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*Aggregatibacter actinomycetemcomitans arc*B INFLUENCES HYDROPHOBIC PROPERTIES, BIOFILM FORMATION AND ADHESION TO HYDROXYAPATITE

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ABSTRACT

The regulation of gene expression in the oral pathogen *Aggregatibacter actinomycetemcomitans* is still not fully elucidated. ArcAB is a two-component system which allows facultative anaerobic bacteria to sense various respiratory growth conditions and adapt their gene expression accordingly. This study investigated in *A. actinomycetemcomitans* the role of ArcB on the regulation of biofilm formation, adhesion to saliva coated hydroxyapatite (SHA) and the hydrophobic properties of the cell. These phenotypic traits were determined for an *A. actinomycetemcomitans arc*B deficient type and a wild type strain. Differences in hydrophobic properties were shown at early and late exponential growth phases under microaerobic incubation and at late exponential phase under anaerobiosis. The *arc*B mutant formed less biofilm than the wild type strain when grown under anaerobic incubation, but displayed higher biofilm formation activity under microaerobic conditions. The adherence to SHA was significantly lower in the mutant when compared with the wild type strain. These results suggest that the transmembrane sensor kinase ArcB, in *A. actinomycetemcomitans*, senses redox growth conditions and regulates the expression of surface components of the bacterial cell related to biofilm formation and adhesion to saliva coated surfaces.

Key words: two component system, gene regulation, adherence, colonization

INTRODUCTION

The gram-negative facultative anaerobic coccobacillus *Aggregatibacter actinomycetemcomitans* is associated with several oral and extra oral infections including localized

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aggressive periodontitis (28). The bacterium produces several virulence factors involved in the colonization of the oral cavity, destruction and inhibition of regeneration of the periodontal tissues and evasion of host defense mechanisms (16, 39). However, the environmental signals leading to the regulation of most of these factors are still unknown.

Bacteria are able to sense environmental conditions such as secondary metabolites, oxygen concentration, ions and regulatory proteins by a variety of systems including the two-component systems signaling in the complex transcriptional regulatory network (3). The Arc (anoxic redox control) two-component system allows facultative anaerobic bacteria to sense various respiratory growth conditions and adapt the expression of their genes accordingly. This system consists of the transmembrane sensor kinase ArcB and the cognate response regulator ArcA (OMPR-like) (11, 23).

Under anoxic growth conditions, ArcB autophosphorylates and transphosphorylates ArcA, which then represses or activates the expression of its target operons. Under aerobic conditions, ArcB acts as a phosphatase that catalyzes the dephosphorylation of ArcA-P releasing its transcriptional regulator (24). Upon cessation of signaling, both the cognate response regulator and the sensor kinase undergo dephosphorylation that results in silencing of the system (29).

In *Escherichia coli*, expression of genes involved in oxygen utilization is down-regulated as oxygen is depleted, and in a reciprocal fashion, expression of genes encoding alternative anaerobic electron transport pathways or genes needed for fermentation are switched on. Many of these metabolic transitions are controlled at the transcriptional level by the two-component ArcAB regulatory system (13, 31, 35). This system is involved in the up-regulation of tri carboxylic acid cycle genes in strains grown on glucose as the sole carbon source (2) and plays a role in the synthesis of polyhydroxyalkanoic acids (PHAs), which accumulate in the cytoplasm and function as a carbon reservoir (27).

However, many other genes in addition to those involved in redox metabolism are the putative targets of ArcAB regulation, such as the *tra* operon for conjugation of resistance plasmid R1 (37), the *psi* site for *Xer*-based recombination in plasmid pSC101 (5), the replication site *oriC* (21), and even operons encoding a putative fimbrial-like protein, Mn-superoxide dismutase and iron uptake system (22). In fact, about 9% of *E. coli* open reading frames comprising 55 Arc-regulated operons implicated in energy metabolism, transport, survival, catabolism, and transcriptional regulation are affected either directly or indirectly by ArcA-P (22). It is predicted that 1139 genes in the *E. coli* genome are regulated either directly or indirectly by ArcA (34).

As a capnophilic microorganism, whose only known habitat is the oral cavity, *A. actinomycetemcomitans* is not subjected to changes in environmental conditions as *E. coli*, since the latter exhibits a much broader spectrum of habitats, facing anaerobic conditions in the gut but also very oxidized niches as a free-living organism. However, by colonizing oral mucosa surfaces and supra and subgingival dental plaque, *A. actinomycetemcomitans* experiences changes in oxygen tension, as well as in pH, bacterial cells density, concentration of nutrients and metabolic end-products, toxic and signaling molecules. These conditions may be sensed by the organism and may influence gene expression including those related to virulence. Inactivation of *arc*B in *A. actinomycetemcomitans* results in decreased expression of *afu*A and *ftn*AB involved in the transport of iron and ferritin (10), and it is likely that this system is also involved in other regulatory pathways.

Since environmental parameters influence bacterial gene expression and the role of the ArcAB two-component system

is not fully elucidated in *A. actinomycetemcomitans*, this study aimed to compare the hydrophobic properties and the abilities to form biofilm and to adhere to saliva coated hydroxyapatite of a wild type and an *arc*B defective *A. actinomycetemcomitans* mutant under different environmental conditions.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Bacterial strains used in this study are listed in Table 1. *A. actinomycetemcomitans* was grown in Trypticase Soy broth added with 0.6% yeast extract (TSB-YE) in a humidified 10%CO_2 incubator at 37^{O}C. Anaerobic incubation was performed in an anaerobic chamber (Plaslabs- MI/USA) in an atmosphere of 85% N_2, 5% H_2 and 10% CO_2 and L-cysteine (Inlab, São Paulo, Brazil) (0.07%) was added to TSB-YE. *E. coli* strains were grown in Luria-Bertani broth at 37^{O}C with constant aeration. For solid medium, 15g agar per liter was added to the liquid medium. When necessary, antibiotics (kanamycin - 100µg mL^{-1}, spectinomycin- 100µg mL^{-1} and rifampicin- 100µg mL^{-1}) were added.

Construction of a defective *arc*B *A. actinomycetemcomitans*

Analysis of the genome from *A. actinomycetemcomitans* (www.genome.ou.edu/act.html) revealed a region of homology with *arcB* from *Haemophilus influenzae* and *Pasteurella multocida*. Several primers were constructed based on this sequence. Primers used in this study are listed in Table 2. Primers arcBforward and arcBreverse were used to amplify the *arcB* region using DNA of *A. actinomycetemcomitans* HK1651 as template. The reaction was performed in a Perkin Elmer Thermocycler (CO/USA) with an initial denaturation period at 94^{o}C (5 min), followed by 30 cycles of denaturation at 94^{o}C (30 sec), annealing at 55^{o}C (30 sec), elongation at 72^{o}C (1.5 min), and a final extension at 72^{o}C (7 min).

The 1861bp *arc*B fragment was ligated to pCR2.1-TOPO vector, and cloned in TOPOF10 cells. Transformants were selected in LB with kanamycin. The p*arc*B identity was confirmed by enzymatic restriction with *Eco*RI, *Mef*I and *Hind*III and sequencing.

The gene *aad*9 encoding spectinomycin resistance was obtained by amplification with specific primers (aad9MfeIforward and aad9MfeIreverse), using pdl269 as template DNA. The PCR was performed with an initial denaturation at 94^{o}C (5 min), 30 cycles of denaturation at 94^{o}C (30 sec), annealing at 55^{o}C (30 sec), elongation at 72^{o}C (1 min), and final extension at 72^{o}C (10 min). The *aad*9 fragment was ligated to pCR2.1-TOPO vector, cloned in TOPOF10 cells and transformants were selected in LB with kanamycin and spectinomycin. Interruption of the *arc*B gene was obtained by ligating the *aad*9 fragment digested with *Mfe*I to p*arc*B digested with the same enzyme. The mixture was electropored in competent *E. coli* JM109. Transformants were selected in spectinomycin LB agar and plasmid containing *arc*B disrupted by *aad*9 (parcB/aad9) was identified by enzymatic restriction with *Eco*RI and *Mfe*I.

The *Eco*RI 2.9kb fragment of parcB/aad9 was ligated with the conjugative plasmid pVT1460 digested with the same enzyme and transformed in *E. coli* DH5α. The plasmid was extracted and transformed in the conjugative *E. coli* SM10(λpir) competent cells. Transformant cells containing a plasmid with the disrupted *arc*B (pVTarcB/aad9) were selected in LB with spectinomycin and plasmid identity was confirmed.

Table 1. Bacterial strains and plasmids used in this study.

Bacterial strains and plasmids	Description	Reference
Aggregatibacter actinomycetemcomitans HK1651	*arc*B donor	www.genome.ou.edu/act.html
A. actinomycetemcomitans VT1169 rif^R/nal^R	*A. actinomycetemcomitans* smooth colony, parent strain	Mintz; Fives-Taylor (2000)[20]
A. actinomycetemcomitans USP71	*Arc*B defective	This study
Escherichia coli TOPOF10	Electrocompetent cells	Invitrogen Life Technologies- Brazil
E. coli JM109	Competent cells.	Promega U.S. (WI/USA)
E. coli DH5α	Competent cells	Promega U.S. (WI/USA)
E. coli SM10(λpir)	Conjugative competent cells	Mintz; Fives-Taylor (2000)[20]
PCR2.1-TOPO vector	*E. coli* clone vector kanamycin and ampicilin resistant	Invitrogen Life Technologies- Brazil
parcB	pCR2.1-TOPO + *arc*B	This study
pDL269	Spectinomycin resistance	Mintz; Fives-Taylor (20)
parcB/aad9	Plasmid pCR2.1-TOPO + *arc*B disrupted with *aad*9	This study
pVT1460	Mobilizable plasmid	Mintz *et al.* (21)
pVTarcB/aad9	Plasmid pVT1460+ *arc*B disrupted with *aad*9	This study

Table 2. Primers used for amplification reactions

Primer	Sequence
arcBforward	5′GTCACGAAATGCTATGAAAAATC3′
arcBreverse	5′ATCAATAACCTGCCAACCAC 3′
aad9MfeIforward	5′CTCCCAATTGATCGATTTTCGTTCGT3′
aad9MfeIreverse	5′CATATGCAAGGGTCAATTGTTTTCT 3′
arcBup	5′ATTGGAACACGCGTTA 3′
arcBdown	5′CATCGGCGTCAACCCTTACTG3′
intspec	5′TCAATGGTTCAGATACGACGACTA3′
RT16SrRNA- forward	5′ ACGCTGTAAACGGTGTCG 3′
RT16SrRNA- reverse	5′ TTGCATCGAATTAAACCACAT3′
RTarcB- forward	5′ GCCAATTTCGCGTTA 3′
RTarcB-reverse	5′ TAACGCTGCCCTGTT 3′

A mutagenesis system based on that described by Mintz; Fives-Taylor (2000)[20] was used to generate an *arc*B isogenic mutant of *A. actinomycetemcomitans* VT1169 rif^R/nal^R (SUNY 465 rifampicin and nalidixic acid resistant). *A. actinomycetemcomitans* VT1169, the recipient strain, and *E. coli* SM10(λpir) pVTarcB/aad9, the donor strain, were grown to OD_{560nm} ~ 0.5 and ~0.3 for donor and recipient strains, respectively.

Cells were suspended in TSB-YE without antibiotic, transferred to the surface of TSB-YE agar, and incubated for conjugation in 10% CO_2 at 37^OC for 5 hours. Transconjugants cells were grown on TSB-YE agar plates containing spectinomycin and rifampicin in 10% CO_2 at 37^OC for 48h. Transconjugants were screened by PCR with primer pairs located upstream and downstream *arc*B (arcBup and arcBdown) and an internal primer to *aad*9 gene (intspec) (described in Table 02).

The transconjugant *A. actinomycetemcomitans* USP71 was selected and disruption of *arc*B by insertion of *aad*9 at the *Mfe*I site, between the sequence of transmitter domain of *arc*B and receiver and phosphoptranfer domains was confirmed by sequencing using primers arcBup (located 641pb upstream *arc*B) and arcBdown (located 149pb dowstream *arc*B) exceeding the cloned region in *E. coli* TOPOF10. The sequencing was performed in MegaBACE100 (GE Health Care) with DYEnamic ET Dye Terminatror Kit in Centro de Estudos do Genoma Humano sequencing facility (IB- University of São Paulo/Brazil).

In order to confirm the absence of an intact *arc*B transcript in the mutant strain, total RNA from microaerophilic grown cultures (OD_{500nm} ~0.5) of the wild and the mutant strains were extracted with Trizol LS Reagent (Invitrogen Life Technologies- São Paulo/ Brazil). After DNAse (Invitrogen Life Technologies) treatment, RNA was purified using RNeasy Min Elute Cleanup kit (QIAGEN-CA/USA) and quantified.

cDNA synthesis was obtained by RT-PCR from total RNA of both strains with random primer by using the Super Script III First Strand Synthesis System (Invitrogen Life Technologies). PCR with primers homologous to *arc*B and *16Sr*RNA (control) (described in Table 02) using cDNA as template were performed, with an initial denaturation step at 94^oC (5 min), followed by 30 cycles of denaturation at 94^oC (30 sec), annealing at 48^oC (30 sec), elongation at 72^oC (45sec) and a final extension at 72^oC (7 min).

Bacterial cultures

Cultures of *A. actinomycetemcomitans s*trains VT1169 (wild type) and USP71 (*arc*B^- mutant) grown in anaerobic and microaerophilic conditions at early and late exponential phase were adjusted to an OD_{500nm} ~0.2 corresponding to 3 X 10^8 CFU mL^{-1}. These standardized cultures were used in the following assays.

Adherence to n-hexadecane

The ability to adhere to n-hexadecane was used to determine the relative surface hydrophobicity as described by Gibbons; Etherden (1983)[14] with some modifications. Bacteria cells grown until early and late exponential phases under microaerophilic and anaerobic conditions were harvested (3,000 x g/ 20 min/ 4^oC), washed three times with PUM buffer (16.94g K_2HPO_4, 7.26g KH_2PO_4, 1.8g urea, 0.2g $Mg_2SO_4.7H_2O$ per liter, pH 7.1) and suspended in the same buffer. The suspensions were adjusted to an OD_{550nm} of 0.85 (~1X10^9 CFU mL^{-1}) and transferred to tubes (10 X 100mm).

The bacterial suspensions were added with 400µl of n-hexadecane (Sigma- Aldrich, MO/USA) and then equilibrated in a water bath at 30^oC for 10 minutes. After

mixing for two 30 seconds periods with 5 seconds in between, the suspensions were allowed to stand for 30 minutes until phase separation. The OD_{550nm} of the lower aqueous phase was determined and the fraction of adherence was expressed as the percentage of bacteria that remained in the aqueous phase compared with the initial value. All experiments were performed in sextuplicate.

Biofilm Formation Assay

Biofilm formation was evaluated by the crystal violet staining method (15). Standardized cell cultures of wild type and *arc*B deficient mutant were obtained in TSB-YE with antibiotics at initial exponential growth phase. Aliquots of each cell suspension, corresponding to 10^6 and 10^7 CFU/well were added to wells of a flat-bottomed polystyrene microtiter plate (Corning Inc., NY/USA). Plates were incubated statically under microaerophilic or anaerobic atmospheres at 37^0C for 14 and 18 hours.

Total growth in the wells was estimated by measuring OD_{490nm} using a microplate reader (Model 680, Biorad, Tokyo, Japan). Biofilms in the wells were washed with PBS (pH7.3), fixed with methanol for 15 minutes and stained with 0.1% crystal violet. The dye was eluted with ethanol, and absorbance measured at 600nm.

Biofilm formation index was the biofilm: planktonic growth ratio between the absorbance value of crystal violet eluted from the biofilm (OD_{600nm}) and the absorbance value obtained from the total growth ($OD_{490\ nm}$). Negative controls contained growth medium without the addition of bacteria suspension. All experiments were performed in sextuplicate.

Adherence to saliva-coated hydroxyapatite (SHA)

The attachment to SHA was performed as described by Fine *et al.* (8) with some modifications, in quadruplicate experiments. Whole paraffin-stimulated saliva samples were collected from five periodontally and medically healthy male adults (27-35 years old). Pooled saliva was clarified by centrifugation (10,000xg/ 30 min/ 4^oC) and the supernatant was heated to 60^oC for 30 minutes to inactivate degradative enzymes. Salivary collection was aliquoted and frozen (-20^oC) until use.

Sterile 50mg spheroidal hydroxyapatite beads (BDH Chemicals, Poole/England) were washed and equilibrated overnight at 37^oC in buffered KCl (0.05mM KCl, 1mM potassium phosphate, pH6.0, 1mM $CaCl_2$, 0.1mM $MgCl_2$). Clarified saliva (800µl) was added to the beads and incubated for 2 hours at 37^oC under slow rotation. Tubes were centrifuged (12,000x g/ 5 min) and the beads were washed three times with buffered KCl.

A. actinomycetemcomitans VT1169 and the isogenic *arc*B$^-$ mutant were grown until early exponential phase in microaerophilic atmosphere and cells concentration adjusted to 3 X 10^8 CFU mL^{-1}, centrifuged and suspended in buffered KCl. Bacterial suspensions (1ml) were added to SHA beads and the mixture was incubated for 2 hours at 37^oC with slow rotation. After incubation, the complex beads/bacteria was washed three times with buffered KCl. Cells attached to the SHA were removed by 30 seconds sonication at low power pulse (Branson Ultrasonic Cleaner, CT/USA). After sonication, the beads were allowed to settle to the bottom of the tube for 2 minutes. Aliquots of supernatant of non sonicated and sonicated mixtures were serially diluted and plated in TS-YE agar with antibiotics for CFU estimation.

The number of unbound cells was estimated as the number of CFU in the supernatant of the non-sonicated tubes. Bound cells were calculated by subtracting the number of cells in the supernatant after sonication from the number of cells in the non sonicated mixtures.

Statistical Analysis

Student t-test was used to compare the ratio between absorbance values of biofilm and planktonic growth, and the number of cells adherent to SHA beads of strains *A. actinomycetemcomitans* VT1169 (wild type) and the isogenic *arc*B deficient mutant (USP71). Two-way analysis of variance with posterior multiple comparisons by Tukey was used to detect differences in mean percent values of cells remaining in the aqueous phase in the hydrophobicity assay. The significance level used was $p<0.05$.

RESULTS

Construction and characterization of an *A. actinomycetemcomitans arcB* deficient mutant

A recombinant strain originated from *A. actinomycetemcomitans* VT1169 was obtained by disruption of *arc*B with a spectinomycin resistance cassette. The mutant was screened by amplification of the *arc*B region, and one mutant (USP71) was selected (Figure 01). Sequencing of the *arc*B region of strain USP71 revealed that *arc*B was disrupted by *aad*9, in the region encoding the histidine kinase signaling and ATP binding site in a single recombination step. Dowstream the *arc*B region there is a ribosomal binding site and an open reading frame site indicating that other genes were not affected by the spectinomycin insertion.

Disruption of gene coding for ArcB in the mutant strain was confirmed by RT-PCR. The *arc*B transcript was absent in the isogenic mutant strain but present in the wild type strain. A transcript of the predicted size for the control gene (*16Sr*RNA) was present in both strains (Fig. 1).

Adherence to n-hexadecane

The adherence to n-hexadecane, representing cell hydrophobicity, was expressed as the percentage of bacteria that remained in the aqueous phase after the treatment compared with the initial value. As shown in Figure 2, the wild type cells were more hydrophobic than the *arc*B$^-$ mutant in the late exponential growth phase under microaerophilic and anaerobic conditions, and these differences were statistically significant. In addition, a small but significant difference in hydrophobic properties between both strains was shown in early exponential phase after microaerophilic incubation, but not after growth under anaerobic conditions.

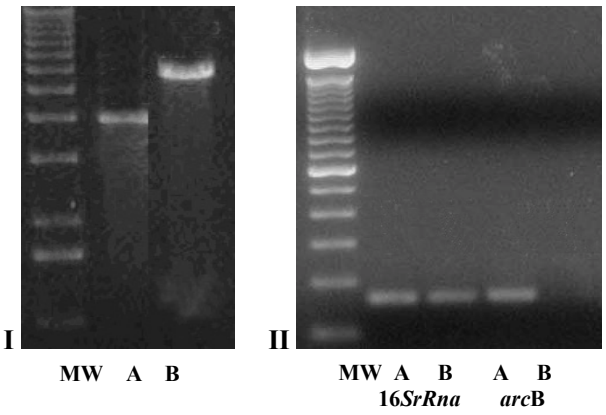

Figure 1. I: 1% agarose gel after electrophoresis of PCR products using template DNA of wild type (VT1169) and *arc*B$^-$ mutant (USP71) *A. actinomycetemcomitans* strains. MW: 1Kb plus DNA ladder (Invitrogen Life Technologies- São Paulo- Brazil) and amplification with primers upstream and downstream of *arc*B (arcBup e arcBdown). A: VT1169 (~3.5Kb amplicon) and B: USP71 (5Kb amplicon). **II**: 2% agarose gel after electrophoresis with MW: molecular weight 100bp molecular weight marker (Invitrogen Life Technologies, São Paulo- Brazil) and RT-PCR products with primers to *16SrRNA* (160bp) and *arc*B (163bp) genes using as template cDNA from A: wild strain (VT1169) and B: mutant strain (USP71).

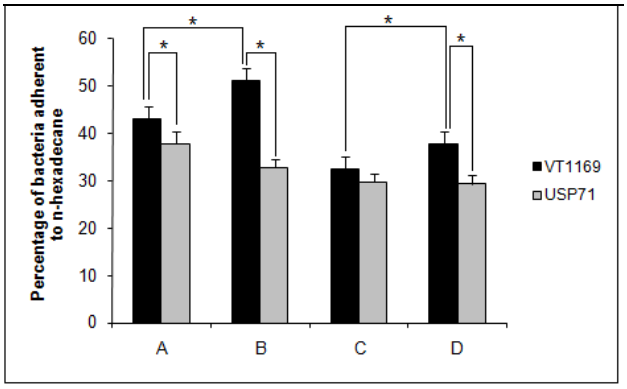

Figure 2. Percentage of bacteria adherent to n-hexadecane of *A. actinomycetemcomitans* VT1169 (wild type) and USP71(arcB$^-$ mutant) grown under microaerophilic incubation until early (A) and late (B) exponential phase, and grown under anaerobic incubation until early (C) and late (D) exponential phase (average and standard deviation of sextuplicate assays). (*statistical significant differences - Student t-test - $p<0.05$).

Multiple comparison analysis (Tukey) showed that the wild type cells grown until the late exponential phase were more hydrophobic than those cells grown in early exponential phase either under anaerobic or microaerophilic atmospheres. The difference in hydrophobicity between early and late exponential phases was not observed with the mutant strain. The hydrophobic properties of the *arc*B$^-$ mutant remained constant throughout the different growth phases under anaerobic and microaerophilic conditions. Interestingly, the cells grown under anaerobic conditions were more hydrophilic than those bacteria grown in the presence of 10% carbon dioxide, especially at late exponential phase.

Biofilm formation

Under microaerophilic growth conditions, there was no difference in the amount of biofilm formed between the wild type and the mutant strains using an inoculum of 10^6 CFU/well after 14 and 18 hours of incubation (Fig. 3) However, unlike the wild type strain where there was greater biofilm formation when grown under anaerobic conditions as compared with microaerophilic growth conditions, the mutant strain formed less biofilm under anaerobic growth.

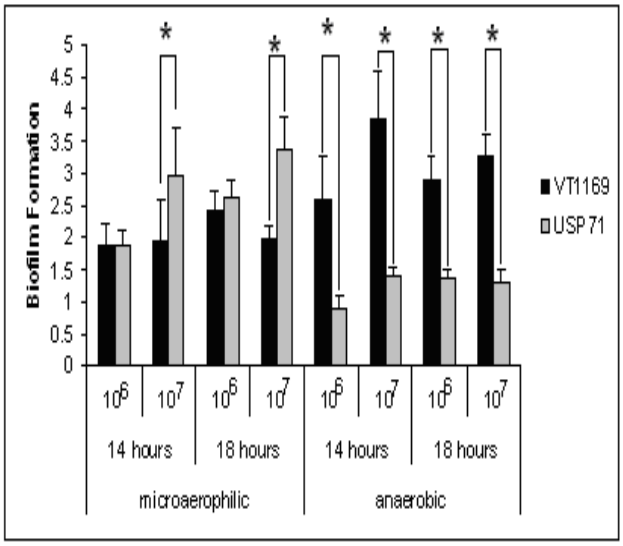

Figure 3. Biofilm formation of strains VT1169 and USP71 grown under microaerophilic and anaerobic conditions, in 14 and 18 hours of incubation and with different initial inoculum (10^6 and 10^7CFU/ml) (mean values). (*statistical significant differences - Student t-test - $p<0.05$).

When a larger inoculum was used to establish the biofilm (10^7 CFU/well), significant differences in biofilm formation between the two strains was

observed under microaerophilic incubation. The *arc*B deficient mutant exhibited a higher biofilm formation index in microaerophilic incubation for 14 and 18 hours. This is in contrast to wild type strain which showed a higher biofilm formation under anaerobic incubation for both time periods and inoculum than the *arc*B$^-$ mutant (Fig. 3).

Adherence to saliva-coated hydroxyapatite (SHA)

The ability to adhere to saliva-coated hydroxyapatite was compared between strains grown until early exponential phase in microaerophilic atmosphere in a quadruplicate assay. The *arc*B deficient mutant showed a significant ($p<0.001$) lower ability (average 3.0 +- 0.16) to adhere to SHA when compared to the wild type (average 20.06 +- 2.99).

DISCUSSION

Bacteria involved in periodontal diseases live in a highly complex and continuously changing microenvironment to which they must rapidly adapt in order to survive (9). Biofilm growth promotes a gradual decrease in the oxygen concentration within time, favoring anaerobic bacteria (4). For an opportunistic periodontopathogen such as *A. actinomycetemcomitans,* the presence of systems responding to alteration in oxygen content may be particularly important, due to differences in redox conditions found between oral habitats such as the mucosa, the supragingival biofilm and the periodontal pocket. These variations are not only spatial but also temporal and it is assumed that the switch from being a commensal organism to a pathogen depends on cues obtained from the host, or from other members of the oral microbiota, or a combination of both.

It has been previously shown that *A. actinomycetemcomitans* cells grown in an anaerobic atmosphere differed from those seen in aerobic cultures, since the presence of oxygen/CO_2 was associated with the upregulation of five surface associated proteins and a decrease in the levels of a 23 kDa protein (9).

In the present study, the *arc*B defective mutant and the wild type strain grew at a similar rate both under microaerophilic and anaerobic atmospheres in planktonic cultures in a rich medium (data not shown). A previous report had also shown that inactivation of *arc*B had little effect on the aerobic growth of *A. actinomycetemcomitans* under iron-replete conditions, but the isogenic strain grew poorly under anaerobic condition. On the other hand, in aerobic condition, the mutant grew poorly under iron-limiting conditions, indicating that the system ArcAB is involved in the iron metabolism (10).

Phenotypic changes associated with oxygen concentration were shown in the wild type strain. Cells grown under aerobic cultures (with CO_2) were more hydrophobic than those grown under anaerobiosis. In addition, cell hydrophobicity increased from early exponential to late exponential growth phases in the wild type cultures, indicating regulation of cell associated proteins of *A. actinomycetemcomitans* according to growth phase and oxygen concentration. Other studies have also reported that hydrophobic properties are influenced by several experimental variables including culture medium composition and age of the culture (14, 19).

These changes in the cell surface according to incubation atmosphere and growth phase were absent in the *arc*B deficient strain indicating that this two-component system is involved in the regulation of expression of cell surface proteins in *A. actinomycetemcomitans.*

Hydrophobic properties have been associated with bacterial interaction with host tissues (14, 19). In biological systems, hydrophobicity depends on the amount of hydrophobic or

nonpolar aminoacids in the surface proteins, determined by the number of hydrogen-carbon bonds that the molecule contains (20). Thus, the more hydrophilic phenotype observed in the *arc*B deficient mutant reflected in the ability of the mutant to adhere to saliva coated hydroxyapatite and to accumulate as a biofilm.

The *arc*B deficient mutant exhibited a reduced ability to adhere to SHA when compared to the wild type. In addition, this strain formed less biofilm than the wild type under anaerobic incubation, but showed higher biofilm formation ability under microaerophilic incubation. Interestingly, the wild type exhibited an opposite behavior, forming more biofilm under anaerobic incubation than under microaerophilic conditions. Differences in biofilm formation and hydrophobicity according to oxygen concentration at the same growth phase points out that ArcB is functional in *A actinomycetemcomitans* and able to sense oxygen concentrations.

The ArcAB system is considered a microaerobic redox regulator (1). However, the differences between wild type and *arc*B deficient mutant strains were observed in *A. actinomycetemcomitans* not only according to the atmosphere of incubation but also regarding to growth phases, indicating that the differential expression of genes induced by ArcAB system may be signaled by other secondary metabolites, as shown for other ArcAB regulated systems.

In *E. coli* certain fermentation intermediates like acetate and pyruvate accelerate the autophosphorylation activity of ArcB (12), and D-lactate acts as a significant effector that amplifies ArcB kinase activity (33). In addition, ArcB not only phosphorylates ArcA, but also the σ^S proteolytic targeting factor RssB, suggesting that the redox state of quinones, which controls autophosphorylation of ArcB, not only monitors oxygen but also energy supply (25). Thus, ArcA, ArcB and RssB can constitute a branched "three-component system", which coordinates *rpo*S transcription and σ^S proteolysis and thereby maintains low σ^S levels in rapidly growing cells (25).

Our data have also indicated that the ability to form biofilm was influenced by the density of the initial inoculum. No differences between the *arc*B deficient mutant and the wild strain were demonstrated in biofilm formed under microaerophilic conditions when 10^6 CFU/ml starting cultures were used. However, when a denser inoculum was used (10^7 CFU/ml), an increased ability to form biofilm was observed for the *arc*B defective strain, when compared to the wild type. On the other hand, under anaerobic conditions, the wild strain formed more biofilm than the *arc*B defective mutant using either 10^6 or 10^7 CFU/ml starting cultures.

Biofilm formation requires a variety of genes including quorum-sensing systems, environmental sensing two-component systems, general stress response pathways, and those encoding surface adhesins involved in cell-cell or cell-to-surface interactions (6, 7, 36, 38).

Initial adhesion to surfaces by *A. actinomycetemcomitans* is dependent on the fimbria encoded by the *tad* locus but the smooth strains used in this study do not express the *tad* fimbriae that are found in clinical isolates (30). Thus, the biofilm studied here was fimbria independent and other components such as PGA (poly-N-acetyl-glucosamine) may play a role in the aggregation of cells and in the detachment of cells from the colony (15, 17, 18).

These differential phenotypes between wild type and the *arc*B deficient mutant are indicative of differences in the transcription of some genes or operons related to cell surface components and biofilm formation directly or indirectly induced by the ArcAB system.

Fong *et al.* (10) observed that LuxS-dependent response in *A. actinomycetemcomitans* may require the ArcB sensor kinase suggesting that it may contribute to the signal

transduction cascade that directs the response of *A. actinomycetemcomitans* to AI-2. This inductor itself is required for adhesion to a saliva coated surface and biofilm growth by *A. actinomycetemcomitans* and it was suggested that redundant mechanisms may exist in this microorganism for interacting with AI-2 (36).

The ability to form biofilms by either rough or smooth phenotypes of *A. actinomycetemcomitans* on a plastic surface is influenced by the presence of haemin (32), thus the interference in iron acquisition by ArcB (10) may have induced the lower biofilm formation phenotype in the *arc*B mutant strain. However, it should be pointed out that the experimental conditions employed in this study were not iron chelating since an iron rich medium (TSB-YE) was used.

Differences shown here between wild type and the *arc*B defective mutant clearly indicated that in *A. actinomycetemcomitans*, the transmembrane sensor kinase ArcB is able to sense oxygen concentration in the environment, and the low redox turns on the ArcAB system, influencing gene expression. They also suggested that ArcB may be involved in sensing other environmental signals as shown for differences in growth culture phases and variation in cells density in the biofilm assay.

In addition, we have shown that ArcB is involved in the regulation of expression of bacterial surface components related to hydrophobic properties, biofilm formation and adhesion to saliva coated surfaces, as demonstrated by differences in these properties between the wild type and the *arc*B deficient mutant. Thus, these data indicated that *A. actinomycetemcomitans* may exhibit different phenotypes according to its microenvironment in the oral cavity.

CONCLUSION

The sensor kinase ArcB, in *A. actinomycetemcomitans*, senses environmental redox conditions and regulates the expression of surface components related to biofilm formation and interaction with salivary proteins adsorbed to surfaces.

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RESUMO

*arc*B em *Aggregatibacter actinomycetmcomitans* influencia propriedades hidrofóbicas, formação de biofilme e aderência a hidroxiapatita

A regulação da expressão gênica do patógeno oral *Aggregatibacter actinomycetemcomitans* não está completamente descrita. O sistema de dois componentes ArcAB permite que bactérias anaeróbias facultativas percebam diferenças nas condições respiratórias durante sua multiplicação e adaptem a expressão de genes à estas condições. Este estudo investigou em *A. actinomycetemcomitans* o papel de ArcB na regulação da formação de biofilme, aderência à hidroxiapatita recoberta por saliva (SHA) e nas propriedades hidrofóbicas celulares. Estas características fenotípicas foram determinadas para uma linhagem de *A. actinomycetemcomitans* deficiente em *arc*B e para uma linhagem selvagem. Foram observadas diferenças nas propriedades hidrofóbicas entre as linhagens quando estas foram cultivadas em ambiente microaerófilo até início e final de fase exponencial e quando foram cultivadas em ambiente

anaeróbio até final de fase exponencial. A linhagem *arc*B mutante formou menos biofilme do que a linhagem selvagem quando estas foram cultivadas sob incubação anaeróbica, porém, apresentou maior formação de biofilme quando a incubação foi realizada em condições de microaerofilia. A aderência à SHA apresentada pela linhagem mutante foi significantemente menor do que a observada pela linhagem selvagem. Estes estudos sugerem que a quinase sensora ArcB, em *A. actinomycetemcomitans*, percebe as condições redox de multiplicação e regula a expressão de componentes de superfície bacterianos relacionados à formação de biofilme e adesão a superfícies recobertas com saliva.

Palavras- chave: sistema de dois componentes, regulação gênica, aderência, colonização

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