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High Throughput Quantitative Method for Assessing Coaggregation among Oral Bacterial Species

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Abbreviated Title: Coaggregation: High-throughput Assessment Method

Significance and Impact of the Study

Coaggregation between bacterial species is integral to multi-species biofilm development. Difficulties in rapidly and reproducibly identifying and quantifying coaggregation have limited mechanistic studies. This paper demonstrates two complementary quantitative methods to screen for coaggregation. The first approach uses a microplate-based high-throughput approach and the other uses a FlowCam™ device. The microplate-based approach enables rapid detection of coaggregation between candidate coaggregating pairs of strains simultaneously while controlling for variation between replicates. The FlowCam™ approach allows for in-depth analysis of the rates of coaggregation and size of aggregates formed.

Abstract

This paper describes a high throughput method that relies upon a microplate reader to score coaggregation 60 minutes post mixing, and use of a high-speed real-time imaging technology to describe the rate of coaggregation over time. The results of visual, microplate, and FlowCam™ aggregation scores for oral bacteria *Streptococcus gordonii, Streptococcus oralis*, and *Actinomyces oris,* whose ability to coaggregate are well characterized, are compared. Following mixing of all possible pairs, the top fraction of the supernatant was added to a microplate to quantify cell-density. Pairs were also passed through a flow cell within a FlowCam™ to quantify the rate of coaggregation of each pair. Results from both the microplate and FlowCam™ approaches correlated with corresponding visual coaggregation scores and microscopic observations. The microplate-based assay enables high throughput screening, whereas the FlowCam™-based assay validates and quantifies the extent that autoaggregation and coaggregation occur. Together these assays open the door for future in-depth studies of autoaggregation and coaggregation among large panels of test strains.

Keywords: Coaggregation, *Actinomycetes*, Biofilms, Biotechnology, *Streptococci*

Introduction

Coaggregation is defined as the highly specific recognition and adherence of different species of bacteria with each other (Kolenbrander 1988; Rickardet al. 2003). Coaggregation typically occurs as a consequence of the expression of protein adhesins on the cell-surface of one bacterial species, and complementary polysaccharide-containing receptors expressed on the surface of the other bacterial species (Kolenbrander 1988; Rickard et al. 2003).

Coaggregation interactions are important for the development of multi-species biofilms (e.g. dental plaque). It contributes to biofilm development via at least two mechanisms: **i**) free floating planktonic cells of one species specifically recognize cells of another species type and co-adhere to the developing biofilm, and/or **ii**) bacterial cells of a planktonic species recognize and coaggregate with cells of another species within an established biofilm community (Rickard et al. 2003; Kolenbrander et al. 2010). It is likely that these interactions are important for adherence and colonization of bacteria to a variety of biotic and abiotic surfaces, and provide selective advantages against non-coaggregated bacterial species contained within a biofilm (Kolenbrander et al. 1990; Busscher and Van Der Mei 1995; Burmølle et al. 2006; Kolenbrander et al. 2010).

The oral microorganisms *Streptococcus oralis, Streptococcus gordonii* and *Actinomyces oris* strongly coaggregate and are considered early colonizers in the process of dental plaque formation. (Kolenbrander 2000). Early colonizers anchor the biofilm to the substratum surface and thereby contribute to recalcitrance of the biofilm to removal (Busscher and Van Der Mei 1995). For example, the presence of *A. oris* greatly reduces the ability of the *S. oralis* to be removed by shear force when compared with direct attachment of *S. oralis* to the pellicle (proteinaceous conditioning film formed on the tooth surface) (Busscher and Van Der Mei 1995). Furthermore, early colonizers provide a foundation for other species to adhere, forming a mature biofilm community (Busscher and Van Der Mei 1995). Through autoaggregation (aggregation within a single species) or coaggregation, organisms can individually and collectively obtain increased resistance towards antimicrobials and shear forces, communicate via cell-cell signaling, and share nutrients (Kinder and Holt 1994; Watnick and Kolter 2000; Rickard et al. 2003). Research using model dental plaque systems has shown nutritionally mutualistic relationships occurring between coaggregating organisms (Bradshaw 1994; Palmer et al. 2001). For example, *A. oris* and *S. oralis* displayed limited to no growth when grown in monoculture with saliva as the nutrient source, but thrived when allowed to coaggregate (Palmer 2001).

Traditionally, coaggregation is assessed using a visual scoring system based on the size of the coaggregates and turbidity of the supernatant fluid (Cisar et al. 1979; Gilbert et al. 2002; Vornhagen et al. 2013). However, as visual scoring is only semi-quantitative, it is subject to inconsistency and bias in scoring (Busscher and Van Der Mei 1995). Another method, measuring the percent change in optical density, provides a quantitative assessment and greatly improves reliability and reproducibility. However, current methods are not amenable to screening of larger numbers of samples simultaneously (Ikegami et al. 2004; Ledder et al. 2008; Nagaoka et al. 2008; Arzmi et al. 2015) and these technological insufficiencies have limited the in depth study of coaggregation (Katharios-Lanwermeyer et al. 2014). The ability to include multiple replicates in a single experiment is highly desirable, as there may be strain variations in coaggregation requiring multiple crosses to determine if the observed coaggregation occurs generally between two species. Furthermore, because bacterial coaggregation is sensitive to a variety of influences including presence of chelating agents (Taweechaisupapong and Doyle 2000), temperature (Postollec et al. 2005), growth media, and pH (Min et al. 2010), and growth phase of the batch culture cells (Rickard et al. 2000), high throughput methods would be highly desirable to improve reproducibility of results.

Cognizant of these issues, we developed a quantitative method for high throughput screening for coaggregation among bacterial species. Our high throughput method allows for simultaneous analysis of multiple replicates so that experimental variation is reduced and possible subjective bias is minimized. This method's accuracy as a preliminary screening tool was validated using confocal microcopy and a recently developed approach using FlowCam™ technology (Segaloff 2014). A FlowCam™ is a dynamic imaging particle analyzer that examines fluid through a microscope and captures images of the particles as they are pumped through a flow cell via a computer controlled syringe pump. Specifically, a FlowCam™ characterizes the particles using a variety of measurements such as area-based diameter. It has been used in a variety of different industries including aquatic research, algae technology, waste management, pharmaceutical, and oil and gases for purposes such as monitoring algae for biofuels, quantifying protein aggregates in pharmaceuticals, and analyzing drilling products (http://www.fluidimaging.com/). In practice, the high throughput method can be used to screen a large panel of test strains for potential coaggregation. Ideally, strains giving a positive result with the high throughput method would then be tested further using either confocal microscopy or FlowCam™.

Without a high throughput, quantitative method for assessing coaggregation, it is difficult to explore the importance of coaggregation for the development of biofilms. A better understanding of coaggregation can provide a deeper knowledge of how organisms interact and biofilms form. For example, the presence of biofilms can result in the corrosion of sewer pipes. An improved understanding of if and how organisms coaggregate in these biofilms could help in developing strategies to reduce the detrimental effects of species in biofilms on pipe surfaces (Jensen et al. 2016). A high throughput screening method would also be of interest to the dental research community as identifying coaggregation between oral bacterial species (beyond those already known) could be an important step in developing a fuller understanding of dental plaque development (Kolenbrander et al. 1990). Coaggregation may also be an important mechanism through which pathogens interact with the host microbiota. Younes et al. demonstrated a rapid anti-pathogen effect of probiotic lactobacilli with toxic shock syndrome toxin 1-producing *Staphylococcus aureus* strains as a result of coaggregation (Younes et al. 2012). High throughput studies of coaggregation between organisms could be useful in identifying probiotic species.

Results and Discussion:

High throughput quantitative method increases validity and reliability of results

Three strains of oral bacteria were used: *Streptococcus oralis* 34, *Streptococcus gordonii DL1*, and *Actinomyces oris* T14V. *S. gordonii* is a primary colonizer in dental plaques and was previously found to coaggregate with both *A. oris T14V and S. oralis 34* (Cisar et al. 1979). All possible pairwise combinations of these three strains were tested, resulting in six potential coaggregative or autoaggregative pairings. Coaggregation was first assessed in a low throughput format and scored using the visual scoring system developed by Cisar and colleagues (Cisar et al. 1979). As shown in Figure 1A, the maximum visual coaggregation (score = 4) is easily distinguished from no coaggregation (score = 0), but visual intermediate scoring is more subjective and as a consequence is less reproducible.

Using the microplate-based approach with OD 620 nm, it was determined that *S. gordonii DL1* + *A. oris T14V*, *S. oralis 34* + *A. oris T14V*, and *S. oralis* 34+ *S. gordonii DL1* all strongly coaggregated (Figure 1B). No autoaggregation was detected for *S. oralis 34* or *S. gordonii DL1*. Some autoaggregation was observed for *A. oris T14V*, although the OD did not differ significantly from that of non-autoaggregating strains.

Within each micro-plate run, pairs were assayed in triplicate. The average coefficient of variation (standard error / mean) for the triplicates was 14% with a median of 9%. The coefficient of variations differed by specific crosses with autoaggregation by *A. oris* being the most variable (ranging from 1-66%). An increase in the coefficient of variation for coaggregation was also observed when *A. oris* was a component of a coaggregating pair.

Many factors contribute to between run variations: bacteria are harvested from separately grown batch cultures, which may differ slightly in length of growth time, exact nutrient content and pH of media, pH of buffers used for washing and re-suspension, number of times strain has been passaged before current growth, and natural biological variation. To minimize these variations, bacteria of a given species were harvested from the same batch culture, although some variation might remain due to the slight variations in timing between admixture and measurement, and true biologic variation in the amount of autoaggregation occurring within each species of a given candidate coaggregative pairing.

Between run and within run variation highlight the need for multiple replicates of each candidate coaggregative pair in addition to replicates of each strain on its own (to assess autoaggregation) within a single run. This is easily possible using the high-throughput 96-well plate method. Quantitatively comparing coaggregative and autoaggregative behavior within a single run also enables more accurate assessments of coaggregation by controlling for any autoaggregation that may occur, and multiple replicates of all strains and strain pairs allow for construction of confidence intervals around the mean OD value for a given strain or strain pair.

FlowCam™ Technology can measure particle sizes and quantify rate of coaggregation

FlowCam™ technology was used to validate the high-throughput 96-well plate system, providing an in-depth analysis of the rates of coaggregation, and visual and quantitative assessment of the size of aggregates formed (Segaloff et al. 2014). The average particle size per minute increased over time for all three coaggregative pairings (Figure 2). By minute three (two minutes post-mixing) all coaggregating strains experienced a statistically significant increase in average particle area per minute as calculated using area-based diameter. Strong coaggregation occurred when *S. gordonii* and *A. oris* were combined, with particles averaging 212 μm^2 per minute and reaching as large as 3,800 μm^2 in area. The coaggregation between *S. oralis* and *S. gordonii* was not as strong. Particle sizes averaged 122 μm^2 by the final minute of data collection and reached a maximum area of approximately 2,950 μm^2, but many

cells did not coaggregate and remained in suspension. This variation in coaggregation between this pair resulted in large confidence intervals around each time point. *S. oralis* and *A. oris* coaggregated strongly with particle sizes averaging 215 μm^2 in area by minute two and reaching sizes as large as 3,180 μm^2. In the autoaggregation assays, the average area of *A. oris* particles (83 μm^2) was significantly larger than those of *S. oralis* (35 μm^2) and *S. gordonii* (51 μm^2), indicating strong autoaggregation in this species. Here, the use of FlowCam™ allowed for quantification of rates of coaggregation and measurement of the particle size associated with coaggregation. Results from FlowCam™ correlated well with the results of the high throughput screen, with coaggregation indicated by increases in particle size over time following the addition of the second organism and autoaggregation indicated by larger particle sizes. FlowCam™ was more useful for detecting autoaggregation than the high throughput screening method on its own, which did not show a statistically significant difference between autoaggregating and non-autoaggregating strains. These results validate the use of the high throughput method as an initial screening step to be followed up with a more confirmatory assay such as FlowCam™ or confocal microscopy.

Confocal microscopy confirms presence of coaggregation

As a further confirmation, the strains were stained using Syto-9 (green) or Syto-61 (red) nucleic acid stains before crossing them for coaggregation, and then visualized using a confocal microscope (Figure 3). Confocal microscopy images confirmed that *S. oralis* and *S. gordonii* do not autoaggregate (Figure 3, A and C). Visualization of *A. oris* alone confirmed strong autoaggregative behavior (Figure 3B). This was not immediately apparent from initial absorbance readings from the high throughput screening method because autoaggregation was not followed by immediate sedimentation (Figure 1B). This finding is consistent with previous reports. Koop and colleagues showed autoaggregation without associated sedimentation could be missed by spectrophotometry (Koop et al. 1989), highlighting the importance of using a combination of methods for detection. The high-throughput 96-well plate method is most appropriately applied as a screen for potentially coaggregating pairs from a large pool of candidates. Potentially coaggregating pairs should be further evaluated using FlowCam™ or confocal microscopy, ideally both. Moreover, if coaggregation is suspected, autoaggregation should be ruled out.

S. oralis + *S. gordonii* showed moderate coaggregation (Figure 3D) while *S. gordonii + A. oris* (Figure 3E) and *S. oralis* + *A. oris* (Figure 3F) showed strong coaggregation. *S. oralis* and *S. gordonii* appeared to coaggregate in a more even manner, suggesting absence of autoaggregation within the two

species (Figure 3D), while *S. oralis* + *A. oris* and *S. gordonii* + *A. oris* showed clumps of the same color (red or green) indicating strong autoaggregative behavior by *A. oris* (Figure 3, E and F).

Summary

Focusing on the interactions between three well-documented coaggregating strains of oral bacteria, we demonstrated that coaggregation can be quantified, and the kinetics of coaggregation and the size of coaggregates formed can be measured. The microplate-based assay enables high throughput screening to identify potentially coaggregating strains, whereas the FlowCam-based assay validates and quantifies the extent that aggregation and coaggregation occur. In the absence of FlowCam™, or in combination with its use, confocal microscopy is a useful tool for confirming the presence or absence of coaggregation following screening of a large panel of strains with the high throughput method. Together these assays open the door for in-depth studies of aggregation and coaggregation among large panels of test strains.

Materials and Methods:

Growth Conditions

S. oralis 34 and *S. gordonii DL1* were incubated aerobically with CO_2 at 37°C in Schaedler's broth for 24 hours. *A. oris T14V* was incubated aerobically with CO_2 at 37°C in Brain Heart Infusion broth for 48 hours. Cells were harvested from batch culture through centrifugation for 12.5 minutes at 3,000 X g and then washed 3 times in coaggregation buffer (Cisar et al. 1979; Rickard et al. 2000). After each centrifugation step, the supernatant was discarded and the pellet was re-suspended in coaggregation buffer. The washed pellets were then suspended in coaggregation buffer to achieve an optical density at 600 nm of 1.5 (±0.1).

Coaggregation and Autoaggregation assays

Coaggregation and autoaggregation were first assessed using a visual coaggregation assay developed by Cisar et al. where visual scores ranged from 0 (no visible aggregates in the suspension) to 4 (large aggregates form and settle leaving a clear supernatant) (Cisar et al. 1979). To assess coaggregation between two strains, 200 µl of each bacterial suspension were combined in a glass culture tube. To assess autoaggregation 400 µl of the single bacterial suspension as placed in a glass

culture tube. The culture tubes were then vortexed for ten seconds and rolled gently for an additional 30 seconds (Rickard et al. 2000). Each pair was assayed in triplicate.

Samples were allowed to sit 60 minutes to let coaggregates settle to the bottom of the tube. Any changes in visual coaggregation score following the 60-minute time period were documented. This endpoint was selected after initial testing of the supernatant at 30 minute time intervals over 3 hours; 60 minutes was ideal for good separation between coaggregating and non-coaggregating strains. One hundred microliters of supernatant were removed from each sample and placed in a 96 well flat-bottom plate. Absorbance of the supernatant was measured at 620 (A) using a PerkinElmer 2030 workstation (PerkinElmer Life and Analytical Sciences, Turku, Finland). Mean OD and associated 95% confidence intervals were calculated over all trials for each of the strain pairs and for each strain alone. Because strains were set to the same optical density (1.5) before they were combined, an expected value for the combined pair was calculated based on the average experimental OD of the two components. The mean OD, 95% confidence interval, minimum and maximum for each pair was then compared with the calculated expected value for the pair. Coaggregation was suspected when the expected value was above the upper limit for the 95% confidence interval and was considered when the mean OD was below the expected value. In all cases meeting these criteria, further screening was conducted using FlowCam™ and confocal microscopy.

As an additional visual test of autoaggregation and coaggregation between strains, 300 µl of each bacterial suspension in coaggregation buffer were stained with either Syto-9 (green: Excitation: 488, Emission: 503) or Syto-61 (red: Excitation: 561, Emission: 645) nucleic acid stains (Invitrogen, Carlsbad, CA, USA). Each bacterial suspension was incubated for 30 minutes at room temperature to allow staining of the cells. Cells were washed three times with coaggregation buffer and collected by centrifugation, as mentioned above. Each bacterial strain was re-suspended in coaggregation buffer and combined for coaggregation. For autoaggregation studies, Syto-9 and Syto-61 stained cells of that species were mixed together. Twenty microliters of each sample were added to the slide and viewed under the microscope. The entire droplet was scanned and a minimum of three representative fields of view were captured for each pair and for each strain alone using Leica confocal laser scanning microscopy (CLSM, SPE, Leica, IL, USA) with a HCX PL APO 40X/0.85 CORR CS objective. Staining and microscopy were repeated twice to ensure consistency of results." Once the microscopy images were

taken, the image files were rendered using Imaris (Bitplane, Zurich, Switzerland) computer imaging software.

FlowCam™ Imaging and Quantification of Coaggregation

To confirm the results of our findings, coaggregation was quantified using FlowCam™ technology (Fluid Imaging Technologies, ME, USA). *S. oralis* 34, *S. gordonii DL1* and *A. oris T14V* were harvested from batch cultures and washed as described above. The washed pellets were re-suspended in coaggregation buffer to achieve an optical density of 1.0 (±0.1) at 600 nm. Prior to loading the cells into the FlowCam™ device, cell suspensions were further diluted 5X in coaggregation buffer to prevent clogging of flow cell. The first species was added to the device and was pumped through until it reached the flow cell. Data collection began once the Olympus UPlanFL N 10X/0.30 objective was successfully focused on the flowing particles. The second species was added to the vessel containing the first species and gently mixed 1 minute after initiation of data collection. FlowCam™ was run for 10 minutes at a flow rate of 0.3 ml/min with images acquired at a rate of 10 frames per second. Flash duration was set to 8 µSec. Particle size was measured using area based diameter (ABD) and a particle filter of 5 to 10000 µm. Visual spreadsheet software was used for data collection. A minimum of 5 FlowCam runs was conducted for each pair with similar results.

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Conflict of Interest:

The authors declare no conflict of interest associated with this research.

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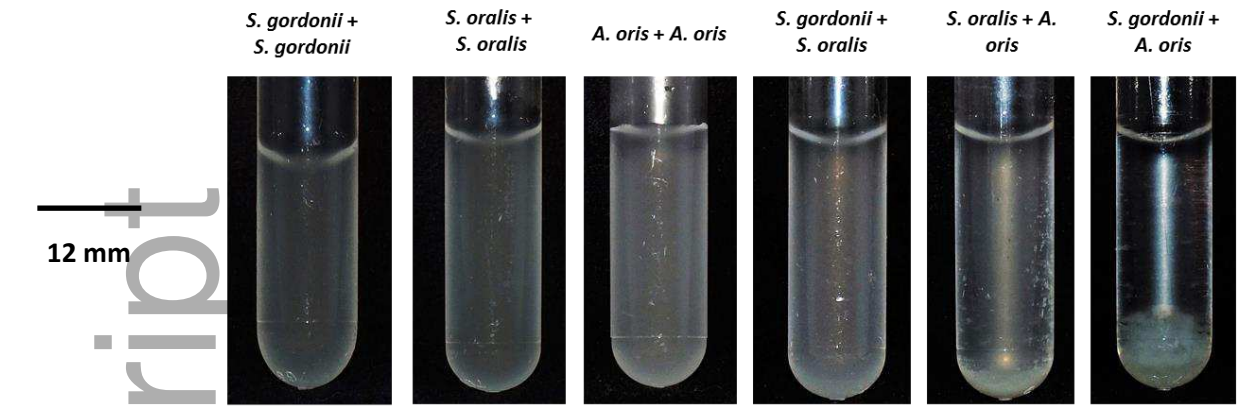
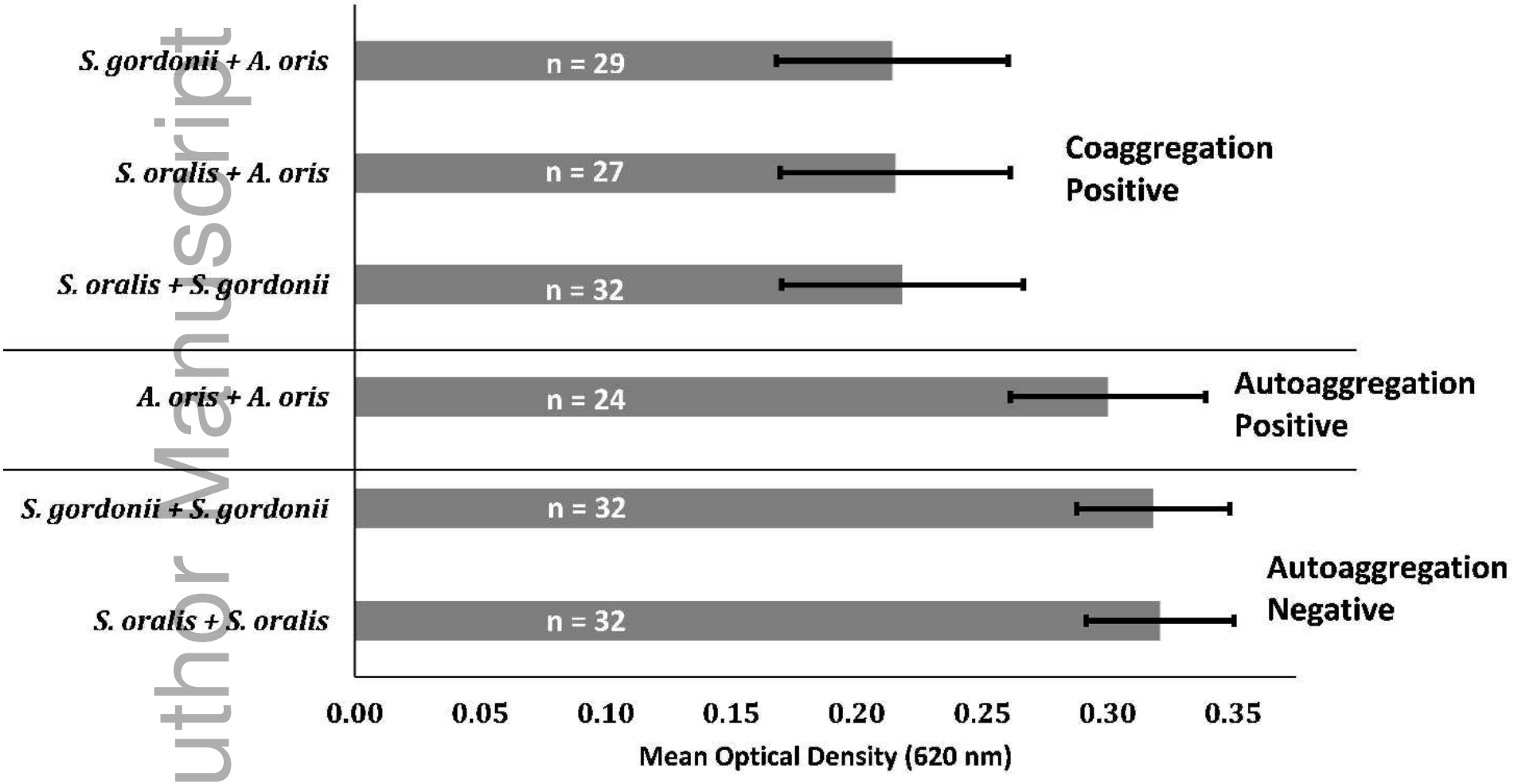
383 **Figures:**

384 **Figure 1.** (**A**) Test crosses of pairwise combinations of S. *gordonii*, *S. oralis*, and *A. oris* with associated
385 visual coaggregation score based on methodology by Cisar et al. 1979. (**B**) Mean Optical Density (620
386 nm) of supernatant with associated 95% confidence intervals of test crosses.

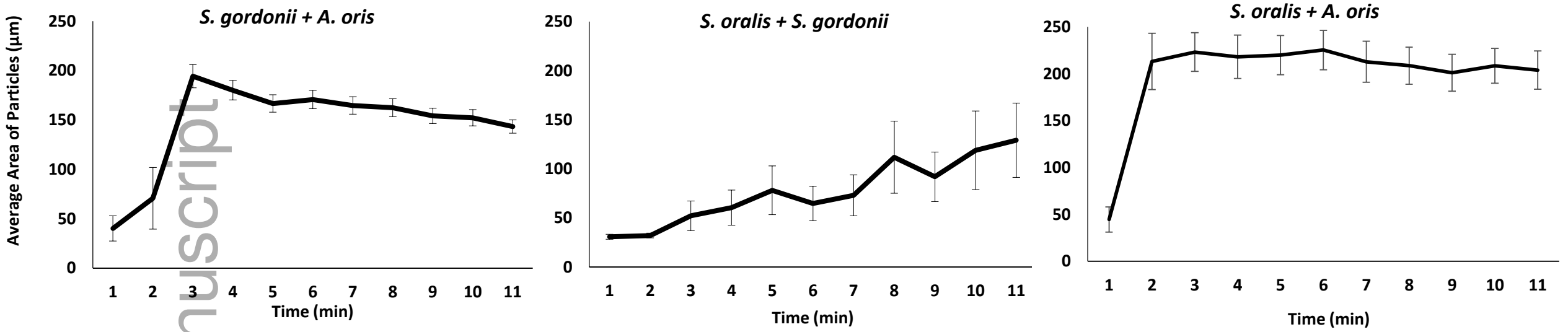
387 **Figure 2.** Change in average particle area (μm) per minute during 10 minute time period with associated
388 95% confidence intervals calculated from number of particles scanned per minute for potential (**A**)
389 coaggregating (*S. gordonii + A. oris, S. oralis + S. gordonii, S. oralis +A. oris*) and (**B**) autoaggregating (*S.*
390 *gordonii, A. oris, S. oralis*) pairs as measured with the FlowCam™ device. Calculations were based on an
391 average of 2,009 particles per minute (median = 707, maximum = 5,867, minimum = 24).

392 **Figure 3. Visualization of selection bacterial pairings using confocal microscopy.** Confocal microscopy
393 images are represented in the x-y plane. Nucleic acid stains syto-9 (green) and syto-61 (red) was used to
394 detect autoaggregation and coaggregation of oral microbes. Bars represent 40 μM.

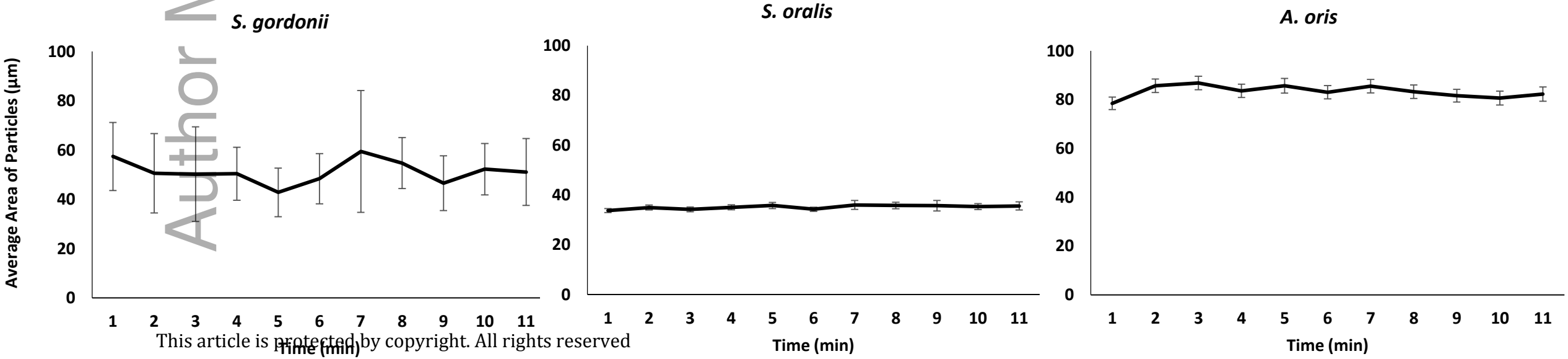


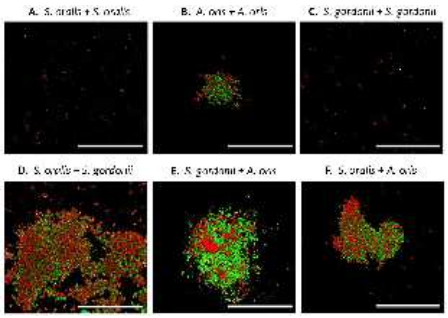


A. Coaggregation pairings



B. Autoaggregation pairings





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