Phenotypic Characterization of *hns* mutants of *Aggregatibacter actinomycetemcomitans*

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ABSTRACT

Aggregatibacter actinomycetemcomitans is associated with aggressive forms of periodontitis. The mechanisms that control the expression of virulence factors are essentially unknown. Histone-like nucleoid structuring protein, H-NS, is a DNA binding protein that has been shown to influence hundreds of genes in Gram-negative bacteria. H-NS usually acts as a transcriptional silencer and has a negative influence on gene expression. H-NS has not been studied in *A. actinomycetemcomitans* before, and its effects on gene expression in this species were lacking. This study aimed to investigate if lack of H-NS expression might result in apparent phenotypical differences regarding gene expression with emphasis on virulence. For this we have used the *A. actinomycetemcomitans* rough-colony serotype a strain, D7S, its smooth-colony derivative D7SS, and *hns* mutants of D7S and D7SS. Our results show that smooth colony strain D7SS releases a larger amount of vesicles as compared to the rough D7S. The D7S *hns* mutant appeared to exhibit pili that were shorter than those of the parental strain, and this was not due to altered expression of RcpA in the *hns* mutant. As judged by Silver-staining, and Western blot analysis, H-NS may influence the level of several proteins. Taken together, our study supports that H-NS is involved in the regulation of virulence factor expression in *A. actinomycetemcomitans*.

INTRODUCTION

Periodontitis is the most common cause of tooth-loss worldwide and affects millions of people each year. It is believed to be a multifactorial disease and it is associated with a defined set of microbes. The contribution of specific bacterial species is poorly understood, and more research is needed (Darveau, 2010).

The Gram-negative bacterium *Aggregatibacter actinomycetemcomitans* is a major species implicated in the development of periodontitis. Studies have shown that in patients with juvenile aggressive periodontitis, 75-100% of infected sites hosted *A. actinomycetemcomitans* (Slots et al., 1999).

The species is divided into six serotypes, a-f and the virulence potential of *A. actinomycetemcomitans* appears to vary among strains and different serotypes. Some of the serotypes are observed in aggressive periodontitis, such as the JP2 clone of serotype b. The fast onset and progression may be due to production of high levels of leukotoxin (Haubek et al., 2008; Kittichotirat el al., 2011). Serotype c, on the other hand is frequently found in periodontally healthy patients (Asikainen et al., 1991).

Initial adherence of the bacterium to the surface of the epithelium is supposedly mediated through receptor binding by the protein Aae (Henderson, 2010). Attachment to hard surfaces is mediated by fimbriae and extracellular carbohydrate polymer, Poly-β-1,6-N-acetyl-D-glucosamine PGA, which probably is the reason that *A. actinomycetemcomitans* can move to the tooth surface and eventually the supragingival plaque. PGA is a hexosamine-containing polysaccharide that mediates adhesion between cells, and a crucial factor in the biofilm matrix (Izano et al., 2008).

Adherence is also mediated by the tight-adherence (*tad*) gene locus, which includes *flp*, *rcpA* and *rcpB*. RcpA (rough colony protein A) is a 43-kDa protein that is expressed at significantly higher levels in rough type colonies. (Saito et al., 2010) Its role is presumably to create a channel in the outer cell membrane through which Flp1-pili from the *tad* locus can pass (Clock et al., 2008; Tomich et al., 2006).

Examples of virulence factors contributing to periodontal tissue destruction include leukotoxin, endotoxin (LPS) and cytolethal-distending toxin (CDT) (Henderson et al., 2010). Leukotoxin acts by selectively killing leukocytes (Johansson, 2011). Today the only typical oral bacterium known to produce leukotoxin is *A. actinomycetemcomitans* (Yamano et al., 2003).

Outer membrane vesicles (OMVs) is a mechanism for release of proteins and cell wall components in gram negative-bacteria. They are created from a part of the cell membrane

(MacDonald et al., 2012; Nowotny et al., 1982). *A. actinomycetemcomitans* OMVs have a role in virulence by delivering effector proteins such as CDT and leukotoxin to target host cells (Rompikuntal et al., 2012). Recent data also indicate that the OMVs from *A. actinomycetemcomitans* can be taken up by non-phagocytic host cells to elicit an inflammatory response by triggering specific pathogen recognition receptors (PRR), NOD1 and NOD2 (Thay et al., 2014).

How the regulation of different virulence factors in *A. actinomycetemcomitans* is mediated is essentially unknown. Histone-like nucleoid structuring protein, H-NS, encoded by the *hns* gene locus is a 15.6-kDa DNA binding protein that has been shown to influence hundreds of genes in Gram-negative bacteria such as *Escherichia coli*. H-NS usually acts as a transcriptional silencer (Filloux, 2012). H-NS binds most strongly but not exclusively to double-stranded, curved DNA and prefers target sites, which are rich in A T bases (Atlung et al., 1997).

Studies in *E. coli* have shown that *hns* regulates genes in the bacteria that are primarily related to adaptation to environmental changes, such as osmolarity and temperature. In *hns* mutant *E. coli* bacterial cells, some genes become upregulated while others are downregulated as compared to the wild type. However, the typical function of *hns* seems to be down-regulating genes such as those encoding major virulence factors, i.e. it mostly acts as a suppressor. For example, *hns* negatively controls the expression of type 1 fimbriae in *E. coli* via suppressing expression of the recombinases FimB and FimE (Müller et al., 2006).

The putative role of H-NS has not been investigated in *A. actinomycetemcomitans* before, and its effects on gene expression in this species, are yet to be discovered. This study therefore aimed to investigate if lack of *hns* expression might result in apparent phenotypical differences regarding gene expression with emphasis on virulence. For this we have used the *A. actinomycetemcomitans* rough-colony serotype a strain, D7S, its smooth-colony derivative D7SS, and *hns* mutants of D7S and D7SS recently generated in this laboratory.

MATERIALS & METHODS

Ethical considerations

The original sample of the strain D7S that we have used in this study was originally cultivated from a subgingival plaque sample of an African American female diagnosed with generalized aggressive periodontitis. Consent that the strain could be used in research was given (Chen et al., 2010). The strain, which was donated to this Department from Dr. Casey Chen,

University of Southern California, USA, is frequently used in studies worldwide. *A. actinomycetemcomitans* strains are considered to be safe to handle in the laboratory. To the best of our knowledge, and as judged by the phenotypes of *hns* mutants generated earlier in other bacterial species, there is little risk that the virulence of the mutant made in D7S is greater than that of the parental strain. We think that by using this strain we can make a contribution to the understanding of the virulence of this bacterial species. Improved knowledge about the role of *A. actinomycetemcomitans* in periodontitis may benefit future diagnostic and treatment strategies.

Literature search

A search for relevant articles was made in the PubMed database. The exact MeSH terms used were ("actinobacillus actinomycetemcomitans"[MeSH Terms] OR ("actinobacillus"[All Fields] AND "actinomycetemcomitans"[All Fields]) OR "actinobacillus actinomycetemcomitans"[All Fields] OR ("aggregatibacter"[All Fields] AND "actinomycetemcomitans"[All Fields]) OR "aggregatibacter actinomycetemcomitans"[All Fields]) AND "virulence factors"[All Fields] NOT JP2[All Fields] AND ("2003/09/24"[PDat] : "2013/09/20"[PDat].

To find articles on *hns* and recent studies conducted on the subject, we searched for studies involving *hns* and *E.coli*. The MeSH terms used were:

"*hns*"[All Fields] AND "e. coli"[All Fields] AND ("pathogenicity"[Subheading] OR "pathogenicity"[All Fields] OR "virulence"[All Fields] OR "virulence"[MeSH Terms]) NOT fur [All Fields] AND ("2003/09/24"[PDat]: "2013/09/20"[PDat]).

Bacterial strains and growth conditions

The parental *A. actinomycetemcomitans* strains used this study were the serotype a smooth colony strain D7SS, and the rough colony strain D7S. When strain D7S is repeatedly streaked on fresh agar medium it spontaneously transforms into the smooth colony type, D7SS that lacks fimbriae (Wang et al., 2002). Moreover, we used *hns* mutant versions of D7S and D7SS that were earlier generated in this laboratory by gene replacement, inserting a kanamycin resistance cassette. *A. actinomycetemcomitans* strains were cultivated for 2 days in air supplemented with 5% CO_2, at both 30°C and 37°C, on blood agar plates (5% defibrinated horse blood, 5 mg hemin/liter, 10 mg vitamin K/liter, Columbia agar base).

Atomic Force Microscopy

A. actinomycetemcomitans bacterial cells and their released OMVs were visualized using atomic force microscopy (AFM) essentially as earlier described (Rompikuntal et al., 2012). Ultrapure water (Millipore, Billerica, MA, USA) was used to dilute OMV for AFM. Directly after diluting, the samples were incubated for 5 minutes at room temperature, after being placed on a mica surface that was freshly cleaved. Before drying it in a desiccator for at least 2 hr the samples were gently washed with ultrapure water. A Nanoscope V atomic force microscope (Veeco Instruments, Plainview, NY, USA) was used for imaging, with tapping mode. The final images, which were plane fitted in both the x and y-axes, are presented in amplitude mode.

Whole cell protein preparation from bacterial cells

After 2 days of growth as described above, bacterial cells were harvested with a sterile cotton swab moistened with PBS (pH 7.3) and suspended in 3 mL PBS. The bacterial cells were then washed by two centrifugations at 5000 g for 5 minutes, and then resuspended into 1 mL PBS (stock suspension). A volume of 0.1 mL stock suspension was diluted 10-fold with PBS and then the OD was measured in the spectrophotometer at 600 nm. A new suspension with OD600 = 2 were made, based on the OD600 value from the stock suspension. This is equal to approximately 2×10^9 colony forming units (CFU)/mL, and a protein concentration of 1 mg/mL. Samples were stored at -80°C until analysis on SDS-PAGE.

Preparation of bacterial supernatant extracts

A. actinomycetemcomitans strains were cultured on agar as described above. On the second day the bacteria were suspended in PBS to a concentration of OD600 = 6, i.e. equal to approximately 6×10^9 CFU/mL. The suspensions were agitated gently for 1 hr at 4°C, to extract bacterial components. Thereafter the suspensions were centrifuged (10,000 g) for 5 minutes at 4°C, and then stored at -80°C until analysis on SDS-PAGE.

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a method often used to detect proteins (Trun et al., 2004). In order to perform this method, the samples were mixed with Laemmli sample buffer 1:1 (Tris-HCl pH 6.8 125 mM, SDS 4%, Glycerol 50%, Bromophenol Blue 0.02% and B-mercaptoethanol 0.2%) (amounts used were 20 µl + 20 µL) and then heated at 99.9°C for 5 minutes. Thereafter, in order to get rid of the condensation,

they were centrifuged for 10 seconds. For reference the Molecular Weight used were Precision Plus Protein All Blue Standards. 5 µL MW was mixed with 15 µL MQ water and 5 µL 2x Laemmli buffer. Running Buffer was made (from SDS 1%, Glycine 1.92 mM, Tris 250 mM) and diluted with ultrapure water 1:10. The samples were loaded in the polyacrylamide gel (Criterion TGX Precast gel 8-16% Tris-HCl). The amounts of sample taken were 10 µL for OD2, and 15 µL for OD6. Thereafter the gel was runned at 150 V, for approximately 90 minutes. The gel was then removed from the cassette and washed in ultrapure water for 2 x 5 minutes.

Silver staining the proteins after sodium dodecyl sulfate-polyacrylamide gel electrophoresis

To visualize the protein bands the gel was then stained. The Pierce Silver Stain Kit from Thermo Scientific was used. The gel was fixed in ultrapure water, 30% ethanol, 10% acetic acid solution, (i.e., 6:3:1) for 2 x 15 minutes. Thereafter the gel was first washed in 10% ethanol solution for 2 x 5 minutes, and finally 2 x 5 minutes in ultra pure water. The gel were then incubated for 1 minute in Sensitizer Working Solution (100 µL Sensitizer and 50 mL ultra pure water), and washed with ultrapure water for 2 x 1 minutes. Thereafter the gel was incubated for 30 minutes in a Stain Working Solution (1 mL of Enhancer and 50 mL of Stain) and then again washed with ultrapure water for 2 x 20 seconds. The Developer Working Solution (1 mL of Enhancer and 50 mL Developer) was then added and the gel was incubated until the protein band appeared, approximately 2 minutes. The gel were thereafter washed and incubated for 10 minutes with Stop Solution (5% acetic acid solution). The gel was then photographed and documented for further analysis.

Western blot analysis

The bacterial samples were prepared and analyzed on SDS-PAGE using Criterion TGX Precast gels (8 - 16%) and the procedures described above. After electrophoresis (60 minutes), transfer of the proteins in the gel to a membrane was done as follows. A pad the same size as the gel was saturated with 1x anode buffer for 5 minutes and placed on the anode. The transfer membrane was equilibrated (10 sec in 100% methanol and 15 minutes in anode buffer) and placed on top of the first pad. Bubbles between membrane and filter paper were removed by rolling a test tube over the surface. The gel was then carefully placed on top of the membrane and the transfer stack was then completed by putting a pad saturated with cathode buffer on top of the gel. To ensure intimate contact between all layers, a test tube was rolled over the stack again. After the proteins were transferred for 60 minutes at 140 mA, the

membrane was placed in a plastic pocket together with 15 mL primary antibody buffer (15 mL TTBS (Tween/Tris-buffered salt)), 0.15 g dry skimmed milk and 1.5 µL of a rabbit antibody specific for whole *A. actinomycetemcomitans* serotype a cells (Saarela et al., 1992). Thus, this antibody was used at a final dilution of 1:1000. Alternatively, an antibody specific for RcpA (Paino et al., 2011) was used instead (final dilution of 1:1000). The membrane was incubated overnight at 4°C. The next day the membrane was rinsed with TTSB, followed by thorough washing on a shaker containing TTBS for 4 x 5 minutes. The membrane was placed in a new plastic pocket and incubated for 60 minutes with 15 mL antibody buffer (0.08 g skimmed milk and 15 mL TTBS) and secondary antibody: donkey-anti-rabbit horseradish peroxidase (HRP)-conjugate (final dilution 1:10 000). Thereafter the membrane was washed with TTBS 4 x 5 minutes plus an additional 30 minutes wash. To remove excess solution the membrane was drained by holding it with forceps over a piece of paper. The detection solution was prepared (2 mL peroxidase and 2 mL substrate) and poured over the membrane, leaving it to soak for 1 minute. The membrane was then placed on plastic and photographed with Bio-Rad ChemiDoc imaging system.

RESULTS

Imaging and estimation of amount of vesicles and pili

To investigate if inactivation of *hns* caused a visible effect on the cell morphology and vesicle production, we did an AFM analysis. For this, *A. actinomycetemcomitans* samples, cultivated on agar at 30ºC and 37ºC, respectively were sent for AFM analysis at the core facility at Department of Molecular Biology. An ocular analysis concerning the amount of pili and vesicles was conducted. According to our results (Fig. 1), at both 30ºC and 37ºC there was no apparent difference in the amount of vesicles released by the wild type and the *hns* mutant in either D7S or D7SS. Thus, we concluded that H-NS did not have an apparent effect on vesiculation under the present laboratory conditions used. However, on the other hand our results clearly supported a larger amount of vesicles released from the smooth colony strain D7SS (Fig. 1E-F) as compared to the rough D7S (Fig. 1A-D). Moreover, AFM revealed that the D7S *hns* mutant (Fig. 1A, and Fig. 1C) appeared to exhibit pili that were shorter than those of the parental strain (Fig. 1B, and Fig, 1D). This observation supports the notion that *hns* may act as an activator of pili production in *A. actinomycetemcomitans*.

Pattern of protein expression

To investigate if *hns* may control the expression of multiple proteins in *A. actinomycetemcomitans*, the patterns of protein expression in whole cell protein extracts and supernatants of D7S, D7SS, and their *hns* mutants were compared by SDS-PAGE and Silver-staining (Fig. 2). Although no dramatic differences were observed, the slight variations in protein banding patterns in both whole cell protein extracts and supernatants suggest that *hns* may influence the levels of several proteins in *A. actinomycetemcomitans*.

Expression of surface epitopes

To investigate if the expression of surface epitopes was altered in the *A. actinomycetemcomitans hns* mutants, we performed Western blot using an antibody specific for whole serotype a bacterial cells. This antibody detects multiple *A. actinomycetemcomitans* surface proteins including GroEL and peptidoglycan-associated lipoprotein (PAL) (Oscarsson et al., 2008). According to our results (Fig. 3), we again detected small differences in expression of surface epitopes between the *hns* mutants and their corresponding parental strains, consistent with a role of *hns* in the expression of proteins exposed on the bacterial surface.

Expression of RcpA

Consistent with the seemingly impaired pili production in the D7S *hns* mutant (Fig. 1), we investigated if this might be a result of reduced expression of RcpA. Although Western blot supported clearly reduced levels of RcpA in the smooth strain D7SS as compared to the rough D7S (Fig. 4), there was no apparent difference between the *hns* mutants and their corresponding parental strains. We therefore concluded that lack of H-NS had no obvious effect on the expression of RcpA in *A. actinomycetemcomitans*. Thus, the reduced pili production seemed not to be mediated via RcpA.

DISCUSSION

This study shows that there are some differences in gene expression between *hns* mutant and wildtype *A. actinomycetemcomitans.* The aim of the study was to make an initial characterization on the role of *hns* in virulence gene expression in *A. actinomycetemcomitans*. For this we used the serotype a rough strain D7S, its smooth colony derivative D7SS, and *hns* mutants constructed in both of these strains. The strains were screened for phenotypical

differences (vesiculation, and pili formation) by AFM, and for their patterns of expressed and secreted proteins using SDS-PAGE and Silver-staining. In addition, we used Western blot and specific antibodies towards whole serotype a *A. actinomycetemcomitans* cells, and RcpA, respectively.

We assessed the bacterial cells grown at both 30ºC and 37ºC. This was done because *hns*-mediated regulation of virulence genes in *E. coli* and *Shigella* spp. was shown to be thermo-regulated (Umanski et al., 2002; Palchaudhuri et al., 1998). This means that the regulation may function differently depending on temperature. For example the regulatory effect of *hns* was shown to be more pronounced at lower temperature such as 30°C in the above studies. This is consistent with our findings that the reduction of the length and amounts of pili was more clearly detected in the bacterial cells cultured at 30°C, compared to 37ºC (Fig. 1A-C). This supports the hypothesis that the regulatory effect of *hns* is thermo-regulated also in *A. actinomycetemcomitans*. The mechanism behind this seemingly positive role of H-NS in pili biogenesis in *A. actinomycetemcomitans* is not known, and will be further investigated in the laboratory. For example, it remains to be investigated whether it is the actual production of the Flp1 pilin structural component that is reduced in the *hns* mutant. This could not be done in the present study due to the lack of available antibodies. However, since RcpA was detected in the same amount in the D7S *hns* mutant as in the parental strain, we could exclude that *hns* has a significant effect on the expression of RcpA in *A. actinomycetemcomitans*. Thus, the reduction in pili production appears not to be due to RcpA. Our finding that RcpA was expressed at much higher levels in the rough strain D7S than in the smooth D7SS is in accordance with earlier reports (Saito et al., 2010).

It cannot be excluded that *hns* represses a yet unknown, negative regulator of pili production in *A. actinomycetemcomitans*. This type of mechanism would be similar to the role H-NS plays in *E. coli* when acting as an activator of flagella production (Krin et al., 2010). It is not impossible to think that *hns* may affect pili production in *A. actinomycetemcomitans* in a manner similar to the flagella of *E. coli*, as it is known to specifically influence outer membrane proteins in other bacteria (Landini et al., 2002). In contrast, previous studies have also provided evidence of the opposite, i.e. a repressing role of H-NS in fimbriae/pili production. For example, *E. coli hns*-mutants are hyper-piliated (Kouokam et al., 2006). Type 1 fimbriae are affected by *hns* in *E. coli*, primarily by preventing transcription of recombinases fimB and fimE, which upon mutagenesis of *hns* led to increased pili production (Müller et al., 2006).

Within the limitations of only a few AFM micrographs analysed in this study, we did not detect an apparent difference between the numbers of OMVs released by the *hns* mutants as compared to their corresponding wild-type strains. This is in contrast to a previous study where

it was shown that H-NS influences vesiculation in *E. coli*. Notably, the *hns* mutant bacterial cells were surrounded by a lot more OMVs than the wild type (Kouokam et al., 2006). The reason for this was not determined, but it was suggested that it might be a result of a destabilized cell envelope in the *hns* mutant. On the other hand, we observed a clear difference between the number of OMVs released by the smooth, i.e. non-piliated (D7SS) and the rough (D7S) strain. More specifically, the smooth strain appeared to produce larger amounts of vesicles. Thus, we note that the findings of our study and those of (Kouokam et al., 2006) are both consistent with the idea that there appears to be a correlation between pili production and amount of vesicles released. Similarly, also the ability to form flagella in *E. coli* affects the production of OMVs (Manabe et al., 2013), however the molecular mechanisms behind such correlations remain to be elucidated. To investigate these hypotheses further, it would be meaningful to quantitate vesicle production using additional analysis methods such as determining their protein amount (Kouokam et al., 2006).

The analysis of the patterns of proteins and surface epitopes expressed and released by the *A. actinomycetemcomitans* bacterial cells revealed only small differences between the *hns* mutants and their parental strains. We are aware of that some of the protein extracts analyzed on the SDS-PAGE gels seem to not have been fully separated, which may be a result of too much extract being loaded. Our results are nevertheless consistent with a role of H-NS in regulating the expression of several genes in this species, which has been shown in previous studies performed in other Gram-negative bacteria, including *E. coli* (Müller et al., 2006). Moreover, our findings are consistent with observations that the proteins that predominantly differed in expression in *E. coli hns* mutants as compared to *hns*$^+$ parental strains were outer membrane proteins, i.e. likely exposed on the surface of the bacterial cells (Landini et al., 2002). As H-NS is a transcriptional regulator, to obtain a more complete survey of the multitude of genes affected by *hns* in *A. actinomycetemcomitans*, we think that it would be of interest to perform an analysis of the levels on all transcribed mRNAs in the *hns* mutants and wild types, respectively. Although this was not possible within the margins of our study, identifying the differentially expressed mRNA sequences can provide important information on how the virulence is regulated in this human pathogen.

Conclusions from this study

AFM revealed that the *hns* mutant derivatives of *A. actinomycetemcomitans* strains D7S and D7SS released similar amounts of OMVs as their parental strains.

According to AFM, the D7S *hns* mutant appeared to produce fewer and shorter pili as compared to the parental strain. This phenotype was more clearly seen at lower temperature, 30°C, suggesting that the regulatory effect of H-NS may be thermoregulated in *A. actinomycetemcomitans*.

Western blot using an antibody specific for RcpA revealed the same expression of RcpA in *hns* mutant and wild type *A. actinomycetemcomitans* strains suggesting that RcpA had no role in the reduced pili production in the H-NS mutant.

Western blot confirmed that RcpA was expressed at much higher level in the rough strain D7S, than in the smooth D7SS.

According to AFM, the smooth strain D7SS released more OMVs than the rough D7S.

Silver-staining and Western blot using an antibody specific for whole *A. actinomycetemcomitans* serotype a cells both supported that H-NS influences the expression of several proteins in this species, including those that are exposed on the surface of the bacterium.

Taken together, our study supports that H-NS is involved in the regulation of virulence factor expression in *A. actinomycetemcomitans,* and that lack of *hns* expression results in phenotypical differences regarding gene expression.

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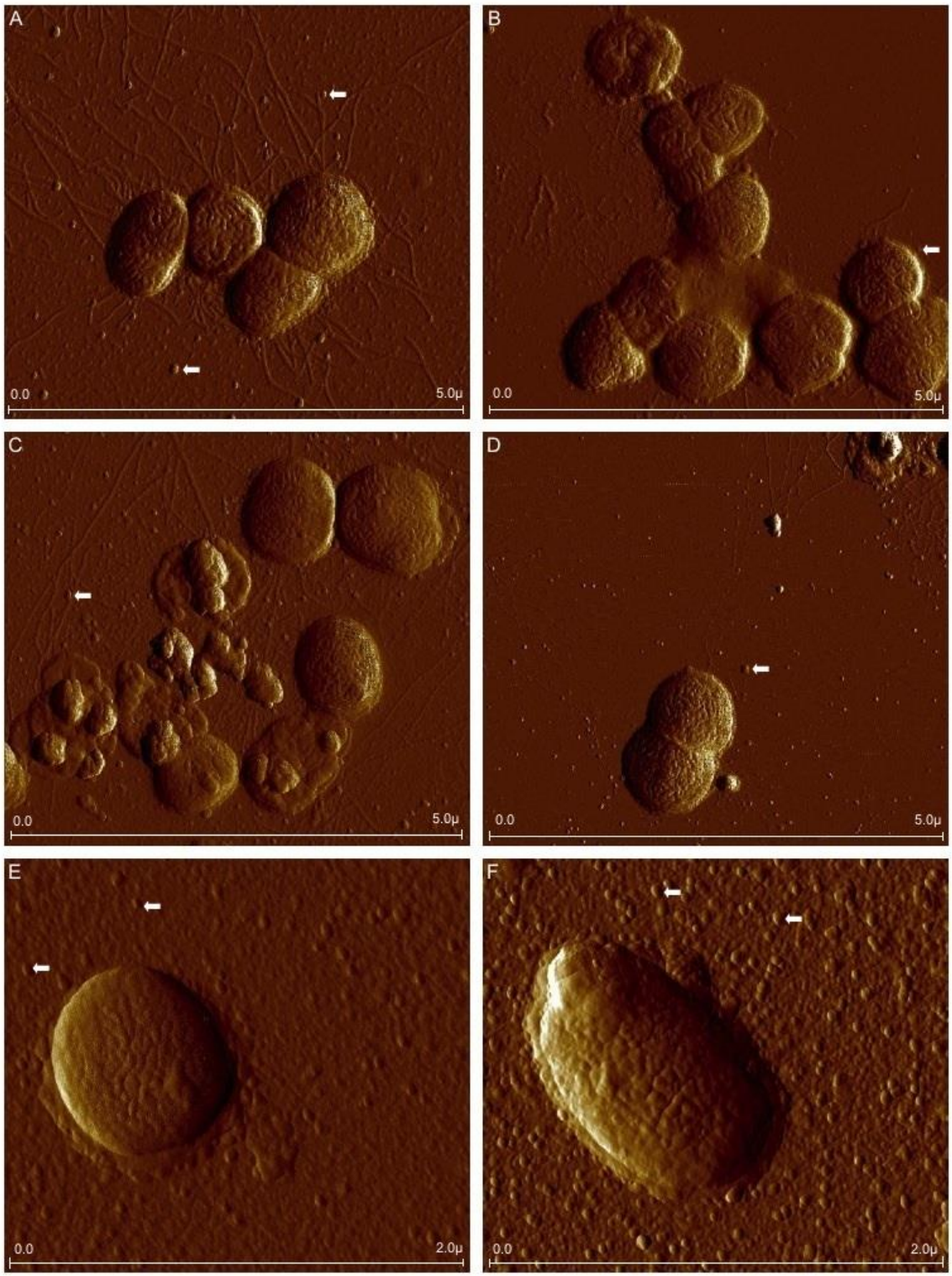

Figure 1. Atomic Force Microscopy analysis of *A. actinomycetemcomitans* strains grown on agar was conducted to visualize pili and OMVs. Arrows indicate examples of the released OMVs. Strains shown and the growth temperatures used are as indicated: Fig. 1a) D7S 30°C, b) D7S *hns* 30°C, c) D7S 37°C, d) D7S *hns* 37°C, e) D7SS 37°C, f) D7SS *hns* 37°C. A Nanoscope V atomic force was used for imaging, with tapping mode. The final images, which were plane fitted in both the x and y-axes, are presented in amplitude mode. The images displaying wildtypes are shown in the left column, compared to *hns* mutants, which are placed to the right. No images of D7SS 30°C and D7SS *hns* 30°C were obtained.

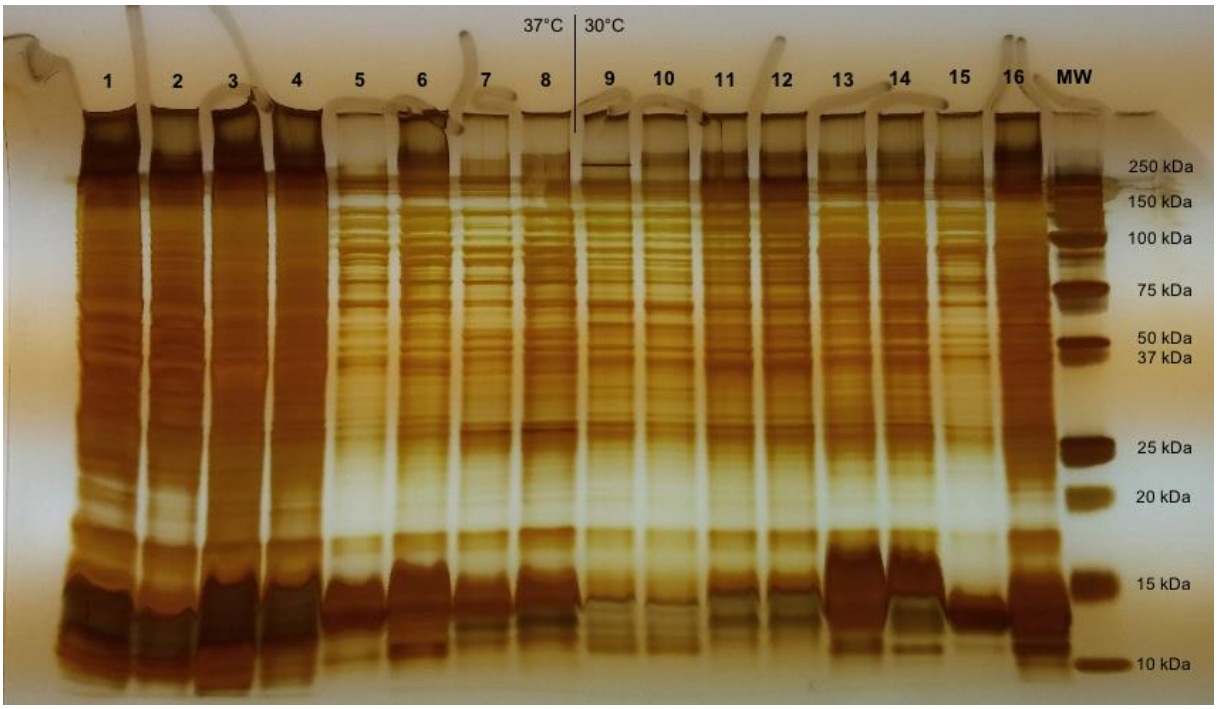

Figure 2. SDS-PAGE analysis of *A. actinomycetemcomitans* whole cell protein extracts (indicated by OD2), and supernatants (OD6) obtained from strains D7S and D7SS, and their respective *hns* mutants as indicated. The strains were cultivated at 37ºC (left side), and 30ºC (right side). The gel was Silver-stained using The Pierce Silver Stain Kit from Thermo Scientific. For reference the Precision Plus Protein All Blue Standards were used to display standard molecular weight, and loaded to the right in this image (MW). The molecular sizes (kDa) of these bands are indicated. 1) D7S OD2, 2) D7S hns OD2, 3) D7SS OD2, 4) D7SS hns OD2, 5) D7S OD 6, 6) D7S hns OD6, 7) D7SS OD6, 8) D7SS hns OD6, 9) D7S OD2, 10) D7S hns OD2, 11) D7SS OD2, 12) D7SS hns OD2, 13) D7S OD 6, 14) D7S hns OD6, 15) D7SS OD6, 16) D7SS hns OD6.

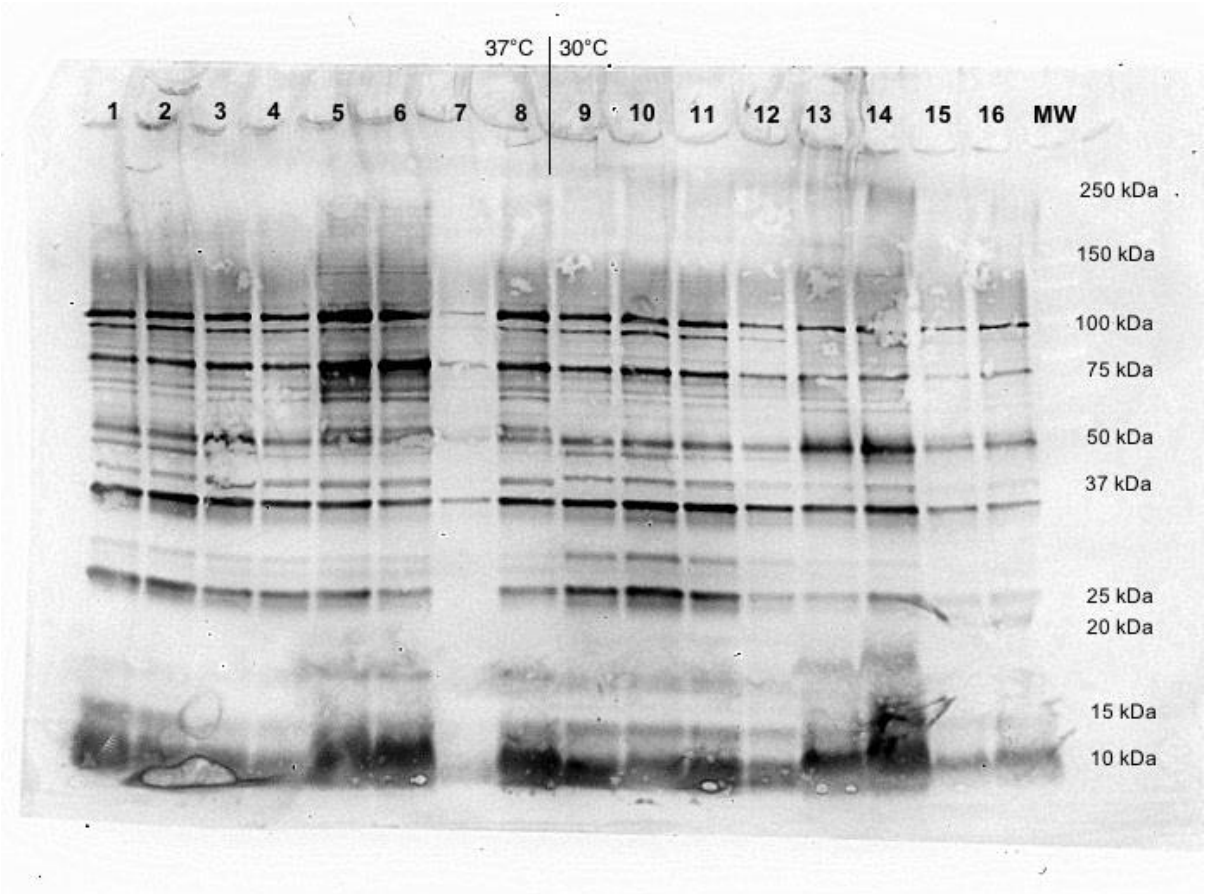

Figure 3. Western blotting using a serotype a-specific antibody. Loaded on the gel are *A. actinomycetemcomitans* whole cell protein extracts (indicated by OD2), and supernatants (OD6) obtained from strains D7S and D7SS, and their respective *hns* mutants as indicated. The strains were cultivated at 37ºC (left side), and 30ºC (right side). Proteins were transferred from Criterion precast gel to a membrane. Donkey-anti-rabbit HRP-conjugate was used as a secondary antibody. 1) D7S OD2, 2) D7S hns OD2, 3) D7SS OD2, 4) D7SS hns OD2, 5) D7S OD 6, 6) D7S hns OD6, 7) D7SS OD6, 8) D7SS hns OD6, 9) D7S OD2, 10) D7S hns OD2, 11) D7SS OD2, 12) D7SS hns OD2, 13) D7S OD 6, 14) D7S hns OD6, 15) D7SS OD6, 16) D7SS hns OD6.

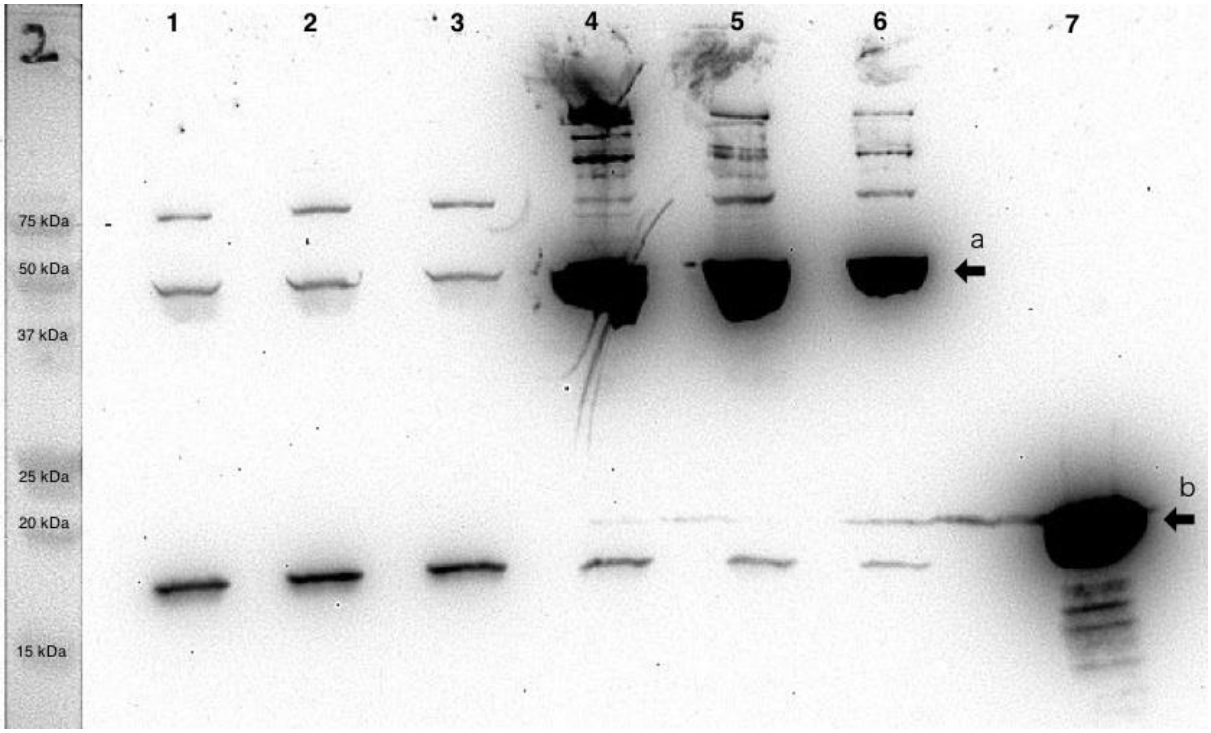

Figure 4. Western blotting using an RcpA-specific antibody. Analyzed on the gel are *A. actinomycetemcomitans* whole cell protein extracts (OD2) from bacteria cultivated at 37°C, which are displayed as follows 1) D7SS, 2) D7SS *hns*, 3) D7SS *hns*, 4) D7S, 5) D7S *hns*, 6) D7S *hns*. An N-terminal fragment of RcpA was used as a positive control (sample 7). RcpA monomers are seen at 43 kDa. For reference the Precision Plus Protein All Blue Standards were loaded to the left in this image. The molecular sizes (kDa) of these bands are indicated. Proteins were transferred from Criterion precast gel to a membrane. Donkey-anti-rabbit HRP-conjugate was used as a secondary antibody. The arrows indicate RcpA monomer (a), and the N-terminal fragment of RcpA (b), respectively. The N-terminal fragment was loaded as a control.