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Gene expression and phenotypic traits of *Aggregatibacter actinomycetemcomitans* in response to environmental changes

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Background and Objective: Periodontopathogens experience several challenges in the oral cavity that may influence their transcription profile and resulting phenotype. This study evaluated the effect of environmental changes on phenotype and gene expression in a serotype b *Aggregatibacter actinomycetemcomitans* isolate.

Material and Methods: Cultures in early exponential phase and at the start of stationary growth phase in microaerophilic and anaerobic atmospheres were evaluated. Cell hydrophobic properties were measured by adherence to n-hexadecane; in addition, adhesion to, and the ability to invade, KB cells was evaluated. Relative transcription of 12 virulence-associated genes was determined by real-time reverse transcritption quantitative PCR.

Results: The culture conditions tested in this study were found to influence the phenotypic and genotypic traits of *A. actinomycetemcomitans*. Cells cultured in microaerophilic conditions were the most hydrophobic, reached the highest adhesion efficiency and showed up-regulation of *omp100* (which encodes an adhesion) and *pga* (related to polysaccharide synthesis). Cells grown anaerobically were more invasive to epithelial cells and showed up-regulation of genes involved in host-cell invasion or apoptosis induction (such as *apaH, omp29, cagE* and *cdtB*) and in adhesion to extracellular matrix protein (*emaA*).

Conclusion: Environmental conditions of different oral habitats may influence the expression of factors involved in the binding of *A. actinomycetemcomitans* to host tissues and the damage resulting thereby, and thus should be considered in *in-vitro* studies assessing its pathogenic potential.

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Periodontitis is an inflammatory disease initiated and maintained by a consortium of microorganisms, including periodontal pathogens, which colonize subgingival sites. Such organisms express virulence factors that enable them to colonize and persist in these habitats, as well as act in cells and host tissues, thus activating the immune system.

A periodontopathogenic organism faces environmental variations in

oxygen concentration, nutrient availability, toxic catabolytes and cell concentration in the oral cavity. Sensing these conditions may lead the organism to express factors needed for survival in different habitats, such as mucosa, supragingival biofilm and periodontal pocket. Recognized periodontopathogenic organisms, including serotype b *Aggregatibacter actinomycetemcomitans*, which is associated with aggressive periodontal disease, may also be present in low levels as resident members of the oral microbiota in healthy subjects (1). Thus, both spatial and temporal variations may affect the expression of virulence factors, leading to an organism, such as *A. actinomycetemcomitans*, to change its phenotype from commensal to pathogen, depending on cues obtained from the host and from other members of the oral microbiota (2,3).

A. actinomycetemcomitans is a periodontopathogen associated with both aggressive periodontitis and extra-oral infections, including brain and lung abscesses, osteomyelitis, endocarditis and other cardiovascular pathologies (4,5). Little is known about the regulation of virulence-related genes in this bacterium *in vivo* as the expression of virulence factors has mostly been evaluated under standard *in vitro* conditions (6–10). Some data indicate that surface characteristics and efficiency of adhesion to epithelial cells may change according to atmosphere and length of incubation (6) and that expression of genes related to biofilm formation is regulated by nutritional and atmospheric environmental conditions (11). Also, the morphological change of *A. actinomycetemcomitans* during *in-vitro* subculture, from rough to smooth colony phenotype, is one of the reasons that makes it difficult to elucidate its virulence (12).

Given the need to understand gene regulation in *A. actinomycetemcomitans*, this study evaluated the effects of growth phase and incubation atmosphere on phenotypic traits such as cell-surface properties, adhesion and invasion ability, as well as on the transcription of 12 virulence-associated genes in a serotype b *A. actinomycetemcomitans* isolate.

Material and methods

Bacterial strain and culture conditions

A. actinomycetemcomitans strain VT1169 was used in this study. This is a spontaneous mutant nalidixic acid/rifampin-resistant strain that was derived from serotype b SUNY465 after successive plating on trypticase soy broth + 0.6% yeast extract (TSB-YE) agar plates containing rifampin and increasing concentrations of nalidixic acid (13). *A. actinomycetemcomitans* strain VT1169 was grown at 37°C in TSB-YE in microaerophilic (10% CO_2 in air; Shel Lab, Cornelius, OR, USA) or anaerobic (85% N_2, 5% H_2 and 10% CO_2) conditions generated in an anaerobic chamber (Plaslabs, Lansing, MI, USA). L-cysteine (Inlab, São Paulo, Brazil) (0.07%) was added to the medium in anaerobic cultures. Cells were cultured and growth phases were determined spectrophotometrically. Cells reached early exponential and early stationary growth phases after 6 and 9 h, respectively, under microaerophilic incubation, and after 9 and 13 h, respectively, under anaerobic incubation.

The phenotypic traits and mRNA transcript levels of 12 virulence-associated genes [measured using real-time RT-PCR (RT-qPCR)] were determined for the following groups of cells: incubation under microaerophilic conditions at the early exponential growth phase (group A) and the early stationary phase (group B); and incubation under anaerobic conditions at the early exponential growth phase (group C) and the early stationary phase (group D). All assays were performed in triplicate, in two independent experiments.

Phenotypic characterization

Adherence to n-hexadecane— The ability to adhere to n-hexadecane was used to determine the relative surface hydrophobicity of *A. actinomycetemcomitans*, as previously described (14), with some modifications. Bacterial cells were harvested (3000 ***g***/20 min/4°C), washed with PUM buffer (16.94 g/L of K_2HPO_4, 7.26 g/L of KH_2PO_4, 1.8 g/L of urea and 0.2 g/L of $Mg_2SO_4.7H_2O$, pH 7.1) and resuspended to an optical density at 550 nm (OD_{550}) of 0.85Å (approximately 1×10^9 colony-forming units/mL). Bacterial suspensions (3 mL) were transferred to tubes (10 × 100mm, gass tubes, Laborglass - São Paulo, Brazil) and the OD_{550} was measured again. An aliquot of 400 μL of n-hexadecane (Sigma-Aldrich, St. Louis, MO, USA) was added to the 3-mL bacterial suspensions , which were then equilibrated at 30°C for 10 min and then mixed. After mixing, the bacterial suspensions were allowed to stand for 30 min and the OD_{550} of the lower aqueous phase was measured. The adherence fraction was expressed as the percentage of bacteria remaining in the aqueous phase in relation to the initial value.

Adhesion to KB cells— Adhesion to KB cells, generously provided by Dr Paula Fives-Taylor (University of Vermont), was performed as described by Meyer and Fives-Taylor (6), with some modifications. Bacterial suspensions (an OD_{500} of approximately 0.2 Å) were added, at a multiplicity of infection of approximately 1 : 100, to monolayers of KB cells (2×10^5 cells/well) in RPMI-1640 (Sigma-Aldrich) together with 5% fetal bovine serum (Cultilab Ltda, São Paulo, Brazil). After 2 h of incubation (at 37°C in an atmosphere of 10% CO_2), the cells were lysed with 100 μL of Triton X-100 (Union Carbide Co., Texas City, TX, USA) and 1.9 mL of phosphate-buffered saline was added. Volumes of 0.1 mL of cell suspension were inoculated onto the surface of TSA-YE agar plates, which were then incubated overnight at 37°C in a 10% CO2 atmosphere. After incubation, the number of viable bacterial cells was determined by colony-forming unit counting. Adhesion efficiency was calculated as the percentage of adherent cells/well.

Invasion of KB cells— Invasion of KB cells was performed as described by Meyer *et al.* (7) with some modifications. Bacterial suspensions were added

to monolayers of semiconfluent KB cells (1×10^5 cells/well) at a multiplicity of infection of approximately 1 : 1000. After centrifugation (900 ***g***/24°C/10 min), the co-culture was incubated for 2 h at 37°C in a 10% CO_2 atmosphere. Extracellular bacteria were killed by incubation with gentamycin sulfate for 1 h, and cells were lysed with 100 μL of Triton X-100. The number of viable bacterial cells was determined and invasion efficiency was calculated as the percentage of internalized cells/well.

Comparison between data on phenotypic traits was performed using ANOVA, and differences were considered significant at $p < 0.01$.

Gene transcription

Bacterial suspensions were adjusted to an OD_{500} of approximately 0.4 Å. Total RNA was obtained using Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. After chloroform extraction, RNA was precipitated with isopropanol and washed with 70% ethanol. Contaminating genomic DNA was removed by digestion with DNase (Invitrogen Life Technologies). The quantity of RNA was determined by spectrophotometry (Nanodrop ND-1000; Thermo-Fisher Scientific Inc., Wilmington, DE, USA). First-strand synthesis was performed with 1 μg of RNA, using the Super Script III First Strand Synthesis System for RT-PCR (Invitrogen Life Technologies) according to the manufacturer's instructions. Real-time PCR was used to screen the transcription of 13 genes, including the internal control gene *gapdH* (*orf*1383) (15). The primers used are described in Table 1. The primers listed as 'this study' were constructed based on the sequence of *A. actinomycetemcomitans* (http://www.genome.ou.edu/act.html). The specificity of each primer was verified by amplification using DNA from *A. actinomycetemcomitans* HK 1651 as template. RT-qPCR was performed with cDNA, SYBR Green (QuantiMix EASY SYG Kit; Biotools Inc., Madrid, Spain), primers and magnesium in a final concentration of 3–4.5 mM in 96-well plates (Axygen Scientific Inc., Edison, NJ, USA) in a thermocycler linked to software (IQ-5 Real Time PCR Detection System, Bio-Rad, Hercules, CA, USA), at 48 cycles of: 95°C for 10 s, 50°C for 1 min and 80–83°C for 6 s. Melting curve analysis was carried out with 10 s of 100 repetitions by increasing the annealing temperature, from 55°C to 95°C, by 0.4°C per step (15). The threshold cycle (C_t) value was determined as the point (cycle) at which the amplification plot crossed the threshold line and was analyzed using REST2005 Beta V1.9.10 (Corbett Life Science, Sydney, NSW, Australia) (16). All samples were examined in triplicate for each primer pair, with parallel control samples without a reverse transcriptase reaction, control samples without template and internal controls of *gapdH* amplification in each experiment. All C_t values were normalized to the C_t values of *gapdH* obtained from their respective templates. The differential value ($\Delta\Delta C_t$) between normalized ΔC_t values was then averaged for the experiments and converted to fold difference ($2^{-\Delta\Delta C_t}$).

Differential normalized ($\Delta\Delta C_t$) values were used to assess the differences in mRNA transcript levels between different environmental conditions using ANOVA followed by a Tukey's post-hoc test. Fold changes in the transcription of each gene were calculated in relation to those of group A (early exponential growth phase after culture in a microaerophilic atmosphere). Differences were considered statistically significant at $p < 0.05$.

Results

Phenotypic traits

A. actinomycetemcomitans cells grown under anaerobic and microaerophilic conditions exhibited differences in phenotypic traits at early exponential and early stationary growth phases.

Cells grown under microaerophilic conditions were more hydrophobic than those cultured anaerobically (Fig. 1), and cells at stationary growth phase were more hydrophobic than those at exponential growth phase, in both incubation conditions. In addition, the ability to adhere to epithelial cells was more prominent in cells cultured microaerophilically at the exponential growth phase than in cells at other growth conditions and growth phases (Fig. 2A). On the other hand, invasion efficiency was higher for stationary-phase cells cultured anaerobically (Fig. 2B).

Transcription of virulence-associated genes

The relative transcription of 12 virulence-associated genes in response to environmental conditions was assessed by RT-qPCR in experiments with RNA obtained independently in two separate assays. The transcription levels of selected genes were calculated in relation to transcription of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*gadpH*). Fold changes of mRNA in cells cultured under different growth conditions were calculated relative to the mRNA of cells in early exponential growth phase cultured microaerophilically (control condition; group A). Cells grown under control conditions (group A) were compared with cells at the early stationary phase in microaerophilic culture (group B) and with cells at early exponential (group C) and early stationary (group D) growth phases of anaerobic culture.

The mRNA levels of *aae*, *ltxA*, *orf859* and *vppA* were not significantly affected by the study conditions, whereas the relative transcription levels of *apaH*, *emaA*, *flp*, *omp29* and *pga* were slightly affected by the varying study conditions (changes of 0.4- to 6.3-fold were observed).

On the other hand, as shown in Table 2, transcription of several genes (*apaH, cagE, cdtB, flp, emaA* and *omp29)* was up-regulated in anaerobic culture compared with microaerophilic culture. Furthermore, environmental conditions significantly affected transcription of *omp100* and *cagE*. The *omp100* gene showed transcriptional up-regulation of 165.8-fold in stationary microaerophilic culture relative to the early exponential phase of

Table 1. Targeted genes, primer sequences and amplicons expected after amplification reactions

Gene	Primer sequence	Amplicon (bp)	References
gapdH (ORF1383)	5′-CCCAAAACATCATCCCATCTTC-3′ 5′-GGAACACGGAACGCCATAC-3′	60	(15)
aae	5′-GGTTTTAGGCGGCACATTTA-3′ 5′-TGCTTGACCAACCATAACCA-3′	152	This study
apaH	5′-CACCTTGGTTTGCCTTGGATA-3′ 5′-TGTCTTCCCAACGTAGCATG-3′	159	This study (17)
cagE	5′-TGGATTGGGACAAGTGAACA-3′ 5′-TACAAAGCCATAAGAGAAAT-3′	190	This study
cdtB	5′-CAACAACACAATTCCAACCC-3′ 5′-GGCGATACCTGTCCATTCTT-3′	94	This study
emaA	5′-CTGCAGCAACCGGGGATTAT-3′ 5′-AATGGATTGGTTGCCTTTAG-3′	110	This study
flp	5′-TCAAAGCAATCGAAGCAATC-3′ 5′-GCAATAGCGATCAAACCGTA-3′	82	This study
ltxA	5′-TTGTCGCAAGTGCCATAGTTATCCAC-3′ 5′-TAGCCCCATGGCAACGGTAGAA-3′	193	This study
omp100	5′-ATCTTCAAGCCAAAACATC-3′ 5′-AAGGCTGCCGACATTAT-3′	169	(9)
omp29	5′-TCTCAACAAGCCATCTCTGC-3′ 5′-CGACCTTTAACTACGTCGCA-3′	80	This study
orf859	5′-CAATCTCACCCAAGCCCTAC-3′ 5′-GCGGCGGAAATATAGAAACTG-3′	83	(28)
pga	5′-GACGGTGATGCGGTATTGG-3′ 5′-GACCGATGATGGAGCTGAA-3′	160	(46) This study
vppA	5′-GGTTACCGGTGGAGTTCG-3′ 5′-CGGGTCGTAATCGTTTGA-3′	190	This study

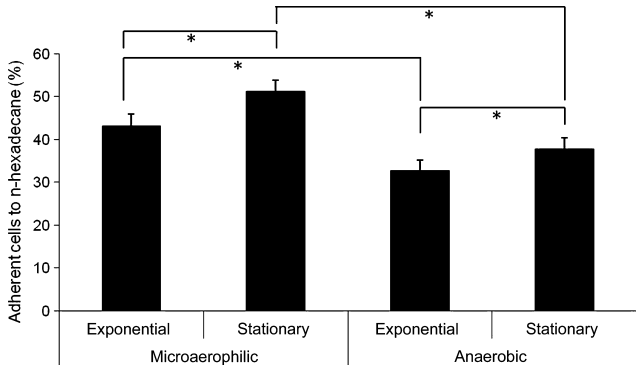

Fig. 1. Percentage of *Aggregatibacter actinomycetemcomitans* cells adherent to n-hexadecane after growth under different conditions (*Statistically significant difference, ANOVA $p < 0.01$).

microaerophilic culture, whereas transcription of *cagE* was 830.3-fold higher in stationary anaerobic culture than in early exponential growth under microaerophilic culture.

Discussion

The present data have clearly shown that both incubation atmosphere and phase of growth affect the ability of *A. actinomycetemcomitans* to adhere to and invade KB cells. This epithelial cell lineage has been extensively used to investigate adherence and internalization of oral pathogens (17–20), especially regarding the interaction of *A. actinomycetemcomitans* and its virulence factors with eukaryotic cells (8,21–23). Environmental changes stimulate alterations to the cell surface of *A. actinomycetemcomitans*, resulting in modifications such as differences in cell hydrophobic properties (Fig. 1). The data on gene transcription reveal that several genes are regulated under different atmospheric culture conditions and growth phases (Table 2).

On the other hand, environmental changes did not affect expression of the gene that encodes the leukotoxin active subunit (*ltxA*); this is in contrast to previous data reporting that ltx is regulated by multiple environmental factors - for example, *ltx* is regulated under anaerobic conditions (24) and by quorum sensing (25). In addition, a recent study reported that the transcription of *ltx* is also regulated by the global regulatory cyclic-AMP receptor protein (26). However, interpretation of the data is difficult because of the complex nature of *ltx* regulation and the differences in the strains studied among studies.

Also, environmental conditions did not affect the transcription of *aae*, *vppA* and *orf859*. Aae is an autotransported protein involved in interaction with oral mucosal cells (8,27), whereas the *orf859* gene is associated with intracellular survival of *A. actinomycetemcomitans* and its product is

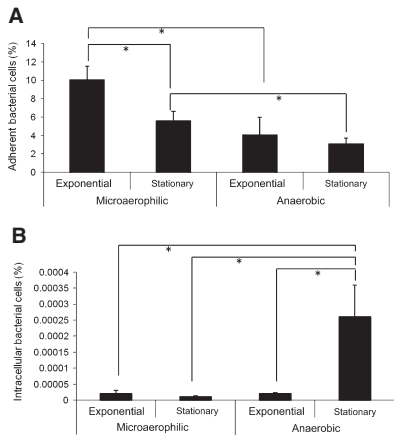

Fig. 2. Percentage of viable *Aggregatibacter actinomycetemcomitans* adherent to (A) and invading (B) KB cells, after growth under different conditions (*Statistically significant difference, ANOVA $p < 0.01$).

detected in the sera of patients with localized aggressive periodontitis, which supports the *in-vivo* expression of this antigen (28) and indicates its role in virulence.

Transcription of *flp* (which encodes a fimbrial subunit) was very low as a smooth phenotype of *A. actinomycetemcomitans* was tested (11,29,30). However, the expression of *flp* was slightly increased under anaerobic culture (Table 2). Previous data have reported that anaerobic environments induce the formation of structurally different fimbriae (31) and higher expression of *tad*V, which is related to fimbriae assembly (11) in *A. actinomycetemcomitans*. Conversion from an afimbriated to a fimbriated phenotype has been shown in *in-vitro* cultures previously (18), and some authors have associated a morphotype change with a mutation in the *flp* region (32). Our data indicate that fimbriae-encoding genes may be transcribed above basal levels under certain circumstances, even in the smooth-colony morphotype.

The ability to form biofilms is also more pronounced in rough strains, and transcription of *pgaC*, which is related to polysaccharide synthesis, was shown to be up-regulated in rough isolates (11). Transcription of *pga*, which is related to the polysaccharide biosynthesis operon, might be down-regulated in the smooth strain tested, but our data indicate that it is regulated in accordance with environmental changes, reaching the highest levels of transcription in late growth phase under microaerophilic culture. Interestingly, cells were more hydrophobic under the same conditions in which *pga* was up-regulated, suggesting signaling for biofilm formation.

The adhesion efficiency, to KB cells, of bacteria grown under the conditions tested in this study was 4-10%. Previous data have reported that *A. actinomycetemcomitans* isolates (not including SUNY465) adhere more efficiently when grown aerobically to exponential phase than when grown aerobically to stationary phase (6), which is in accordance with the data presented here. However, it should be noted that anaerobic incubation led to a less adherent phenotype.

Adherence to KB cells was not correlated with the ability to invade (Fig. 2), confirming that one step is not the consequence of the other; rather, invasion is an active process in *A. actinomycetemcomitans*, requiring a high rate of protein synthesis (27,33,34). Invasion efficiencies were very low, but anaerobically grown cells at stationary phase were about 10 times more invasive than cells grown in the other study conditions.

At early stationary phase, especially in low oxygen concentrations, as found in the periodontal pocket (24), transcription of several virulence-associated genes, such as *cdtB*, *cagE*, *apaH*, *omp29* and *emaA*, is up-regulated. In biofilms, as well as in stationary phase, bacterial cells exhibit slow growth because they are exposed to challenges such as low level of nutrients, the presence of inhibitory metabolites and high cell density (35). Transcription of the genes *cdtB*, *cagE*, *apaH, omp100* and *emaA* was strongly up-regulated at stationary phase, although differences in regulation related to the incubation atmosphere could be shown.

Both cytolethal distending toxin and CagE are factors that mediate interaction with host cells. The cytolethal distending toxin is formed by three subunits – CdtA, CdtB and CdtC – encoded in the same operon (36) and may be part of a genomic island (37). Cytolethal distending toxin is toxic to periodontal epithelial cells as a result of its ability to interrupt the cell cycle and promote apoptosis (38). CagE is not widely found among *A. actinomycetemcomitans* isolates, but rather is restricted to certain serotype b isolates (37). It is a putative virulence factor associated with a

Table 2. Differentially expressed genes involved in virulence of *Aggregatibacter actinomycetemcomitans* in response to environmental alterations

	mRNA fold-change				
	Microaerophilic		Anaerobic		
Gene	Exponential (group A)	Stationary (group B)	Exponential (group C)	Stationary (group)D	Significant differences ($p < 0.05$)
apaH	1	0.4	0.6	1.8	A > (B = C) < D
cagE	1	121.2	130.3	830.3	A < (B = C) < D
cdtB	1	2.4	3.3	6.5	A < (B = C) < D
emaA	1	2.2	3.1	6.3	A < (B = C) < D
flp	1	1.4	2.0	2.3	(C = D) > (A = B)
omp100	1	165.8	9.7	56.5	(A < B) ≠ (C < D)
omp29	1	1.2	6.0	0.5	(A = B) ≠ (C > D)
pga	1	4.7	4.1	2.3	(A < B) ≠ (C > D)

bacterial type-4 secretion type, which is able to promote membrane disruption of KB cells, inducing host-cell apoptosis in a process distinct from bacterial invasion-associated apoptosis, and may be involved in tissue destruction, inflammation and subsequent adverse immunity in perio dontal pathogenesis (39). The coincident higher expression of *cdt* and *cagE* observed under the conditions at which invasion occurs may not be incidental, but rather indicates that multiple potentially destructive mechanisms are expressed by bacteria subjected to anaerobiosis and the stressful challenge presented at the stationary phase.

The highest efficiency of invasion of KB cells was observed for cells grown anaerobically to stationary phase, which is also coincident with the highest transcription levels of *emaA* and *apaH*. EmaA is required for collagen binding (40), whereas *apaH* encodes a diadenosine tetraphosphatase, which is associated with an invasive phenotype in other pathogenic bacteria (41–44), and possibly with the invasion process in *A. actinomycetemcomitans* (17). These data strongly suggest that the periodontal pocket would be the ideal environment for occurrence of the invasive phenotype.

The gene encoding Omp100 was also up-regulated in the anaerobic stationary-phase culture, but the highest mRNA levels were found under microaerophilic conditions. Omp100 is an immunodominant outer membrane protein involved in adhesion to (9,27), and invasion of (34), host cells. On the other hand, another immunodominant outer membrane protein, Omp29, involved in the invasion of, but not in the adherence to, epithelial cells (45) was up-regulated when cells were cultured anaerobically to early exponential phase. These data indicate that different regulatory systems are involved in the expression of Omp29 and Omp100, suggesting that these proteins may participate in different steps of *A. actinomycetemcomitans* infection.

Substantial genomic differences have been shown among *A. actinomycetemcomitans* strains, even among those from the same serotype (37). Hence, the transcriptomes of different strains may differ widely. Our data reveal that transcription of virulence factors associated with colonization and maintenance of *A. actinomycetemcomitans* in the host are regulated by environmental changes. Thus, different factors may be expressed in different niches; for example, factors expressed by *A. actinomycetemcomitans* in the more aerophilic environment of the outer surface of supragingival dental plaque may be different from those expressed by *A. actinomycetemcomitans* in the more challenging anaerobic environment found in deep pockets. These observations should be taken into account when evaluating phenotypic traits and the transcriptional profile of different strains.

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