Oral Microbiology Immunology 2005: 20: 274–281
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Oral Microbiology and Immunology

Influence of genetic background on transformation and expression of Green Fluorescent Protein in *Actinobacillus actinomycetemcomitans*

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Teughels W, Sliepen I, De Keersmaecker S, Quirynen M, Lippmann J, Pauwels M, Fives-Taylor P. Influence of genetic background on transformation and expression of Green Fluorescent Protein in Actinobacillus actinomycetemcomitans.
Oral Microbiol Immunol 2005: 20: 274–281. © Blackwell Munksgaard, 2005.

Background/aims: The development of an electro-transformation system and the construction of shuttle plasmids for *Actinobacillus actinomycetemcomitans* have enhanced the molecular analysis of virulence factors. However, inefficient transformation is frequently encountered. This study investigated the efficiency of electro-transformation and expression of Green Fluorescent Protein (GFP) in 12 different *A. actinomycetemcomitans* strains. The influence of the plasmid vector, serotype, and phenotype were the major factors taken into consideration.

Material and methods: Twelve serotyped *A. actinomycetemcomitans* strains were independently electro-transformed with two different *Escherichia coli–A. actinomycetemcomitans* shuttle plasmids (pVT1303 and pVT1304), both containing an identical *ltx-GFPmut2* gene construct but a different backbone (pDMG4 and pPK1, respectively). The transformation efficiency, transformation frequency, and electro-transformation survival rate were determined by culture techniques. GFP expression was observed at the colony level by fluorescence microscopy.

Results: All strains could be transformed with both plasmids. However, major differences were observed for the transformation efficiency, transformation frequency, and electro-transformation survival rate between strains. The data demonstrated that plasmid vector, serotype, and phenotype are key players for obtaining a successful transformation. An inverted relationship between the electro-transformation survival rate and tranformation frequency was also observed. GFP expression was also influenced by phenotype, serotype and plasmid vector.

Conclusions: The serotype of *A. actinomycetemcomitans* has an important influence on its survival after electro-transformation and on transformation frequency. The expression of GFP is strain and plasmid vector dependent.

Key words: *Actinobacillus actinomycetemcomitans*; electro-transformation; Green Fluorescent Protein; virulence

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Accepted for publication March 1, 2005

Actinobacillus actinomycetemcomitans is one of the primary pathogens in localized juvenile periodontitis and rapidly progressing periodontitis and is a key pathogen in chronic adult periodontitis (22, 32–34, 38, 41). Moreover, *A. actinomycetemcomitans* can also be associated with extraoral infections such as endocarditis and subcutaneous and brain abscesses (5, 25, 30, 42). This gram-negative, capnophilic pathogen produces a wide variety of virulence factors such as leukotoxin, immunosuppressive factors, lipopolysaccharides, and adhesins (7). Both bacterial and host factors play a role in the clinical outcome

of subgingival colonization by *A. actinomycetemcomitans*. The exact mechanisms of its pathogenesis are not fully understood due to the genetic diversity of the *A. actinomycetemcomitans* strains as reflected in interstrain differences in the expression of some virulence factors (6, 14, 16, 17, 20).

A. actinomycetemcomitans strains appear as two distinct colonial morphologies when cultured on solid media. Most clinical isolated strains have rough phenotypes characterized by a translucent, circular colony with irregular borders and a star-shaped or cross-cigar-shaped pattern in the center of the colony (15, 26, 27, 29). They grow in broth as granular, autoaggregated adherent cells that form pellets and leave a clear broth. Many of these rough type *A. actinomycetemcomitans* strains develop a smooth phenotype upon successive rounds of *in vitro* subculturing. Sometimes smooth phenotypes are isolated directly from the oral cavity. These smooth phenotype strains grow in broth as a homogeneous suspension with minimal adherent cells (11, 12, 15, 21).

A. actinomycetemcomitans interstrain genetic diversity is widely accepted; however, only a limited number of papers have taken this into consideration.

The development of an electro-transformation system and the construction of shuttle plasmids for *A. actinomycetemcomitans* (37) have enhanced the molecular analysis of virulence factors (4, 11, 18, 23, 24, 36, 37). Unfortunately, inefficient transformation is frequently encountered (9, 21, 37). Several factors that influence the efficiency of transformation, such as host modification, plasmid DNA concentration, bacterial cell density, pH, length of recovery time, freezing, and plasmid size have been identified (9, 37). Moreover, it has been observed that phenotypic variation enhances the electro-transformation efficiency of the strains, suggesting that rough isolates are more difficult to transform than smooth variants (9). As a consequence, rough *A. actinomycetemcomitans* have been refractory to genetic analysis (39) and virulence factors have been almost exclusively investigated in smooth strains. Few differences between rough and smooth variants have been described to date and, as such, the potential importance of these differences has been overlooked (43, 44).

It is conceivable that the composition of the bacterial membrane is an important factor for successful electro-transformation. The existence and importance of differences in bacterial cell membrane composition for *A. actinomycetemcomitans* strains is reflected in their serological classification and correlation between the *in situ* colonization with specific serotypes and the clinical situation (1). Very little is known about the impact of phenotype and serotype on the expression of virulence factors and the use of laboratory research techniques. It is very likely that these factors are influenced by serotype and/or phenotype.

Despite a reasonable knowledge of the genome of *A. actinomycetemcomitans* and of its physiology in terms of gene regulation and structural features, many cellular processes remain largely unknown (43). The Green Fluorescent Protein (GFP) technology derived from eukaryotic biology can contribute to the understanding of *A. actinomycetemcomitans* biology. GFP has become a popular reporter system for use in both prokaryotes and eukaryotes. In prokaryotes, GFP has been primarily used as a reporter for promoter activity and as a fluorescent label to monitor interaction with eukaryotic hosts (2). GFP has already been successfully expressed in *A. actinomycetemcomitans* SUNY465 under the control of the leukotoxin promoter of *A. actinomycetemcomitans* JP2 (19). This system allows noninvasive, real-time monitoring of *A. actinomycetemcomitans* adhesion to and interaction with epithelial cells. However, some assays, e.g. real-time monitoring of gene expression and protein localization, have not been utilized in the study of *A. actinomycetemcomitans*. A more generalized use of GFP in *A. actinomycetemcomitans* requires a better understanding of the possible genetic and culture conditions that affect its expression, particularly in view of its genetic diversity *A. actinomycetemcomitans*.

This study investigated the efficiency of electro-transformation and expression of GFP in 12 different *A. actinomycetemcomitans* strains. The influence of the plasmid vector, serotype, and phenotype were taken into consideration.

Material and methods

Bacterial strains and growth conditions

The 12 different *A. actinomycetemcomitans* strains (see Table 1) used in this study were previously described and serotyped (1, 28). They were grown in Trypticase soy broth (Difco Laboratories, Detroit, MI) supplemented with 0.6% (w/v) yeast extract, 0.04% (w/v) $NaHCO_3$, bacitracin (75 μg/ml), and vancomycin (5 μg/ml) (TSBYE). Spectinomycin (50 μg/ml) was used to select for transformed *A. actinomycetemcomitans* because the plasmid that codes for GFP also includes a spectinomycin resistance marker. For some experiments, strains were grown on nonselective blood-agar (Blood Agar Base II; Oxoid, Basingstoke, England), supplemented with hemin (5 mg/ml), menadione (1 mg/ml), 5% sterile horse blood, and 0.8% (w/v) Bacto Agar (Difco Laboratories). Unless otherwise indicated, all strains were grown at 37°C in an atmosphere containing 5% CO_2. *Escherichia coli* JM109 (transformed with pVT1303 or pVT1304) was grown aerobically at 37°C in Luria-Bertani (LB) (Difco Laboratories) supplemented with spectinomycin (50 μg/ml). When solid media were needed, 1.5% Agar (Difco Laboratories) was added.

Table 1. Overview of the *A. actinomycetemcomitans* strains used in this study. The strains were a kind gift of S. Asikainen who serotyped them. Classification was based on serotype and colonial morphology

Serotype	Smooth strain	Rough strain
A	ATCC 29523	4418
B	ATCC 43718	1398
	JP2	1029
C	ATCC 33384	
D	IDH 781	685
E	IDH 1705	2751
X		1016

Plasmids

pVT1303 (19) and pVT1304 (this work) were used as *E. coli–A. actinomycetemcomitans* shuttle vectors for expression of GFP in *A. actinomycetemcomitans*. In brief, pVT1303 was constructed using plasmid vector pDMG4 with a spectinomycin selective marker. A 1.2 kb construct containing the leukotoxin promoter (*ltxP*) from *A. actinomycetemcomitans* JP2 (0.5 kb) and the *GFPmut2* gene (0.7 kb) was subcloned into pDMG4 (3.4 kb) (11) at the *Bam*HI and *Sal*I sites and electro-transformed into *E. coli* JM109 (VT1303) (19). To create pVT1304, the same DNA fragment comprising *ltxP* and *GFPmut2* was subcloned into pPK1 (3.4 kb) (27) also at the *Bam*HI and *Sal*I sites. This construct was electrotransformed into *E. coli* JM109 to generate VT1304. Both plasmids have similar copy numbers (10). Fluorescence was determined using a hand-held fluorescent light with a wavelength of 488 nm. The plasmid from a fluorescent colony was analysed by restriction mapping and the construct was confirmed by cycle sequencing (19).

Electro-transformation

Prior to electro-transformation of *A. actinomycetemcomitans,* pVT1303 and pVT1304 were purified from an overnight culture of *E. coli* VT1303 and VT1304 using spin columns according to the manufacturer's instructions (Qiagen Plasmid Mini Kit; Qiagen Inc., Valencia, CA) and diluted to a concentration of 1 μg/ml plasmid DNA (A260/A280: 1.79) (spectrophotometer (Bio-Rad, Hercules, CA). Electro-transformation was carried out as described by Sreenivasan et al. (1991) with the following parameters (the bacterial strains were grown to an early logarithmic phase). After harvesting and washing in ice-cold electroporation buffer (EPB) at pH 5.5, bacteria were resuspended in EPB to obtain a final optical density (OD) of 6.0 at 600 nm. To determine the concentration of viable bacteria (C^i), serial dilutions were prepared after thorough pipetting and vortexing of the initial suspensions. The dilutions were plated onto blood agar plates by means of a spiral plater (Spiral Systems Inc., Cincinnati, OH). After electro-transformation (Easyject, Equibio, Ashford, UK), bacteria were immediately incubated in TSBYE without antibiotics for a 3-h recovery period. Afterwards, dilutions of transformed cultures were plated on TSBYE plates containing vancomycin, bacitracin and spectinomycin (C^t) and on nonselective blood agar plates (C^r) by means of a spiral plater. Successful transformants and nonselective grown controls were evaluated after 24 h by phase-contrast/fluorescence microscopy (Leica DMLS, Leica, Brussels, Belgium), a catalase test, and enzymatic tests (Api Zym, Bio Mérieux, Marcy-l'Etoile, France). The enzymatic tests checked for hydrolyzation of 2-naphthyl-phosphate (present), 2-naphthyl-butyrate (not present), 2-naphthyl-caprylate (not present), 2-naphthyl-myristate (not present), L-leucyl-2-naphthylamide (present), L-valyl-2-naphthylamide (present), L-cystyl-2-naphthylamide (not present), N-benzoyl-DL-arginine-2-naphthylamide (not present), N-glutaryl-phenylalanine-2-naphthylamide (not present), naphtol-AS-BI-phosphate (not present), 2-Br-2-naphthyl-αD-galactopyranoside (not present), 2-naphthyl-βD-galactopyranoside (not present), naphtol-AS-BI-βD-glucuronide (present), 2-naphthyl-αD-glucopyranoside (not present), 6-Br-2-naphthyl-βD-glucopyranoside (not present), 1-naphthyl-N-acetyl-βD-glucosaminide (not present), 6-Br-2-naphthyl-βD-mannopyranoside (not present) and 2-naphthyl-αL-fucopyranoside (not present). Transformation frequencies, efficiencies, and electro-transformation survival rates were determined as an average of at least three experiments, as elaborated below.

Image acquisition

Images were taken from 20 colonies that were grown for 24 h using a phase-contrast/fluorescence microscopy (Leica DMLS) equipped with a conventional camera. The shutter time of the camera was controlled by a computer and depended on the fluorescence intensity of the colony. Highly fluorescent colonies induced shorter shutter times (darker environment around the colony) and low fluorescent colonies induced longer shutter times (green-reddish environment around the colony).

Electro-transformation survival rate, transformation frequency and efficiency

The electro-transformation survival rate (ESR) was determined as the proportion of viable bacteria after the recovery period by comparing the concentration of viable bacteria before electro-transformation (C^i) with the concentration of viable bacteria after the recovery period (C^r): $ESR = (C^r/C^i) \times 100$.

Transformation efficiency was calculated as the number of transformants per microgram of DNA.

Transformation frequency (TF) was calculated in two different ways. TF^i was calculated as the percentage of transformants per initial colony forming unit (CFU) by comparing the concentration of viable bacteria before electro-transformation (C^i) to the concentration of transformed bacteria after the electro-transformation (C^t): $TF^i = (C^t/C^i) \times 100$.

TF^r was calculated as the proportion of transformants per CFU after the recovery period by comparing the concentration of viable bacteria after the recovery period (C^r) to the concentration of transformed bacteria after the electro-transformation (C^t): $TF^r = (C^t/C^r) \times 100$.

Statistical analysis

An ANOVA was carried out for electro-transformation survival rate, transformation efficiency, TF^i and TF^r with morphology, serotype and plasmid as main factors, including their interaction effect. When the interaction effect was significant, multiple comparisons were calculated for one factor per level of the other factor. A correction for simultaneous hypothesis testing was applied according to the Tukey-Kramer method.

Results

Electro-transformation and expression of GFP by different *A. actinomycetemcomitans* strains

None of the *A. actinomycetemcomitans* strains used in this study was spectinomycin resistant prior to electro-transformation as determined by the absence of growth when the strains were subcultured on TSBYE plates supplemented with spectinomycin. Each of the *A. actinomycetemcomitans* strains was electroporated three times with the same plasmid. Transformations with pVT1303 and pVT1304 were performed on the same batch of competent cells to avoid differences caused by environmental variation. The strains to be transformed on the same day were chosen in a random order. Although all the strains could be transformed after three attempts, not all experiments were equally successful. When strains IDH781 and 1016 were transformed with pVT1303, and strains JP2, 1398, 1029, 685 and 1016 were transformed with pVT1304, one of the three attempts failed to produce selectable transformants (defined as colonies that were spectinomycin resistant). Only strain 4418 could not be transformed with pVT1304 when the electro-transformation protocol was followed. To enhance the chance for transformation for this strain with this plasmid, a minor adjustment to the protocol had to be made. The bacterial concentration prior to electro-transformation was increased to an optical density of 8 at 600 nm for strain 4418. This adjustment allowed successful transformation of the strain with pVT1304.

Major interstrain differences in the number of successful transformants were observable, although the bacterial concentrations prior to electro-transformation were spectrophotometrically identical ($OD_{600} = 6$ and 8 for strain 4418 when transformed with pVT1304).

All transformed strains exhibited green fluorescence when observed by fluorescence microscopy, indicating that spectinomycin resistance was not due to mutation. Intra-strain differences in fluorescence were minimal. However, major interstrain differences in fluorescence intensity and pattern could be observed (Fig. 1). Smooth strains exhibited a more radial, star-shaped fluorescence pattern, whereas rough strains showed a more peripheral pattern. Even the typical morphologic central star was sometimes

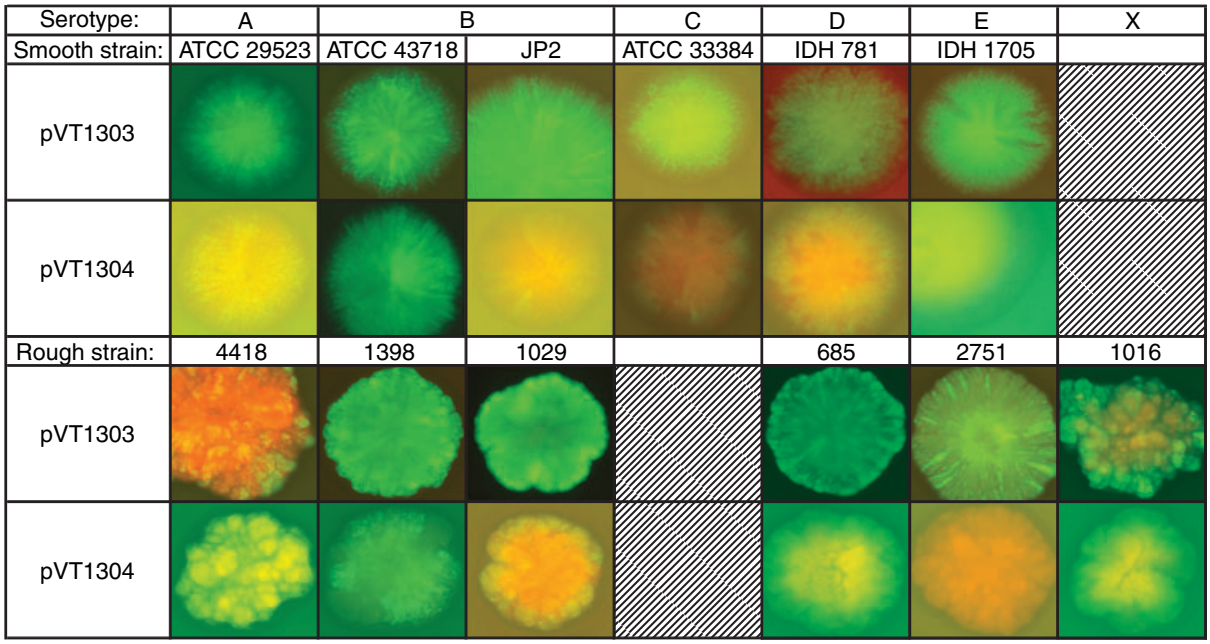

Fig. 1. Illustration of the wide variety in GFP expression in 48-h-old *A. actinomycetemcomitans* colonies.

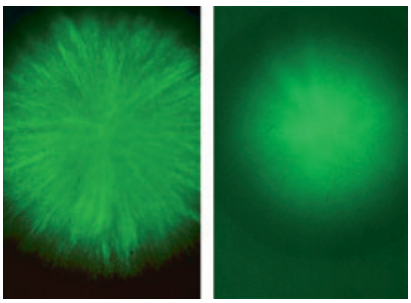

Fig. 2. Representative image of an *A. actinomycetemcomitans* strain JP2 colony with fading fluorescence (GFP) intensity over 2 days. Panel a: a colony 2 days after electro-transformation. Panel b: the same colony 4 days after transformation.

detectable by fluorescence. Visually, strains transformed with pVT1303 exhibited a higher fluorescence intensity than the same strains transformed with pVT1304, although this was not quantified. In the latter strains, the GFP could be observed as diffuse spots within the colony on a reddish background. The fluorescence intensity had the tendency to fade in a few days (Fig. 2). *A. actinomycetemcomitans* strains not transformed but grown on TSBYE plates without spectinomycin, as a control, did not exhibit any fluorescence.

No alterations in morphologic appearance, biochemical properties or catalase activity due to the transformation or GFP expression could be observed (data not shown).

Transformation efficiency of *A. actinomycetemcomitans*

In order to understand why some of the strains (IDH781, JP2, 1398, 1029, 685, 1016, 4418) could not be transformed consistently, we assayed the number of bacteria surviving after the recovery period (C^r). No colonies could be isolated in these strains after electro-transformation, even on nonselective media, suggesting that the strains did not survive the electrical pulse (data not shown).

As shown in Table 2, the average of the transformation efficiency was highly strain and plasmid dependent. No relationship could be found between the transformation efficiency and the serotype or colonial morphology of the strain, although we could detect a higher transformation efficiency when pVT1304 was used. The electro-transformation of *A. actinomycetemcomitans* 4418 with pVT1304 was not taken into consideration because no transformants could be detected when electro-transformation was carried out at an optical density of 6.

Transformation frequency of *A. actinomycetemcomitans*

The culture data collected from the samples prior to electro-transformation indicated a high interstrain variability in the number of CFU, although the bacterial concentrations were set spectrophotometrically at an optical density of 6. In brief, for smooth *A. actinomycetemcomitans* strains a range of 2 log scales could be observed for the same optical density. For rough strains, a range of 1.5 log scales was observed. In general, slightly more of the smooth strains than of the rough strains were cultured, but there was no relationship between serotype and culture data after adjustment for optical density (data not shown). To avoid a possible concentration bias, transformation frequencies (TF^i) were determined (Table 3). For pVT1303, no relationship between TF^i and the serotype or colonial morphology of the strain was detected. No correlation between concentration determined by culture prior to electro-transformation and TF^i could be detected. The same was true for pVT1304, although there was a tendency for rough isolates to transform at a higher frequency than smooth strains. In addition, transformations with pVT1304 always resulted in higher transformation frequencies than tranformations with pVT1303.

Electro-transformation survival rate of *A. actinomycetemcomitans*

The electro-transformation survival rate was related to *A. actinomycetemcomitans* serotype (Table 4). Between serotypes, there was a significantly higher survival rate for smooth strains than for rough strains. No relationship could be seen between initial concentration (culture data) and the electro-transformation survival

Table 2. Efficiency of transformation

TE for pVT1303					TE for pVT1304				
Mean	SD	Strain	S	M	Mean	SD	Strain	S	M
3.33×10^{1}	1.44×10^{1}	ATCC 29523	A	S	3.30×10^{3}	1.43×10^{3}	ATCC 29523	A	S
3.97×10^{1}	1.53×10^{1}	685	D	R	2.13×10^{4}	1.03×10^{4}	IDH 781	D	S
4.29×10^{1}	2.02×10^{1}	IDH 781	D	S	2.57×10^{4}	7.75×10^{4}	ATCC 33384	C	S
1.71×10^{2}	4.95×10^{2}	2751	E	R	2.60×10^{4}	1.70×10^{4}	685	D	R
2.10×10^{2}	1.15×10^{2}	ATCC 33384	C	S	5.46×10^{4}	1.76×10^{4}	ATCC 43718	B	S
2.89×10^{2}	1.17×10^{2}	1029	B	R	1.71×10^{5}	3.95×10^{4}	2751	E	R
3.33×10^{2}	1.67×10^{2}	1398	B	R	2.17×10^{5}	1.05×10^{5}	IDH 1705	E	S
3.49×10^{2}	1.58×10^{2}	ATCC 43718	B	S	2.69×10^{5}	2.67×10^{5}	1398	B	R
5.81×10^{2}	1.57×10^{2}	4418	A	R	3.19×10^{5}	4.38×10^{4}	1029	B	R
2.51×10^{3}	1.01×10^{3}	1016	X	R	6.00×10^{5}	5.66×10^{4}	1016	X	R
1.09×10^{4}	1.90×10^{3}	IDH 1705	E	S	2.30×10^{6}	4.24×10^{5}	JP2	B	S
2.73×10^{4}	7.57×10^{3}	JP2	B	S					

Ranking of efficiencies of transformation (mean for three independent experiments and standard deviation (SD)) for each strain from lowest to highest. TE = efficiency of transformation, S = serotype (A, B, C, D, E, X (nontypable)), M = colonial morphology (R = rough, S = smooth).

Table 3. Frequency of transformation (TF^{i})

Tf^{i} for pVT1303 (%)					$Tf^{i\ fo}$for pVT1304 (%)				
Mean	SD	Strain	S	M	Mean	SD	Strain	S	M
4.76×10^{-6}	1.29×10^{-6}	418	A	R	8.98×10^{-4}	4.35×10^{-4}	IDH 1705	E	S
4.87×10^{-6}	2.30×10^{-6}	IDH 781	D	S	1.50×10^{-3}	6.48×10^{-3}	ATCC 29523	A	S
1.05×10^{-5}	3.02×10^{-6}	2751	E	R	2.11×10^{-3}	6.35×10^{-3}	ATCC 33384	C	S
1.52×10^{-5}	6.56×10^{-6}	ATCC 29523	A	S	2.42×10^{-3}	1.17×10^{-3}	IDH 781	D	S
1.72×10^{-5}	9.46×10^{-6}	ATCC 33384	C	S	7.01×10^{-3}	2.26×10^{-3}	ATCC 43718	B	S
1.85×10^{-5}	9.30×10^{-6}	1398	B	R	8.05×10^{-3}	5.69×10^{-3}	4418	A	R
2.89×10^{-5}	1.17×10^{-5}	1029	B	R	1.04×10^{-2}	2.41×10^{-3}	751	E	R
3.42×10^{-5}	1.32×10^{-5}	685	D	R	1.06×10^{-2}	1.95×10^{-3}	JP2	B	S
4.48×10^{-5}	2.02×10^{-5}	ATCC 43718	B	S	1.50×10^{-2}	1.48×10^{-2}	1398	B	R
4.51×10^{-5}	7.86×10^{-6}	IDH 1705	E	S	2.24×10^{-2}	1.46×10^{-2}	685	D	R
1.25×10^{-4}	3.47×10^{-5}	JP2	B	S	3.19×10^{-2}	4.38×10^{-3}	1029	B	R
4.19×10^{-3}	1.68×10^{-3}	1016	X	R	1.00×10^{0}	9.43×10^{-2}	1016	X	R

Means of three independent experiments and standard deviations of the frequencies of transformation calculated upon initial concentration (TF^{i}) for each strain. TF^{i} were ranked from low to high.
TF^{i} = frequencies of transformation calculated upon initial concentration, S = serotype (A, B, C, D, E, X (nontypable)), M = colonial morphology (R = rough, S = smooth).

rate. Nor could any statistically significant differences be observed between the electro-transformation survival rate when using pVT1303 or pVT1304. When frequency of transformation (TF^{r}) was calculated based upon the concentration of surviving bacteria, a high degree of correlation was observed between TF^{r} and plasmid, serotype, colonial morphology, and electro-transformation survival rate. Only *A. actinomycetemcomitans* strain 1016, the serotypically untypable strain, was not correlated with the latter variable. The differences between rough and smooth strains of the same serotype were statistically significant for both plasmids ($P < 0.05$). Rough strains exhibited a higher TF^{r} which could be as high as 100-fold. Only the difference between *A. actinomycetemcomitans* strains 2751 and IDH1705 (both E serotypes) did not reach this level of statistical significance. Electro-transformation with pVT1304 always produced a significantly ($P < 0.001$) higher number of transformants in comparison with pVT1303 when serotype and colonial morphology were taken into consideration. To compare the influence of the serotype, TF^{r} of both rough B serotypes and both smooth B serotypes were averaged. In general, X serotypes yielded the highest TF^{r}, followed by B and E serotypes. Differences between A, C, and D serotypes were minimal. Many of the differences were statistically significant, although the differences were sometimes only a tendency. An inverted relationship between electro-transformation survival rate and TF^{r} was observed. Only *A. actinomycetemcomitans* strain 1016 exhibited a high electro-transformation survival rate and a high TF^{r}.

Discussion

Electro-transformation has become an important biotechnological tool for investigation of bacterial virulence factors through their manipulation at the molecular level. Although the research described in this paper was primarily intended to express GFP in different strains of *A. actinomycetemcomitans*, additional measurements of efficiency of transformation, frequency of transformation and electro-transformation survival rate provided new insights into the use of electro-transformation as a molecular biological tool for *A. actinomycetemcomitans.*

Twelve strains of *A. actinomycetemcomitans* could be transformed by the above described technique with a GFP encoding plasmid. All of the strains produced fluorescent proteins, although there were striking differences between fluorescence intensity at the colony level. This suggests that there are interstrain differences in GFP expression for *A. actinomycetemcomitans*. These differences could result from interstrain variations in specific transcription factors. Our data are consistent with the observations of Kolodrubetz and coworkers, who demonstrated that *trans*-factors play an important role in the expression of virulence factors for *A. actinomycetem-*

Table 4. Electro-transformation survival rate and frequency of transformation (TF^r)

	Smooth					Rough				
Serotype	ESR (%) Mean	SD	TF^r pVT1303 (%) Mean	SD	TF^r pVT1304 (%) Mean	SD	ESR (%) Mean	SD	TF^r pVT1303 (%) Mean	SD	TF^r pVT1304 (%) Mean	SD
X							10.08	4.32	5.92×10^{-2}	4.13×10^{-3}	7.78	1.37
B	5.24	0.49	1.52×10^{-3}	7.87×10^{-4}	1.68×10^{-1}	6.00×10^{-2}	0.56	0.29	4.64×10^{-3}	9.34×10^{-4}	3.72	2.10
E	6.26	0.92	7.41×10^{-4}	7.30×10^{-5}	1.35×10^{-2}	5.20×10^{-3}	1.08	0.30	9.38×10^{-4}	2.86×10^{-4}	1.05	1.07×10^{-1}
A	26.53	6.71	6.29×10^{-5}	2.57×10^{-5}	5.05×10^{-3}	9.79×10^{-4}	1.25	0.82	6.41×10^{-4}	7.96×10^{-5}	4.76×10^{-1}	1.90×10^{-1}
C	47.16	6.69	4.04×10^{-5}	2.21×10^{-5}	3.98×10^{-3}	8.75×10^{-4}						
D	50.45	12.06	9.21×10^{-6}	1.41×10^{-6}	4.69×10^{-3}	1.40×10^{-3}	5.52	1.23	5.88×10^{-4}	2.84×10^{-4}	4.59×10^{-1}	2.18×10^{-1}

Means and standard deviations of the frequencies of transformation calculated upon concentration after recovery period (TF^r: three independent experiments) and of the electro-transformation survival rates (ESR: six independent experiments). For the B serotype strains, the results were averaged. The serotypes were ranked based on TF^r for pVT1303 from high to low.

comitans (17). On the other hand, GFP fluorescence is dependent on certain parameters including oxygen availability and pH levels (13). As GFP requires oxygen for chromophore formation (40), it is conceivable that differences in oxygen diffusibility may be related to the interstain variation in GFP expression. It should be pointed out that GFP requires oxygen in stoichiometric amounts rather than catalytically, and efficient fluorophore formation occurs at low levels of oxygen (35). Additionally, Hansen and coworkers reported that for *Lactococcus lactis*, acidification of the growth medium rather than oxygen limitation is the primary cause of fluorescence reduction (13). To cope with an extracellular acidic environment, e.g. that caused by acid production, bacteria adapt their intracellular pH accordingly to avoid loss of energy caused by a too high proton gradient (31). In response, they drop their intracellular pH, which could reduce GFP fluorescence. In the case of *Aequorea* GFP, for a pH decrease from 6.5 to 4.5, this loss is relatively modest (3). This aspect of intracellular pH variations in *A. actinomycetemcomitans* strains still needs further research.

When looking at the GFP fluorescence patterns of transformed colonies, two different patterns could be observed. The radial, star-shaped pattern was seen in smooth phenotypes; a peripheral pattern could only be seen in rough phenotypes. Some rough isolates even showed the phenotypical 'cross-cigars' pattern when observed by fluorescence microscopy. These differences might be a consequence of differences in colony growth between rough phenotypes and smooth phenotypes. Differences in the viability of bacteria within the colony, thickness of a colony, or oxygen gradient within a colony might also contribute to these patterns.

When the intrastrain fluorescence intensities after transformation with pVT1303 were compared to pVT1304, striking differences could be observed even though the promoter-gene construct was identical in both shuttle plasmids. These differences can not be attributed to differences in plasmid copy number (10). However, we cannot rule out differences in expression of the GFP gene by a different regulation of the leukotoxin promoter in the different strains (17). This suggests that plasmid dependent *cis*-elements other than the promoter sequence play a role in the expression of GFP. It is therefore necessary to carefully choose the bacterial host and plasmid when wishing to use GFP as a marker.

The ability of bacteria to take-up plasmid DNA by electro-transformation is almost always expressed as the transformation efficiency, referring to the number of transformants per microgram of plasmid DNA. As was shown in Table 2, no clear correlations could be shown between serotype or phenotype and the number of transformants by using this technique. Although this is a generally accepted and widely used technique to compare the uptake of different plasmids by the same bacterial strain, there is a fundamental drawback to its use for making comparisons between different strains. It is biased by the effect of the electrical pulse on the viability of the bacteria.

To our knowledge, this aspect has never previously been reported for *A. actinomycetemcomitans*. In the present study, when the electro-transformation survival rate was defined and calculated for these *A. actinomycetemcomitans* strains, important differences could be seen between the strains (Table 4). Not all strains recover from the electrical pulse as easily. Rough strains seem to be more sensitive than smooth strains. It is known that the electrical pulse provokes a rupture of the bacterial cell membrane in order to promote the uptake of new molecules. One can hypothesize that the ability of a bacterium to reseal the formed pores or to recover from the imposed stress is reflected in its survival. This will influence the maximum number of transformants that can be achieved afterwards. Such data might provide us with an answer as to why it is more difficult to transform rough strains by electro-transformation (9).

To correct for these losses in viability, we defined a new method to calculate and compare transformation in different strains. Transformation frequency is a convenient way to compensate for differences in the initial number of viable bacteria prior to electro-transformation (TF^i) but does not correct for loss in viability due to the electrical pulse. Therefore we redefined transformation frequency (TF^r) based upon the number of surviving bacteria after the electrical pulse. The results shown in Table 4 clearly indicate that serotype as well as the colonial morphology influences the outcome of electro-transformation. For the strains used in this study, one can conclude that nontypable strains (X serotypes) are more frequently transformed than B and E, and the cluster of A, C, and D serotypes. This association can not be seen when using the conventional method for calculating transformation frequency.

The serologic specificity is defined by the polysaccharides on the surface of *A. actinomycetemcomitans*. It is conceivable that the surface composition of a bacterium is an important factor for a successful electro-transformation. To account for these surface differences, this study used different serotypes of *A. actinomycetemcomitans*. This serotype specificity also seems to be important for natural transformation, a process by which *A. actinomycetemcomitans* can take up extracellular DNA and incorporate it in its genome. As recently shown by Fujise and coworkers, the natural competence of *A. actinomycetemcomitans* can be linked to serotype (8). Serotypes A, D, and E seem, in particular, to harbor competent *A. actinomycetemcomitans* clones. Although the end result might be the same both for electro-transformation and natural transformation, e.g. the uptake of extracellular DNA, both processes are completely different. This makes it is very difficult to compare them. For natural competence, the serotype specificity is related to active DNA uptake and homologues recombination, whereas for electro-transformation, the DNA uptake is passively provoked by the electrical pulse, so that serotype specificity is related to bacterial fitness where the transformation is determined by the delicate balance between rupturing and resealing of the bacterial surface.

The observation of the inverted relationship between TF^r and electro-transformation survival rate may support the hypothesis that during the electrical pulse a gap is created in the bacterial cell membrane. The bigger this pore is, the greater the possibility for a plasmid to enter the bacterium but also the greater the chance that the bacterium will not be able to close it and will die. Therefore, in order to achieve high numbers of transformants by electro-transformation, the strain selection is an important factor.

This study has created new insights and opportunities to use electro-transformation for transformation of *A. actinomycetemcomitans.* Our results show that the selection of the strain is an important step in obtaining sufficient efficiency/frequency of transformation and expression of virulence factors (because the *GFP* was under control of an *ltx* promoter, one of the best known virulence factors of *A. actinomycetemcomitans*). They also show that rough strains, formerly believed to be more difficult to transform, are in fact easier to transform and that earlier data are a result of not accounting for the differences in recovery after electro-transformation.

Acknowledgments

Supported by OT 03/52 research grant of the Catholic University Leuven, grant G.0240.04 of the National Fund for Scientific Research Flanders, Dentaid, and GABA.

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