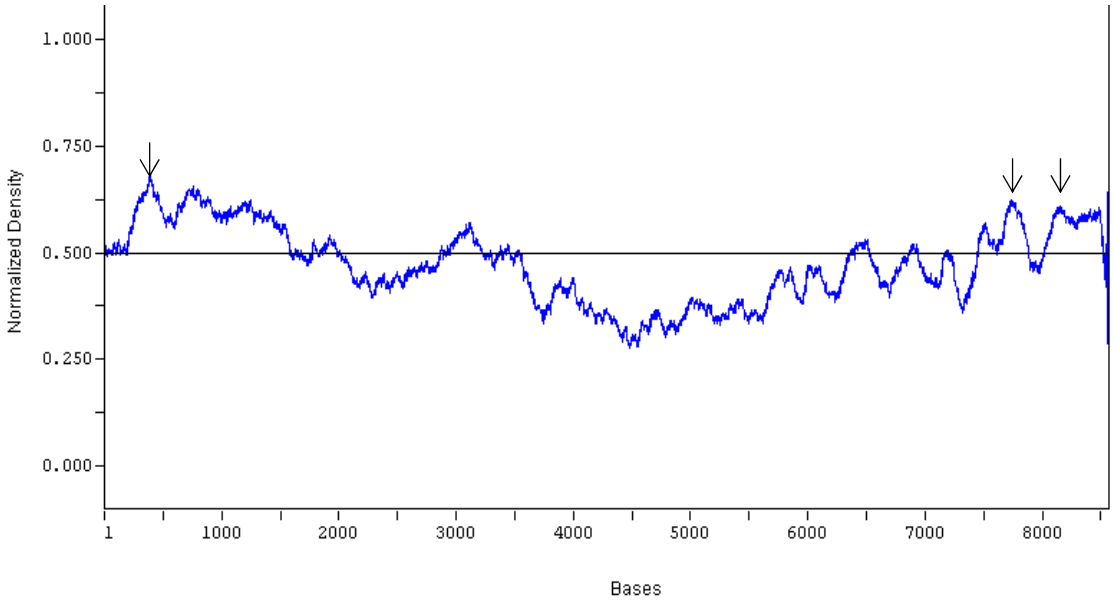


Fig 2.2. G+C density of *Desulfovirbio* SR-1 plasmid (pDaf). Mean G+C is 47.2% and arrows denote G+C-rich peaks.

Nine potential open reading frames (Orfs) were identified within the 8568 bp of pDaf. The Orfs were numbered by their length rather than a successive order in the plasmid sequence. The largest Orf was designated as Orf 1 and the smallest one as Orf 9

(Fig 2.3). Six Orfs are transcribed in a clockwise orientation, while the remaining three Orfs are transcribed counterclockwise. The plasmid sequence contains two non-coding regions located between Orfs 3 and 8, and Orfs 6 and 8. The overlapped Orfs 6, 7 and 2 are organized into an operon. The adjacent Orf 5 and Orf 9 are also organized into an operon with an intergenic distance of 21 nucleotides. The plasmid sequence also revealed the presence of inverted and directed repeat sequences upstream of Orf 6, directed repeat sequences upstream of Orf 1 and inverted repeat sequences downstream of Orf 9.

BLASTP searches of all Orfs are depicted in Table 2.6. The Orfs 2 and 6 bear similarity to genes that are involved in plasmid mobilization. The protein encoded by Orf 2 is 44% identical to pGNB2-relaxase over a stretch of 261 amino acids and 27% identical to a pPLV22a-mobilization protein from *Bacteroides fragilis*. The protein encoded by Orf 6 is 38% similar to a pGNB2-mobC accessory mobilization protein over a stretch of 86 amino acids. The protein encoded by Orf 7 had no similarity to database entries. The protein encoded by Orf 5 showed significant similarity (30% to 35%) to ATPases involved in partitioning systems of plasmids and more often in bacterial chromosomes. Protein encoded by Orf 9 had no similarity to database entries. The protein encoded by Orf 4 is 40% similar to a hypothetical protein predicted as transcriptional regulator. The protein encoded by Orf 1 showed significant similarity (31% to 32%) to plasmid replication proteins from *Neisseria*, *Pseudomonas* and *B. methanoliticus*. The protein encoded by Orf 3 is 37% similar to a hypothetical protein that belongs to a large protein family of unknown function. The protein encoded by Orf 8 showed no similarity to database entries. Based on gene similarity search results, functions associated with

Orfs may be classified into the following categories (Fig 2.4): replication (Orf 1), mobilization (Orf 6, Orf 7 and Orf 2), and plasmid stability (Orf 5 and Orf 9).

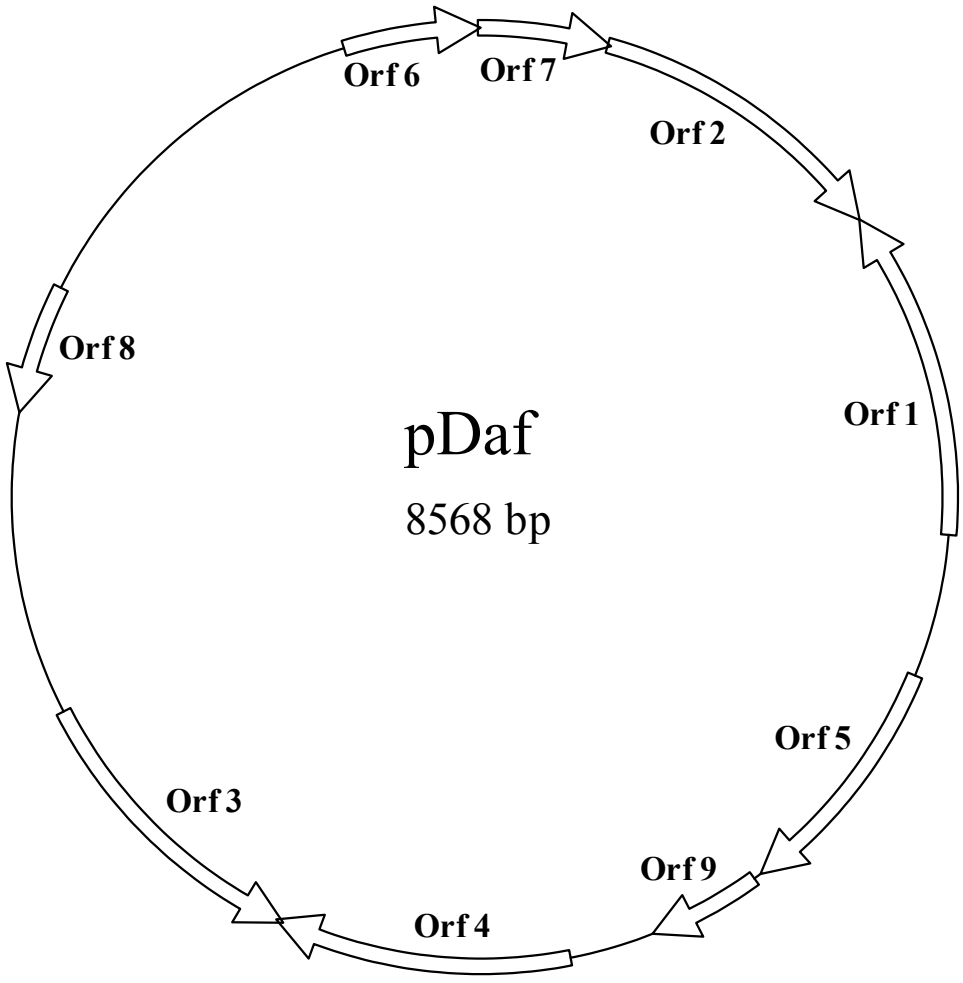


Fig 2.3. Organization of the Open reading frames (Orf) in *Desulfovibrio* plasmid.

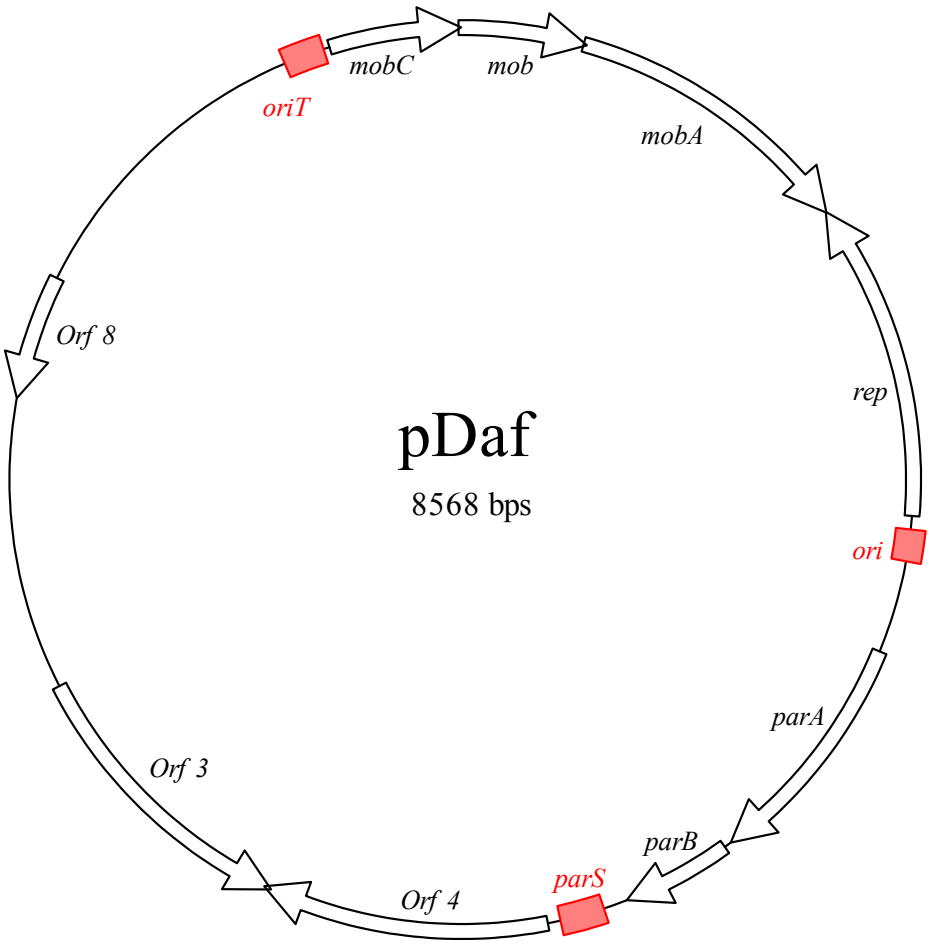


Fig 2.4. Genetic organization of *Desulfovibrio* SR-1 plasmid pDaf. Genes and open reading frames are indicated by arrows. Genes of unknown function are labeled Orf followed by a number. Other gene designations are associated with a potential function of corresponding gene products as listed in Table 2.6.

Table 2. 6. General features of the open reading frames (Orf) in pDaf plasmid and proposed functions for their products.

Orf	Sequence Position	Amino Acids	Function	Organism and protein or plasmid with protein database similarity (accession number).	% amino acid Identity[a]
Orf 1 (*rep*)	2252-1278	324	Replication	Plasmid pSJ5.2, replicase A (ABD61075); plasmid pFA3, replication protein (P17492). *Neisseria gonorrhoeae*	73/233 (31%)
				Plasmid replication protein (AAD34467). *Pseudomonas*.	81/247 (32%)
				Plasmid pBM19, putative replication initiator protein (NP_957662). *Bacillus methanoliticus.*	76/256 (29%)
Orf 2 (*mobA*)	373-1281	302	Mobilization	Plasmid pGNB2, relaxase/ mobilization domain from fusion protein (ABE98193). Experimental evidence.	117/261(44%)
				Plasmid pLV22a, plasmid mobilization protein (AAA82753). *Bacteroides fragilis*. Experimental evidence.	54/199 (27%)
Orf 3	5779-4877	300	Unknown	Hypothetical protein (NP_886930), DUF1814 domain. *Bordetella bronchiseptica* RB50	109/289(37%)
Orf 4	4022-4900	292	Unknown	Hypothetical protein (NP_886931). Predicted transcriptional regulator. *B. bronchiseptica* RB50.	120/295(40%)
Orf 5 (*parA*)	2674-3414	246	Partitioning	ATPase involved in chromosome partitioning (YP_001181521). *Caldicellulosiruptor saccharolyticus.*	91/254 (35%)
				Plasmid pJM1, putative protein involved in partition (NP_943574). *Listonella anguillarum.*	78/259 (30%)
Orf 6 (*mobC*)	8164-8567	134	mobilization	Plasmid pGNB2, auxiliary mobilization protein C (ABE98194)	33/86 (38%)
Orf 7	8559-383	130	Unknown	No similarity	
Orf 8	7056-6673	127	Unknown	No similarity	
Orf 9	3436-3774	112	Unknown	No similarity	

[a] Shown as number of identical amino acids/total number of amino acids in the region of homology identified by BLASTP.
Hypothetical protein: No substantial similarity to any other sequenced protein.

The computer analysis of the pDaf DNA sequence revealed the presence of 36 GATC sites not uniformly distributed along the DNA plasmid. GATC clusters are in coding regions rather than intergenic sequences, and they are predicted to be methylated by the methylases from restriction modification systems (R-M). Several naturally occurring restriction recognition sites for several endonucleases were also detected (Fig 2.5). They were confirmed by restriction enzyme digestion of both the native plasmid and the plasmid derivatives.

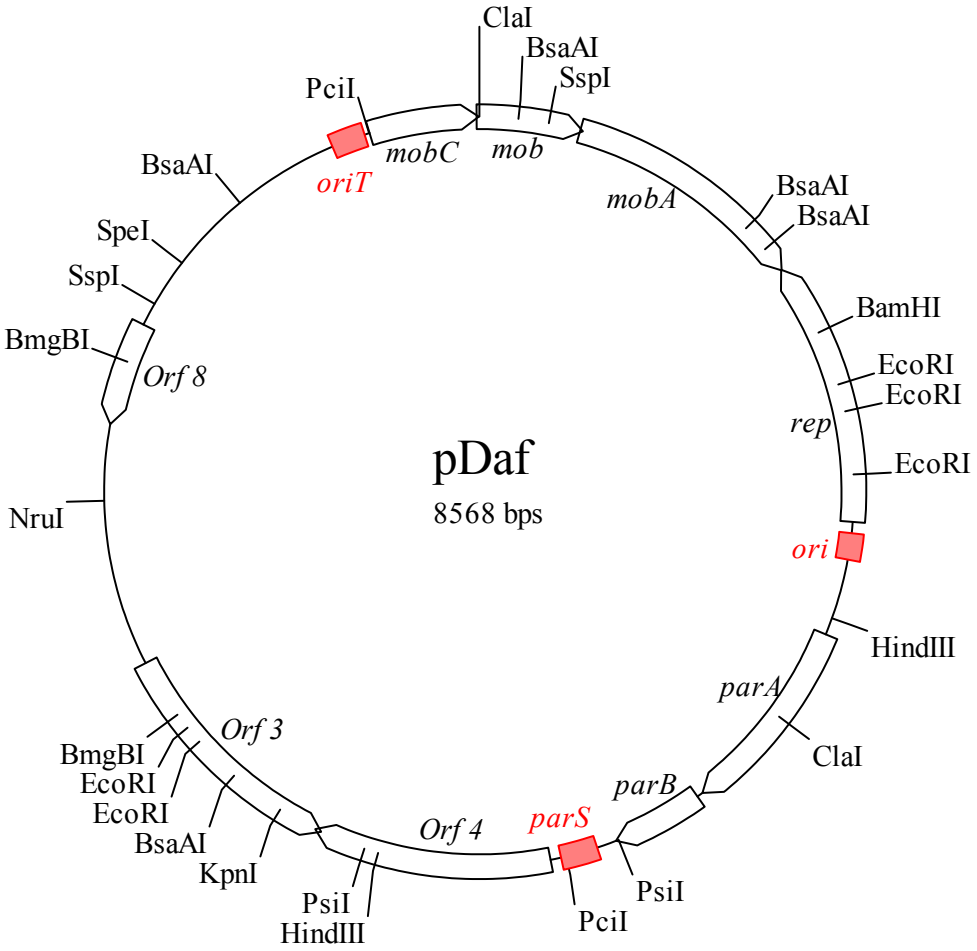


Fig 2.5. Restriction recognition enzymes on pDaf. Restriction recognition sites detected by computer analysis and confirmed by restriction digestion.

Relative plasmid copy number determination

Specificity of real-time PCR products resulted in single 143-bp products. No primer dimers were generated during the 40 amplification cycles. The dissociation curves showed single product specific melting temperatures of about 83°C for Daf-REP and 88°C for *FdIII* (Fig 2.6). The standard curves of Ct against log (DNA molecules per reaction) were linear, with correlation coefficients $r^2 = 0.99$ for both the plasmid and the reference gene assays. The slope of the standard curve line equation for the plasmid was -3.320759 (Fig 2.7) and for the reference gene -3.333353 (Fig 2.8), with an amplification efficiency of 100% and 99.5%, respectively. The estimated relative copy number of pDaf was 6.08 SD ±0.26 (Table A1) which correlates to densitometric analysis of Southern blot hybridization bands (Fig 2.9). *Desulfovibrio* plasmid (pDaf) replicates at a copy number of 6 in the native host *Desulfovibrio* SR-1.

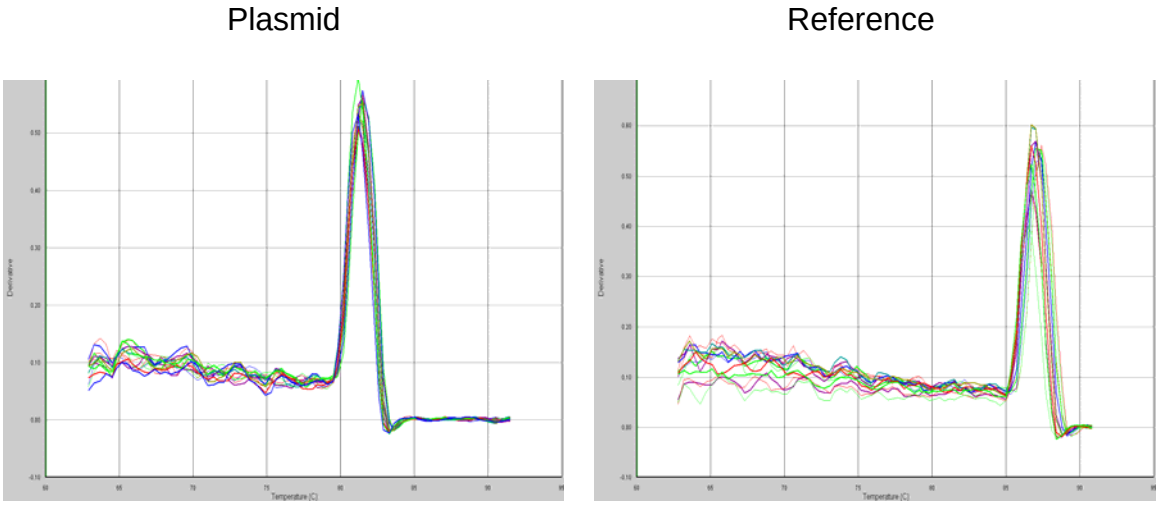


Fig 2.6. Specificity of single PCR products of plasmid and chromosomal reference genes. Plasmid: Daf-REP with83°C melting temperature. Reference: FdIII with 88°C melting temperature.

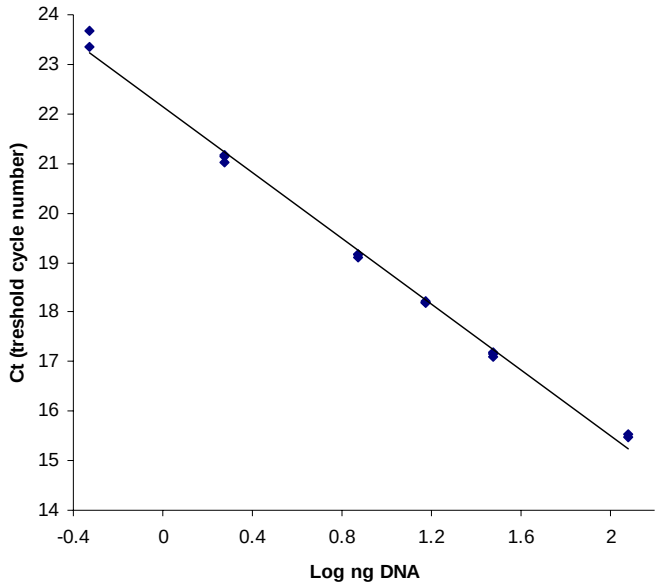


Fig 2.7. Standard curve for the real-time PCR amplification of plasmid (Daf-REP). Slope: -3.320759, y-intercept: 22.176767, r^2: 0.9982. E = 100%

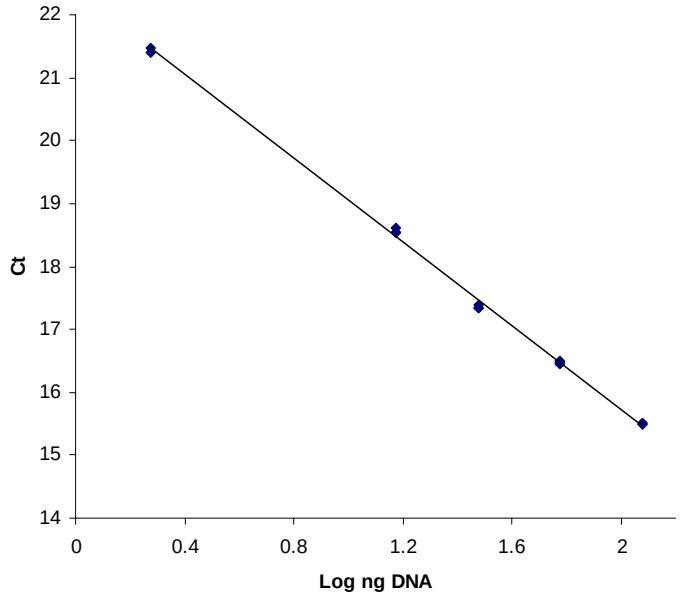


Fig 2.8. Standard curve for the real-time PCR amplification of reference (FdIII). Slope: -3.333353, y-intercept: 22.39545397, r^2: 0.9986. E = 99.5%

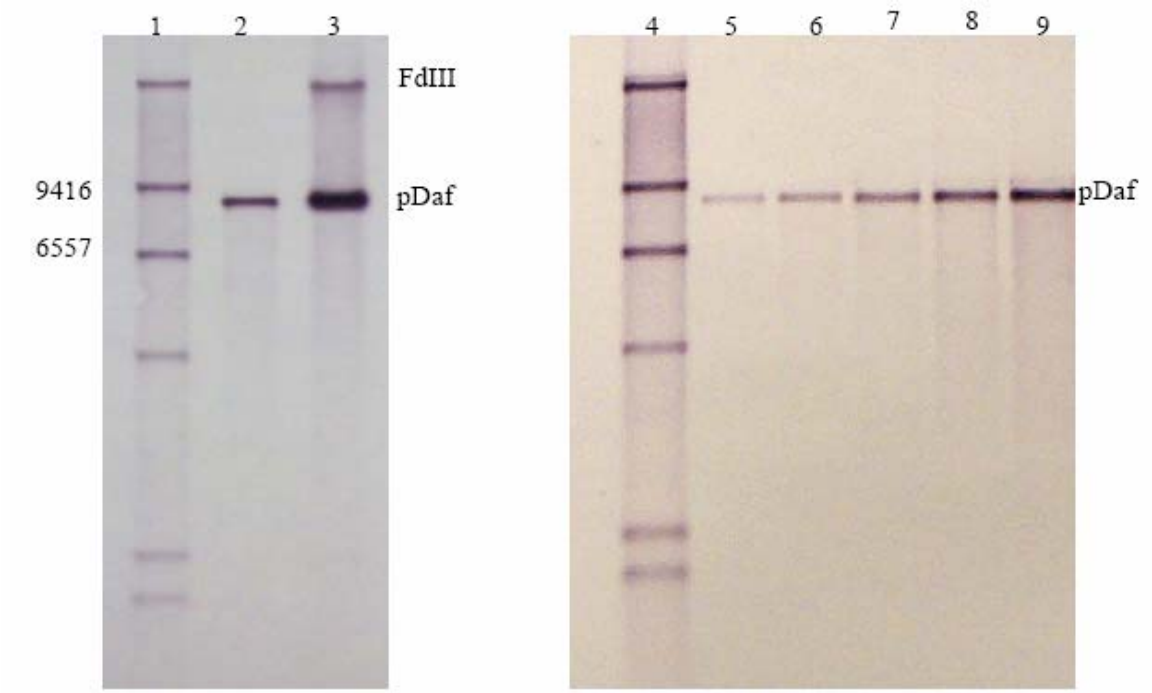


Fig 2.9. Copy number of *Desulfovibrio* plasmid in its native host assayed by Southern blot hybridization. BamHI-digested chromosomal DNA of *Desulfovibrio* SR-1 (lane 3). kpnI-digested pDaf (lane 2). Two-fold increase of kpnI-digested plasmid concentrations (lanes 5-9). Molecular weight marker (lane 1).

Host range of pDaf

The pDaf with a kanamycin gene inserted into a coding or non-coding sequence did not transform either *E. coli* dam- or *E. coli* dam+. The *ColE1*-based pDaf derivatives were used for the transformation of several bacterial hosts.

Electroporation of pDaf into several bacterial strains

D. africanus km^r transformants were obtained with unmethylated and methylated plasmid DNA (Fig 2.10). The insertion of a selectable marker into either an intergenic region or a coding region (Orf 3) did not affect plasmid replication. The copy number of

pBRR1 and *ColE1* origin of replication had no effect on transformation of *D. africanus*. Since shuttle vectors containing *ColE1-ori* are suicide vectors in *P. aeruginosa* PA14, transformation of this bacterium was performed with pDaf-ColE1 derivatives. *P. aeruginosa* PA14 km^r transformants were obtained with unmethylated and methylated plasmid DNA (Fig 2.11). The other bacterial species could not be transformed by either unmodified or modified pDaf plasmid. Since pDaf only replicates in *D. africanus* (δ-proteobacteria) and *P. aeruginosa* (γ-proteobacteria), it can be considered as a narrow host range plasmid.

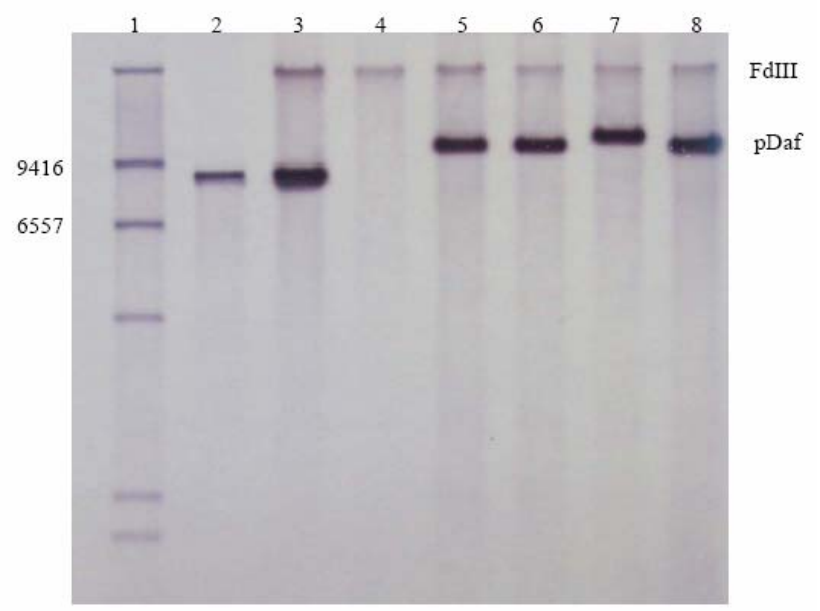


Fig 2.10. Southern Blot of *D. africanus* DSM2603^T transformants. Positive controls: kpnI-digested pDaf (lane 2) and pDaf from *Desulfovibrio* SR-1 (lane 3). Negative control: *D. africanus* DSM2603^T (lane 4). Plasmid derivatives: pDaf-Spe2 (lane 5), pDaf-Spe2 (lane 6), pDaf-BBR (lane 7) and pDaf-T1 (lane 8). DNA molecular weight marker II (lane 1).

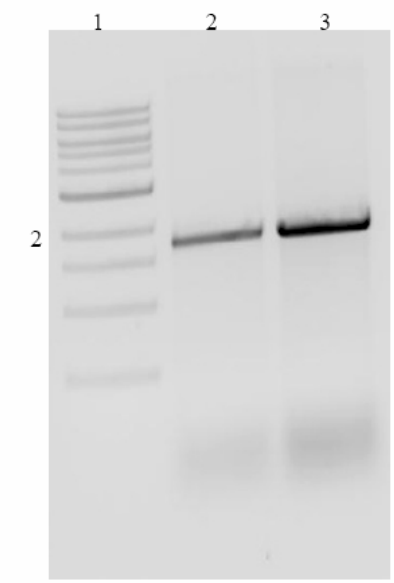


Fig 2.11. Amplified km-ori PCR products from *P. aeruginosa* PA14 transformants. Plasmid derivatives: pDaf-Spe5 (lane 2) and pDaf-Spe2 (lane 3). 1kb DNA ladder (lane 1).

Functional characterization of Orfs in plasmid biology

It seems that the Orf 3, Orf 4, and Orf 8 have no role in the plasmid biology. The individual deletion of these Orfs and deletion of these three Orfs from pDaf did not affect any plasmid function (Table 2.7).

Table 2.7. Role of each gene in pDaf plasmid biology.

Derivative	Plasmid function that is affected by specific deletions of Orfs and regions
pDaf-ΔH	Loss of plasmid stability under non-selective pressure, no plasmid could be isolated from *D. africanus* after 40 generations. Role of *par* operon in segregational stability
pDaf-ΔS	Plasmid could not be transferred from *E. coli* to *S. oneidensis*. The Orfs 6 and 7 are accessory genes required for plasmid mobilization
pDaf-ΔB	Deletion of Orf 8 and Orf 3 did not affect the genes associated with plasmid functions.
pDaf-ΔP	Loss of plasmid stability under non-selective pressure, no plasmid could be isolated from *D. africanus* after 40 generations. Intact *par* operon is required for plasmid stability
pDaf-ΔPP	The putative *oriT* is localized upstream of *mobC* gene. Plasmid could not be transferred from *E. coli* to *S. oneidensis*.
pDaf-ΔE	Genetic evidence of the role of *rep* gene in plasmid replication. The partial deletion of *rep* gene from pDaf abolish its replication in *D. africanus*
pDaf-ΔparS	The partitioning-like site was identified. Plasmid could be transferred from *E. coli* to *S. oneidensis* but it was not stably maintained under non-selective pressure.

Molecular and functional characterization of the plasmid structural genes

Replication

The pDaf sequence was examined for characteristics of plasmid replication origins. The presence of an A+T rich region of 63% followed by four contiguous direct repeated sequences upstream of Orf 1 (*rep*) were detected. The direct repeats termed iterons are 22-bp long with the consensus pattern "AATGGCTACAAATTTCCGGTCA". However, translation initiation signals, GATC sites and binding sites for host proteins

were not detected. By analogy with other *Neisseria* plasmid replicons, this region contains the origin of replication (Fig 2.12).

A

AAGCTTGCGCCTTAGCTAGAAGAATGTCTTTTTGAGTCTTCGCCCTTTCGCCTTTTTTAC
TTTATGGCCTGCCTTTACTTTCTTGTGCCCTTAGCGTGCGGGTTAGCCCGGATTCGATGA
GCTTTGTTCCTCTCTTTCTTTCTTTCTTTCTTCTTTTTCTTCTTTTAGCTGCTTTTAATA
CTTTTAGGCGGTGGTTTGGCCTTGTCATTTCTGGCTTGAACAGCGGAATGCGCTACAAAT
TTCCGGTCAAATGGCTACAAATTTCCGGTCAAATGGCTACAAATTTCCGGTCAAATGGCT
ACAAATTTCCGGCATAATGCTACATTCTTGGCATCTGTAGCAAGTTGGGTTACAAGACTT
TCCC**ATG**

B Consensus (22-bp): AATGGCTACAAATTTCCGGTCA

C

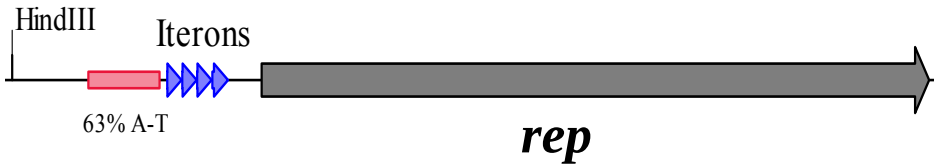


Fig 2.12. *Desulfovibrio* plasmid replicon. **A**. Putative origin of replication containing 109 bp region with 63% A-T residues (red line) followed by four 22-bp directly repeated sequences (arrows), box indicates a HindIII recognition site. **B.** 22-bp Iteron consensus sequence. **C**. Physical map of plasmid replicon.

D. africanus was transformed by the pDaf-km^r with a transformation efficiency of 1.5 x 10^3 CFU/µg DNA. Plasmid isolation from kanamycin resistant transformants indicates plasmid replication rather than integration into the genome. Isolation of pDaf-

ΔH-carrying transformants indicates that replication and partitioning systems function independently. Since the plasmid rep gene is partially deleted in pDaf-ΔE, this derivative did not transform *D. africanus*.

D. africanus DSM2603 was transformed by a plasmid derivative which contains only genes associated with plasmid functions and a kanamycin gene. Plasmid isolation from *D. africanus* transformants indicates that pDaf is a self-replicating plasmid (Fig 2.13).

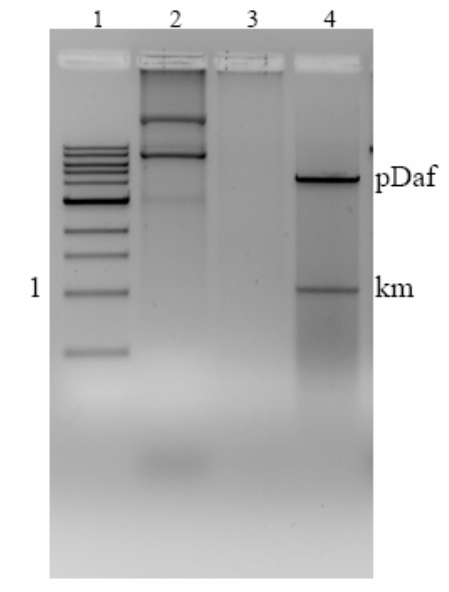


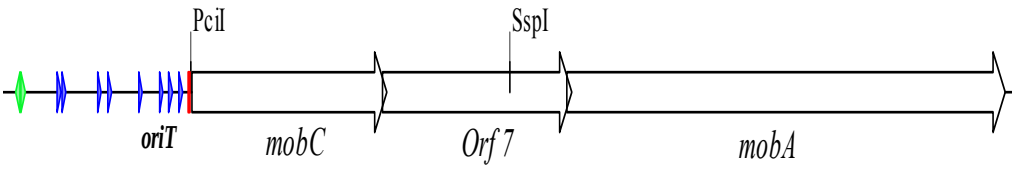
Fig 2.13. KpnI-restriction pattern of pDaf-km^r isolated from *D. africanus* DSM2603^T transformants. 1kb DNA ladder (lane 1). pDaf in the native host *Desulfovibrio* SR-1 (lane 2). No plasmid is present in *D. africanus* before transformation (lane 3). KpnI-fragments: 4951-bp pDaf and 1717-bp km fragments (lane 4).

Mobilization

The organization of the overlapped Orf 6, Orf 7 and Orf 2 into an operon and characteristics of plasmid transfer origins were examined. The presence of directed and inverted repeats in a 485-bp region upstream of Orf 6 correlates with the main features of

plasmid transfer origins (*oriT*). The putative RBS (GGAGG) and promoter sequences were detected upstream of *mobC*. The Orf 6 overlaps 10 nucleotides of Orf 7, and Orf 7 overlaps 11 nucleotides of Orf 2. These features indicate that the functional mobilization operon of pDaf is composed of Orfs 6, 7 and 2 with a putative origin of transfer located upstream of Orf 6 (Fig 2.14).

A



B

TGTTTCTTGGAAGTGTGAGGACCTCTTTGTTTTCTGTGAGGGCTATATTCCCTGCCTGTGAGAAT

TTTCTTGTTTCTTGTGAGGGCTTCATTTCTTGCTGTGAGGGTCCCATTTTGGGCTGTGAGGTAAT

TTTCAGGGAGGCAC**ATG**

C

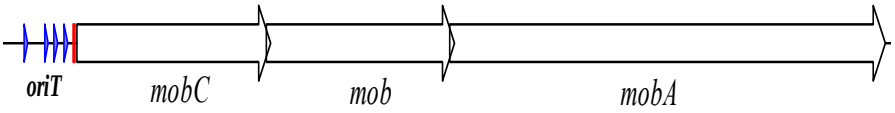


Fig 2.14. Mobilization operon organization. **A**: Mob operon containing the putative OriT. **B**: Putative *oriT* within a 143-bp region containing four direct repeats. **C**: Mob operon with a minimal region containing *oriT*. Gene designations are associated with potential functions corresponding with gene products with known functions.

No transconjugants were recovered from conjugation between pDaf-Spe2-carrying *E. coli* and *S. oneidensis* MR-1, which indicates that pDaf is a non self-transmissible plasmid. Conjugation only occurred in the presence of self-transmissible helper plasmid pRK600. Plasmid DNA from transconjugants was resistant to digestion by ClaI (Fig 2.15). The derivatives pDaf-ΔB, pDaf-ΔparS and pDaf-mob-par were also isolated from transconjugants. As expected, the derivatives pDaf-ΔS and pDafΔPP could not be transferred by triparental mating indicating that Orf 6, Orf 7 and the upstream region to Orf 6 are necessary for plasmid mobilization. *Desulfovibrio* plasmid was transferred when the *tra* functions of the IncP RK2 plasmid was provided in *trans*. The transfer frequency was 4.8 x 10^{-4} transconjugants/donor. Our results indicate that pDaf can be transferred between gram-negative bacteria at high frequency.

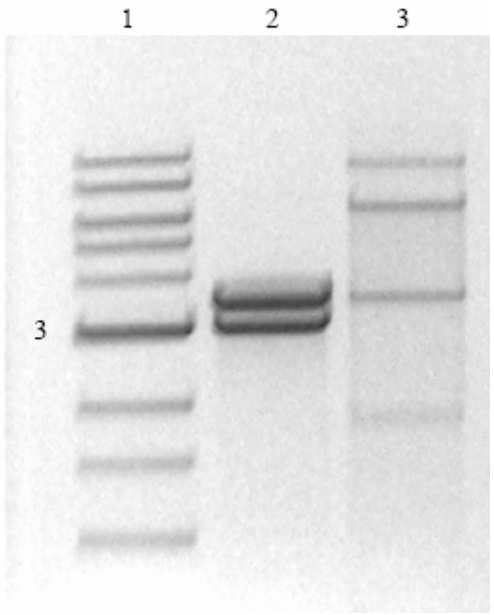


Fig 2.15. ClaI restriction pattern of pDaf-BBR2 isolated from a kanamycin resistant *S. oneidensis* transconjugant. Lane 1: 1kb DNA ladder (lane 1). ClaI-digested pDaf from donor *E. coli* GM2163 (lane 2). Plasmid DNA isolated from recipient strain is resistant to ClaI restriction digestion due to DNA methylation.

Partitioning (Plasmid stability)

The organization of adjacent Orf 5 and Orf 9 into an operon and characteristics of plasmid partition sites (*parS*) were examined. The putative RBS (GGAGG) and promoter sequences were detected upstream of Orf 5 *(parA*). The intergenic distance between *parA* and Orf 9 is 21-bp long and contains a RBS (GGAGA) within 10-bp of the start site of Orf 9. The centromere-like site (*parS*) is contained within a 136-bp region carrying inverted repeats that lies downstream of Orf 9. The predicted partitioning operon of pDaf is comprised of Orf 5 (*parA*), Orf 9 and *parS* (Fig 2.16).

A

TAATATGGCTAGTTATGGCCTAACTCAGCCTATCTTAAAAATTGACTCAGGTTGAGTTGA

CAAATTGTTTATTGTGTGGTATTAAAAAGTTCATGAACTTTTTAATACCAGCAAAGCTGA

TGCCCAAGGCCGGATGTGTTTGATTTCATTACTACGAAATCAAACACATCCGGGCAATTC

GCTATAAGCAAGTACATGTACTTGCTTATAGCATGAAGGAAGCAAAAGATCAAGTTTCCG

GGGAGAGCGC**ATG**

B

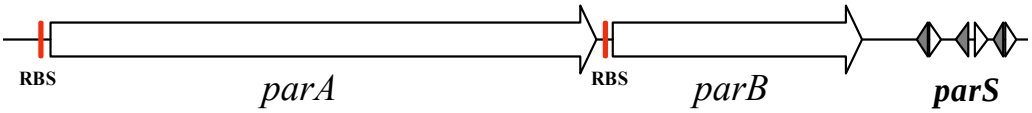


Fig 2.16. Partitioning operon organization.A: Dowstream sequence region of *parB* that contains the partitioning-like site *parS* (inverted repeats). B: Complete partitioning operon. Gene designations are associated with potential functions corresponding with gene products with known functions.

Plasmid stability was assayed in *E. coli* GM2163. The segregational stability of pDaf carrying or not carrying the intact or partially deleted partitioning region was compared. Plasmids containing an intact partitioning operon (pDaf-T1 and pDaf-mob-par) were found to exhibit high segregational stability (91-100%) in the absence of selection pressure. The deletion (pDaf-ΔH) or truncation (pDaf-ΔP, pDaf-ΔparS) of the partitioning operon eliminated the stability effect (Fig 2.17). Similar plasmid stability was observed in *D. africanus*. A small amount of plasmid (pDaf-ΔH and pDaf-ΔP) was recovered after 20 generations (Fig 2.18), and these plasmids could not be isolated from 10-ml cultures of *D. africanus* transformants after 2 weeks. pDaf containing an intact *par* operon was relatively stable in *S. oneidensis* MR-1; the plasmid was maintained in 40% of the colonies after 20 transfers under non-selection pressure. The partitioning system from pDaf stabilized the *ColE1-ori* and pBBR1-*rep* derivatives at the same rate. It is known that *ColE1* shuttle vectors are not stably maintained in *S. oneidensis* with 80% of the colonies losing *ColE1*-plasmids after 20 generations under no selection pressure.

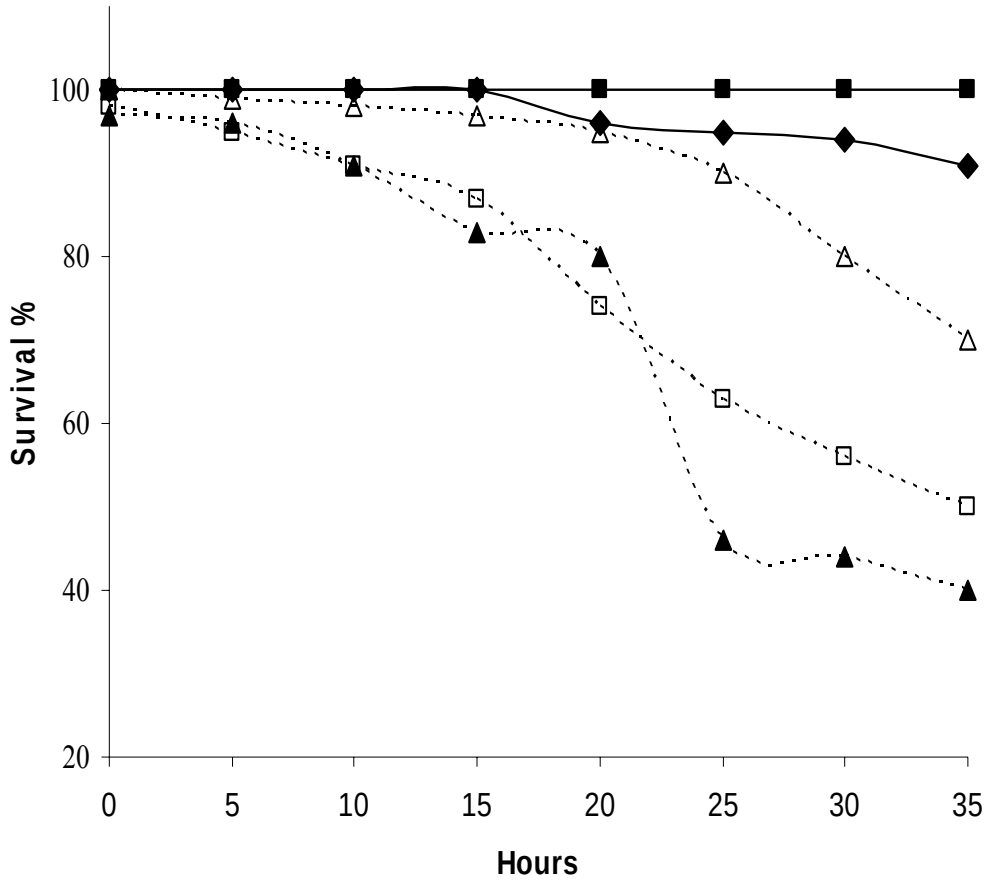


Fig 2.17. Stability of pDaf plasmid assayed in *E. coli* GM2163. Plasmid stability is reported as survival percentage. ♦ pDaf-T1 (complete plasmid), ■ pDaf-BBR2 (only structural genes), pDaf-ΔparS (partitioning operon lacks partitioning binding site S), □ pDaf-ΔH (no partitioning operon), ▲pDaf-ΔP (truncated partitioning operon).

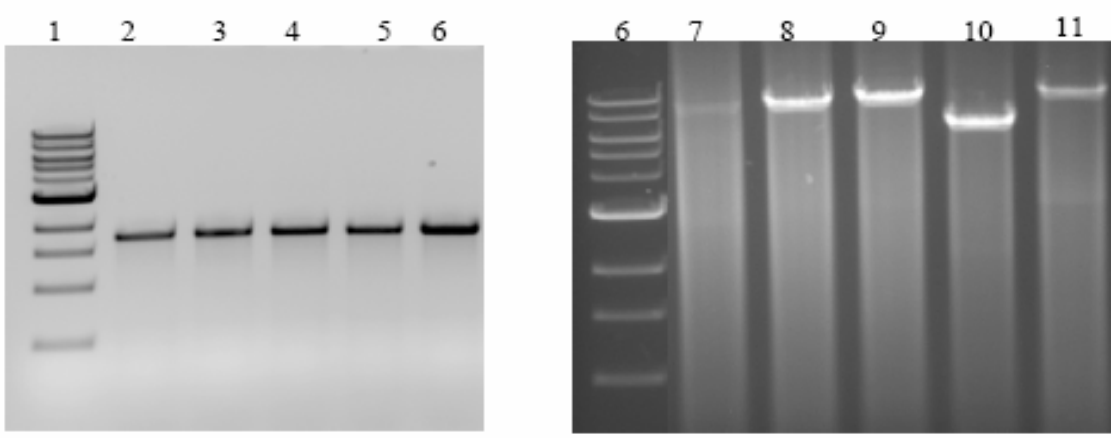


Fig 2.18. Plasmid stability in *D. africanus*. Amplified km-ori PCR products from transformants after 20 generations under non-selection pressure (lanes 2-6). Plasmid isolated from transformants after 20 generations under non-selection pressure. Lane 7, pDaf-H Δ(*parA-parS*). Lane 11, pDaf-ΔP Δ (*parB*'-*parS*). Plasmid derivatives par^{+}(lanes 8-10). 1kb DNA ladder (lanes 1 and 6).

The functional characterization of plasmid structural genes confirmed that pDaf is a self-replicating plasmid with active mobilization and partitioning systems. The plasmid backbone comprised 53.6% of the plasmid. Replication, mobilization and partitioning genes in addition to origin of transfer (*oriT)*, origin of replication (*ori*), and partitioning-like site (*parS*) were contained in a 4591-bp plasmid fragment (Fig 2.19).

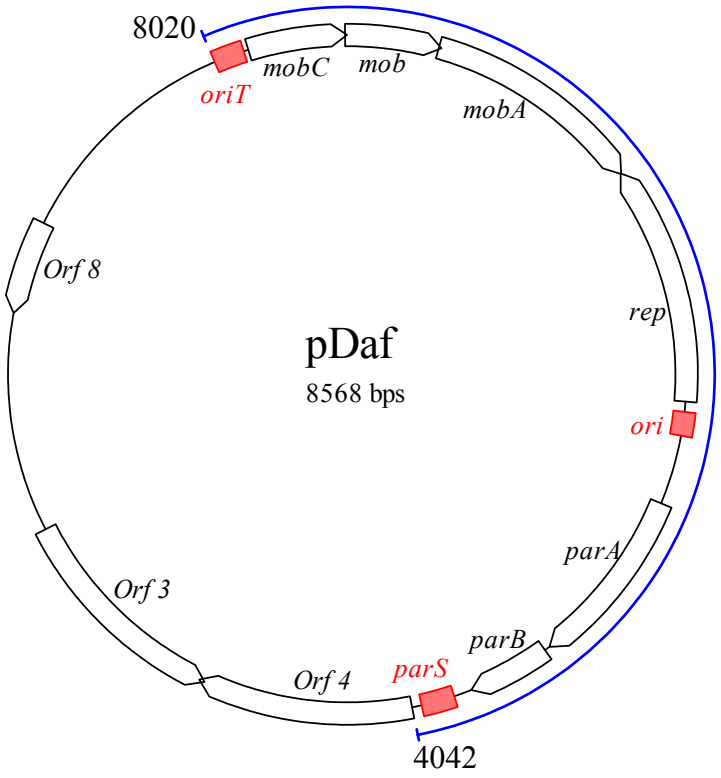


Fig 2.19. *Desulfovibrio* plasmid backbone structure. The blue line represents the 4951-bp fragment (52% G+C content) that contains the features for mobilization, replication and stability. Gene designations are associated with experimental evidence.

Construction of pDaf-MCS cloning vector

The basic cloning vector pDaf-MCS was constructed in an effort to evaluate its usefulness as a genetic tool in *D. africanus*. The main features include: The cloning site contains convenient restriction sites to clone DNA fragments with either blunt or overhanging ends; low copy number; it is mobilizable by IncP RK2 plasmid at high transfer efficiency; the partitioning operon confers high stability to *ColE1-ori* in *E. coli* and *S. oneidensis*; they contain kanamycin as a selectable marker (Fig 2.20).

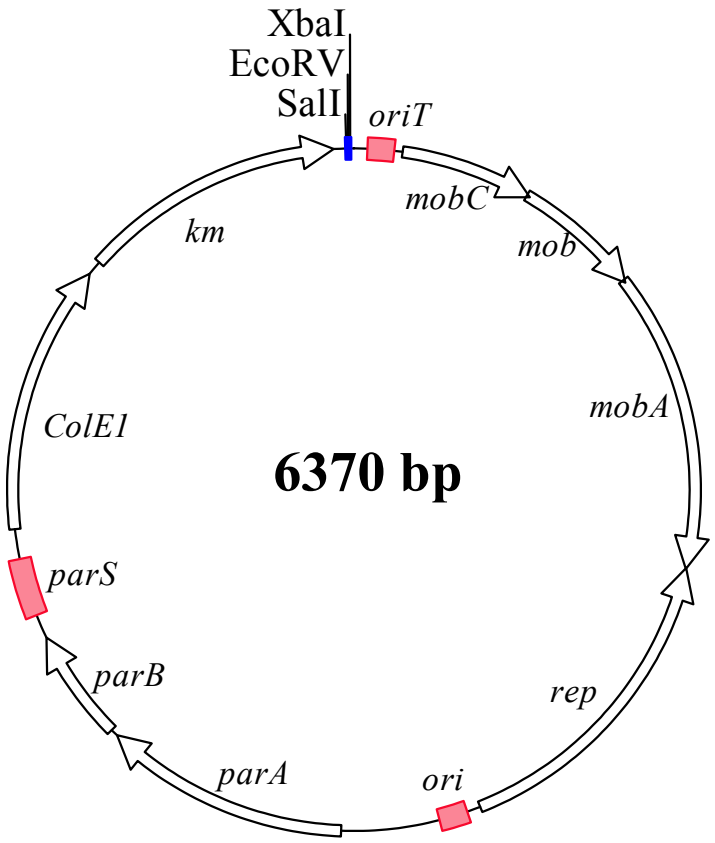


Fig 2.20. Genetic map of the plasmid cloning vector pDaf-MCS. The multiple cloning sequence contains unique sites for the restriction endonucleases XbaI, EcoRV and SalI. The plasmid contains a kanamycin resistance gene, an origin of replication that functions in *E. coli*, and *rep* gene that functions in *D. africanus* and *P. aeruginosa*.

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Discussion

In this study, we report the characterization of a small plasmid endogenous to *Desulfovibrio* SR-1. Our results elucidate plasmid-associated function genes that will form the basis for new genetic tools in *D. africanus* based on native vectors.

The G+C content of *Desulfovibrio* plasmid was found to be lower than that of its native host *Desulfovibrio* SR-1. However, the G+C density plot revealed high G+C regions in the mobilization gene *mobC*. In general, most plasmids have a lower G+C content than their corresponding host chromosome. It has been suggested that the lower G+C content may prevent the integration of plasmids into the host chromosome (van Passel et al., 2006).

The low copy number of the pDaf plasmid in *Desulfovibrio* SR-1 correlates with the presence of a partitioning system, which is characteristic in low copy number plasmids. Most plasmids depend on the host replication machinery and possess partitioning genes. These properties confine plasmids to a limited range of hosts, yielding a close and stable relationship between plasmid and host (van Passel et al., 2006).

Most plasmids of gram-negative bacteria are limited in their host range because replication initiation is dependent on specific host-encoded enzymes (Kües and Stahl, 1989). Notable exceptions are the well-studied plasmids RSF1010 and RK2, which have an extended host range among gram-negative bacteria (Scholz et al., 1989; Rawling and Tietze, 2001). By contrast, plasmid pWCFS103, native to *Lactobacillus plantarum* WCFS1, replicates only in the closely related *L. plantarum* NC8 (Kranenburg et al., 2005).

DNA restriction and modification (RM) systems prevent genetic exchange between groups of bacteria by enabling the host to recognize and cleave foreign DNA (Jeltsch, 2002). The functional role of R-M systems as barriers in bacterial transformation has been reported (Lefrançois and Sicard, 1997; Donahue et al., 2000). There is evidence that restriction-modification system differences are barriers to interstrain plasmid transfer (Ando et al., 2000). Genome sequences of *D. desulfuricans* G20 and *D. vulgaris* Hildenborough have revealed the presence of Type I restriction modification and restriction endonuclease systems which are predicted as defense mechanisms. If these are functional, they may represent a host barrier in bacterial transformation. Since the *Desulfovibrio* plasmid sequence presents specific targets recognized by restriction modification systems, plasmid DNA was protected from restriction by specific *in vivo* methylation and by *in vitro* protection with "ocr" phage protein. This protein inhibits type I DNA restriction enzymes (Murray, 2000) by preventing them from binding to their DNA target (Walkinshaw et al., 2002). Neither the two modified plasmids nor the unmodified plasmid could transform most *Desulfovibrio* species. Only *D. africanus* was transformed by both the unmethylated and the modified pDaf plasmid with similar transformation efficiencies. In addition, the plasmid DNA purified from *D. africanus* transformants was sensitive to ClaI cleavage, which suggests that *Desulfovibrio* SR-1 and the closely related *D. africanus* do not have strain-specific R-M systems. It is likely that other host factors in addition to R-M mechanisms prevented pDaf replication in the other *Desulfovibrio* species. The pBG1 plasmid native to *D. desulfuricans* G100A replicates in pBG1-cured *D. desulfuricans* G20 and *D. fructosivorans*, but it is not stably maintained

in the latter strain (Wall et al., 1993). Plasmid loss from *D. fructosivorans* might be a consequence of differences in the R-M system.

P. aeruginosa was transformed by unmethylated and methylated *ColE1*- based pDaf derivatives. Similar results in electrotransformation of *P. syringae* by both types of plasmid DNA have been reported (Bassett and Janisiewicz, 2003). It is likely that *P. aeruginosa* PA14 does not have strain-specific R-M systems that could prevent the replication of pDaf. The amplification of the *ColE1-km* fragment from neomycin-resistant *Pseudomonas* transformants indicates that host-encoded proteins were involved in the initiation of pDaf replication. Furthermore, *ColE1* origin of replication is not functional in the *Pseudomonas* species (Hickman et al., 2005) because the whole replication process of *ColE1* requires proteins from its host *E. coli* (Kües and Stahl, 1989).

The host range of pBG1 has not been documented, but the pBG1 replication was limited to two *Desulfovibrio* spp including the pBG1-native host (Wall et al., 1993). By contrast, *Desulfovibrio* plasmid pDaf was able to replicate in bacteria that belong to γ-proteobacteria (*P. aeruginosa*) and δ-proteobacteria (*D. africanus*). Although pDaf replicates in bacteria that belong to different groups, pDaf can be considered as a narrow host range plasmid. It is important to note that many plasmids can replicate only in one or a few closely related hosts (Kües and Stahl, 1989).

The basic replicon of a plasmid consists of a replication initiation site (origin) and one or more adjacent elements for controlling the frequency of initiation from this site. *Desulfovibrio* plasmid pDaf is an iteron-containing plasmid that contains the gene encoding the initiator protein Rep and four contiguous directly repeated sequences, 22-bp

each termed iterons, similar to those found in theta replicating plasmids from gram-negative bacteria (Gilbride and Brunton, 1990; Rawlings and Tietze, 2001; Brautaser et al., 2004; Scharbaai-Vázquez et al., 2007). By analogy with other plasmid replicons, the A-T rich sequence contiguous to the iterons is most probably the place where DNA synthesis starts (del Solar et al., 1998). Dam methylation sequences, which are found at the origin of replication of some plasmids (Bergerat et al., 1989; Hénaut et al., 1996), were not detected in pDaf. No sequences resembling DnaA or integration host factor binding sites could be identified (Dasgupta and Løbner-Olesen, 2004).

It is well known that replication initiation proteins for iteron-containing plasmids are autoregulatory in the control of their expression, and iterons control plasmid copy number (Chattoraj, 2000). Orf 4 encodes a protein which is predicted as a transcriptional regulator, but the corresponding deletion did not affect pDaf copy number in *D. africanus* transformants. Genetic evidence that Orf 1 encodes the replication protein was obtained when a plasmid derivative with a partial deletion in Orf 1 did not transform *D. africanus*.

Conjugal transfer is an important characteristic for plasmids. Self-transmissible conjugative plasmids have the ability to form effective cell-to-cell contact, while mobilizable plasmids are only able to prepare their DNA for transfer. Genetic organization of pDaf mobilization region comprised one relaxase-encoding gene and two accessory mobilization genes. Proteins encoded by *mobA* and mobC were similar to mobilization proteins from plasmid pGNB2 (Bönemann et al., 2006) and plasmid pLV22 from *B. fragilis* (Novicki and Hecht, 1995). To date, no conjugative *Desulfovibrio* plasmids have been reported. By analogy with other plasmid mobilization regions, the upstream region of *mobC* containing direct repeats was considered as a potential *oriT*

(Kehrenberg et al., 2005). No similarity between the protein encoded by Orf 7 and other Mob proteins in the database could be detected. However, deletion of Orf 7 abolished the plasmid transfer from *E. coli* to *S. oneidensis*. A similar result was observed when the upstream region of *mobC* was deleted from pDaf. This indicates that the accessory *mobC* and *mob* (Orf 7) genes are required for plasmid mobilization. Deletion of Orf 8, which is divergently transcribed from *mobC,* did not affect plasmid mobilization. It is important to note that the absence of *mobC* in other systems lowers the frequency of mobilization (Rawlings and Tietze, 2001).

In addition to the basic replicon, naturally occurring plasmids have an active partition system that confers segregational stability (Higara, 2000). Partition operons are a characteristic feature of low copy number plasmids. The active partition systems described for plasmids consist of an operon of two structural genes and a *cis*-acting, centromere-like site (*parS*) consisting of an array of sequence repeats. Transcription of *par* operons is tightly autoregulated by the Par proteins themselves. Generally, *par* loci function as cassettes independently of the replicon on which they reside. The first gene of a *par* operon encodes an ATPase; the second gene encodes a DNA-binding protein that recognizes varying numbers of direct or inverted repeats within a cognate centromere-like site. The partitioning complex is the substrate for plasmid segregation. All three elements are essential for plasmid stability (Ho and Yarmolinsky, 2002; Ebersback and Gerdes, 2005).

The pDaf *par* locus encodes a partition system that was fully functional in *E. coli*, *D. africanus* and *S. oneidensis*. The partition system of pDaf contributes to plasmid stability as deletion of either *parA* or Orf 9 (*parB*) had a destabilizing influence on

plasmid maintenance. This is in agreement with the role of the two genes as an active partitioning system (Higara, 2000; Kalnin et al., 2000; Di Lorenzo et al., 2003). It is important to note that the role of Orf 9 as a *parB* was inferred from its location immediately downstream of *parA* and its effect on pDaf stability. Stability assays in *D. africanus* confirmed that the partitioning system functions independently from plasmid replication. A plasmid derivative lacking the whole operon was able to replicate in *D. africanus*, but it was lost after a few generations under non-selective pressure.

Several factors influence the genetic stability of prokaryotic plasmids. One important factor is copy-number control (Pogliano et al., 2002; Ebersbach and Gerdes, 2005). Stable maintenance of the high copy plasmid *ColE1* is dependent on a multimer resolution system consisting of host-encoded proteins acting at the plasmid carried *cer* site, which is absent in *ColE1*-based cloning vectors (Summers and Sherratt, 1984). Thus, these vectors are relatively unstable, being lost at frequencies of 10^{-2} -10^{-5} per cell per generation. The importance of the addition of the active partitioning system of F plasmid to *ColE1* plasmid stability has been reported (Yao et al., 2007). Therefore, stable maintenance of the *ColE1*-based pDaf derivative in *E. coli* was a consequence of the active partitioning system of pDaf.

The essential genes of pDaf plasmid were defined by in vivo experiments in *Desulfovibrio africanus*, *Pseudomonas aeruginosa* PA14, *Shewanella oneidensis* MR-1, and *Escherichia coli*. No phenotypic trait that might represent a selective advantage to the native host was detected. The name of *Desulfovibrio africanus* plasmid (pDaf) refers to the other *Desulfovibrio* species where it replicates rather than its native host. The

cryptic pDaf contains enough genetic information to be mobilized, to display a low copy number, and to be maintained stably in the bacterial hosts tested.

Advances in the understanding of *Desulfovibrio* genetics, as well as in the concomitant generation of new genetic tools, will make important contributions to studies of gene regulation. The results of this study provide potential avenues for genetic tool development. pDaf is small, is native to *Desulfovibrio* species, is mobilizable, and is stably maintained, making pDaf-based derivatives attractive as vectors for stable introduction of genes for mutant complementation or gene expression studies. pDaf-derived plasmid vectors could be especially useful, because the pBG1 *Desulfovibrio* plasmid and the few broad-host-based vectors currently in use have limited stability during growth under non-selective pressure.

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Appendix

Table A1. Plasmid Copy number calculation using the Relative Standard Curve Method (User Bulletin #2 ABI Prism 7700 SDS pp. 8).

	Af	Fd III
Slope	-3.320759	-3.333353
Intercept	22.176767	22.39545397

Condition	1/8 dil

Ct Af	Ct Fd III	Log input amount Af	Log input amount Fd III	ng DNA Af	ng DNA Fd III		Norm
17.80	20.58	1.318002	0.544616	20.79706	3.504418		
17.76	20.64	1.330047	0.526616	21.38196	3.362142		
17.74	20.53	1.33607	0.559616	21.68054	3.62757		
							Norm
			avg	21.28652	3.498043		6.085265
			SD	0.449406	0.132829	SD	0.264385

Af : plasmid target gene

Fd III: chromosomal reference gene

Condition: amount of unknown total DNA within the range tested (1/8 dilution = 30 ng)

Ct: threshold cycle number

Norm: normalized amount of plasmid target gene.

a. Calculate the **log input amount** (x) from the standard curve line equation **y = mx + b**.

Where, **y** = Ct value, **m** = slope of standard curve line, and **b** = y-intercept of standard curve line.

b. Calculate the **input amount** (ng DNA).

$10^{\wedge}$ [log input amount]

c. Divide the amount (ng DNA) of plasmid target gene by the amount of FdIII gene to determine the normalized amount of plasmid, which is the equivalent to plasmid copy number.

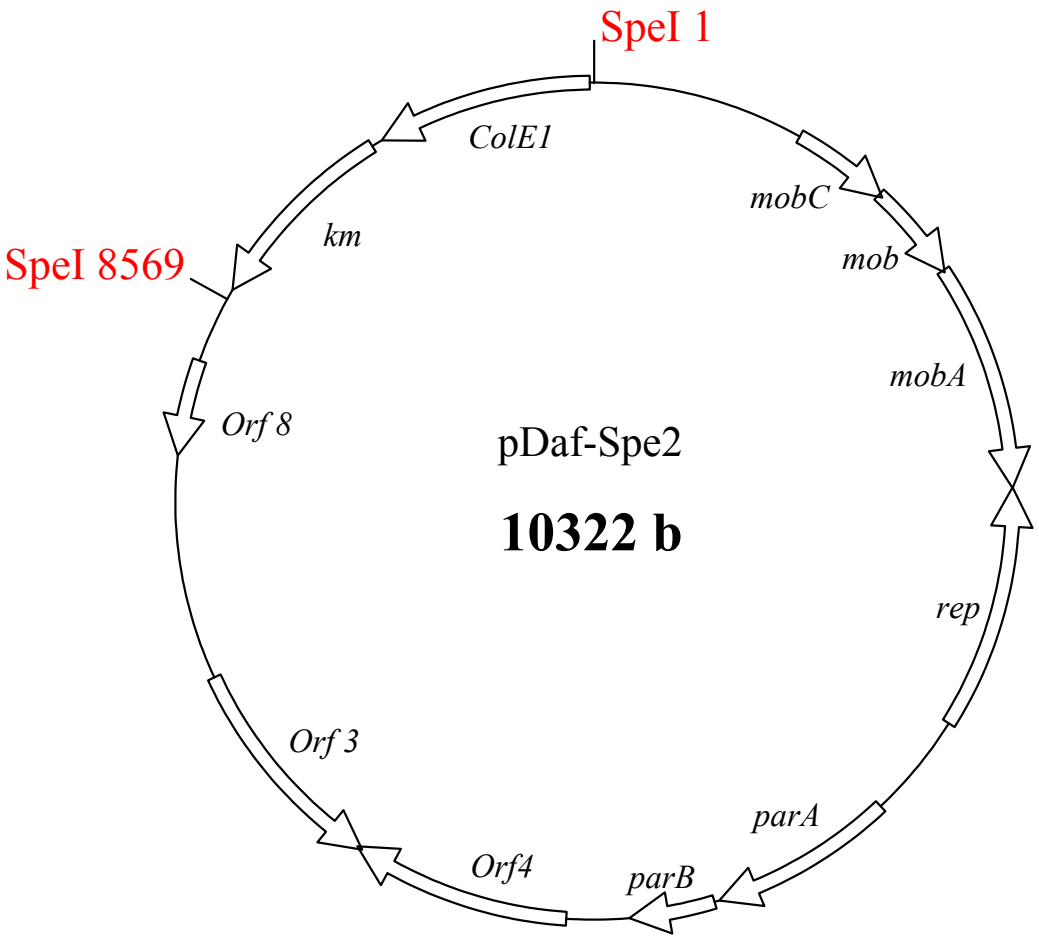

Fig.A1. *Desulfovibrio-ColE1* Shuttle vector with a selectable marker in a non-coding region.