***Streptococcus mutans* membrane vesicles, containing insoluble glucan synthase and extracellular DNA, contribute to the promotion of initial attachment and colonization of *Actinomyces oris***

Running title: Promotion of INAC of *A. oris* by *S. mutans* MVs

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**Abstract**

Oral biofilms contribute to oral infectious diseases such as dental caries and periodontal disease. *Actinomyces oris*, an initial attachment colonizer, uses fimbriae (FimA, FimP, and FimQ) presented on the surface layer of the bacteria for biofilm formation. *Streptococcus mutans*, a cariogenic bacteria, produces membrane vesicles (MVs) with insoluble glucan synthases (GtfB and GtfC) and extracellular DNA (eDNA). These MVs promote biofilm formation. However, few reports are available on the relationship between *S. mutans*-produced MVs and *A. oris*. Herein, we investigated the effect of *S. mutans*-produced MVs on the initial attachment and colonization, (INAC), and biofilm formation of *A. oris*. MVs at a final concentration of 3.125 µg/mL promoted the INAC formation of WT, Δ*fimA*, Δ*fimP*, and Δ*fimQ* of *A. oris*. However, MVs from *S. mutans* Δ*gtfBC*, lacking insoluble glucan synthase, did not induce the INAC formation in *A. oris* wild type and mutants. Deoxyribonuclease inhibited INAC formation of *A. oris* WT and Δ*fimA* produced by MVs. The present study indicated that *S. mutans*-produced MVs induced fimbriae-independent INAC formation of *A. oris* via insoluble glucan synthase. The formation of insoluble glucan-dependent INAC in *A. oris* via *S. mutans*-produced MVs may result from the interaction between *A. oris* type 1 fimbriae with eDNA from MVs, potentially contributing to the stabilization of the INAC structure.

**Introduction**

More than 700 species of bacteria exist in the human oral cavity (1). Approximately 400 are found in periodontal pockets and 300 in the tongue, buccal mucosa, caries cavity, and periapical lesions (2). Oral bacteria form biofilms in the oral cavity, contributing to oral infections such as dental caries and periodontal disease (3, 4). Oral biofilms are initially formed by the interaction of salivary pellicles and oral bacteria on tooth surfaces (e.g., enamel), where initial

colonizers attach and aggregate. Middle and late colonizers subsequently attach and co-aggregate, forming a mature oral biofilm (5). This mature biofilm releases factors that destroy epithelium, damage connective tissue, exhibit cell toxicity, and induce inflammation, contributing to the initial development of periodontal disease (6, 7).

Initial attachments and colonization are caused by *Streptococcus oralis*, *Streptococcus gordonii*, *Streptococcus mitis*, and *Actinomyces oris* (5). *A. oris* is a facultative anaerobic gram-positive bacterium, formerly classified as *Actinomyces naeslundii* genospecies 2 (8). *A. oris* has two types of surface fimbrillin: Type 1 fimbriae, composed of shaft fimbrillin FimP and tip fimbrillin FimQ, facilitates initial attachment. FimQ primarily interacts with high proline proteins (PRPs) in tooth surface salivary pellicles (9, 10). Alternatively, Type 2 fimbriae, composed of shaft fimbrillin FimA and tip fimbrillin FimB and CafA, contributes to aggregation and coaggregation with other oral bacteria. FimA primarily mediates biofilm formation of *A. oris* and CafA facilitates coaggregation with oral streptococci (11, 12). *A. oris* promotes initial attachment and colonization (INAC), and biofilm formation via short-chain fatty acids metabolized by *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, both associated with periodontal diseases (13, 14). *Actinomyces* spp. have been detected in saliva and plaque in the human oral cavity, and are more frequently detected than *Streptococcus* spp. (15). *Actinomyces* spp. have also been reported to be present in the oral cavity of 6-month-old children (16). Moreover, *A. oris* has been detected in infective endocarditis (17).

Membrane vesicles (MVs) are spherical, bilayer-structured materials released from bacteria, with sizes ranging from 20 to 400 nm (18). MVs contain DNA, RNA, and various proteins, playing roles in cell-cell interaction, cargo delivery, bacterial growth, and bacterial survival (18-20). *Streptococcus mutans*, a cariogenic gram-positive bacterium, produces MVs with Glucosyltransferase B and C (GtfBC) adhered to their surface. GtfB synthesizes of insoluble glucan (21), whereas GtfC synthesizes insoluble and soluble glucan in conditions including

sucrose (22). The synthesis of insoluble glucan induces biofilm formation by initial colonizers such as *A. oris* and *Candida albicans* (23-25).

The extracellular DNA (eDNA) produced by bacteria plays a role in biofilm formation, being one of the extracellular polymeric substances in bacterial biofilms (26, 27). Deoxyribonuclease I (DNase I) inhibits biofilm formation in *S. mutans* and *Staphylococcus aureus* (28, 29). In addition, eDNA is crucial for rapid formation and structural stability of short-term biofilms (30, 31). Furthermore, *S. mutans*-produced MVs also contain eDNA (32).

Recent reports highlight the promotion of oral biofilm formation by MVs produced by several bacteria (18, 23-25). MVs, particularly those containing eDNA, may have multiple effects on the INAC and the biofilm formation. However, the effect of *S. mutans*-produced MVs on INAC of *A. oris* remains unclear. Therefore, this study investigated the effects of MVs and elucidated the contributions of pathogenic factors, such as *A. oris* fimbriae, insoluble glucan synthase, and eDNA from *S. mutans* to INAC formation in *A. oris*.

**Material and Methods**

*Bacterial strains*

*A. oris* MG1 wild type (*A. oris* WT) (13), *A. oris* MG1 *fimA* mutant (*A. oris* Δ*fimA*) (11), *A. oris* MG1 *fimP* mutant (*A. oris* Δ*fimP*) (10), *A. oris* MG1 *fimQ* mutant (*A. oris* Δ*fimQ*) (10), *S. mutans* UA159 wild type (*S. mutans* WT) (23), and *S. mutans* UA159 *gtfBC* mutant (*S. mutans* Δ*gtfBC*) (23) were incubated overnight in brain-heart infusion (BHI) broth at 37 °C for in an aerobic atmosphere of 5 % $CO_2$ (AnaeroPack, Mitsubishi Gas Chemical Co., Tokyo, Japan).

*Human saliva collection and sterilization*

Human stimulated saliva samples were collected from three healthy volunteers (25–36 years

old). The volunteers chewed paraffin gum for up to 3 min, and the pooled samples were placed in sterile centrifuge tubes and centrifuged at 3,000 × $g$ for 10 min at 4 °C. Supernatants were transferred into new sterile centrifuge tubes and sterilized using 0.45- and 0.22-micrometer Millex-GP filters (Merck Millipore, Bedford, MA, USA). Sterilized human saliva samples were stored at -20 °C until use.

*Membrane vesicles extraction*

The MVs extraction method was partially modified from a previous procedure (23). *S. mutans* WT and Δ*gtfBC* were incubated overnight in 500 mL BHI broth at 37 °C in a 5 % $CO_2$ aerobic atmosphere. First, bacterial culture was ultra-filtrated using VIVASPIN20 (Sartorius, Göttingen, Germany) at 3,000-4,000 × $g$ for 20 min at 4 °C. Thereafter, the concentrated solution was sterilized using 0.45- and 0.22-micrometer Millex-GP filters and ultracentrifuged using a Beckman SW 41 Ti swinging bucket rotor with a Beckman optima L-90k ultracentrifuge (Beckman Coulter, South Kraemer Boulevard, CA, USA) at 150,000 × $g$ for 2 h at 4 °C. The pellets of MVs were subsequently suspended in sterile phosphate-buffered saline (PBS). Finally, protein concentration of MVs sample was determined using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). MV samples were stored at -20 °C until use.

*INAC formation assay*

INAC formation was assayed using the method described by Suzuki et al. (14) with minor modifications. Briefly, sterile glass-bottom dishes (IWAKI; AGC Techno Glass Co., Shizuoka, Japan) were coated with human saliva for 1 h at 4 °C and washed with sterile PBS. Each strain of *A. oris* was centrifugated at 3,000 × $g$ for 10 min at 4 °C, and the pellet was rinsed with sterile PBS. The bacterial suspension was centrifugated again. The pellet of *A. oris* was diluted using sterile PBS. The concentrations of bacterial cells were adjusted to an optical density of 600 nm

$(OD_{600}) = 0.4$ using Tryptic Soy Broth with 0.25 % sucrose (TSBs). TSBs (180 µL) and 20 µL of *A. oris* dilutions were applied to saliva-coated glass-bottom dishes. To facilitate adherence to the bottom of the glass, bacterial dilutions were pre-incubated for 3 h at 37 °C in 5 % $CO_2$ environment. Following pre-incubation, the bottoms of the dishes were gently washed with sterile PBS to remove non-attached bacteria. Thereafter, 180 µL of fresh TSBs and 10 µL of MVs derived from *S. mutans*, with a final concentration 100 units (33) of DNase I (Takara Bio Inc., Otsu, Japan), were applied to bacteria-attached glass-bottom dishes. The bacterial cultures were subsequently incubated for 1 h at 37 °C in a 5 % $CO_2$ environment. Following incubation, the bottoms of the dishes were gently washed with sterile PBS to remove non-attached bacteria.

*Observation of INAC formation*

The INAC formation of *A. oris* were observed using the FilmTracer LIVE/DEAD Biofilm Viability Kit (Molecular Probes Inc., Eugene, OR, USA). Briefly, SYTO 9 and propidium iodide at final concentrations of 5 µM and 30 µM, respectively, were prepared in sterile PBS.

The mixture was gently applied to attached and colonized cells in a glass-bottom dishes. After 30 min of light-shielding and incubating at room temperature, excess staining was gently washed away with sterile distilled water (DW). Finally, fresh sterile DW was immediately added to prevent sample drying. Samples were imaged using Confocal Laser Scanning Microscopy (CLSM) (LSM700; Carl Zeiss, Oberkochen, Baden-Württemberg, Germany) with the ×10 objective. Three independent experiments were performed, and three random locations were scanned for each INAC sample. CLSM images of INAC were acquisitioned and processed using ZEN analysis software (Carl Zeiss). ImageJ 1.53k software (NIH, Bethesda, MD, USA) was used to calculate the percentage of live and dead cells areas in entire images of INAC formation.

## Results

*Effect of MVs from S. mutans on INAC formation of A. oris*

First, we investigated the effect of MVs derived from *S. mutans* on INAC formation of *A. oris*. MVs derived from *S. mutans* were used to induce INAC formation in *A. oris* WT, adjusted to final concentrations of 0.03125, 0.3125, 3.125, and 31.25 µg/mL. In CLSM images, the quantity of dead cells increased with the addition of MVs and peaked at 3.125 µg/mL (Fig. 1a). However, when 31.25 µg/mL of MVs was used, the quantity of dead cells decreased compared to 3.125 µg/mL MVs (Fig. 1a; H, K). There was no difference in the quantity of live cells at each concentration of MVs (Fig. 1a; A, D, G, J). Thereafter, the area numbers of live or dead cells in the CLSM images were quantified, revealing a significant increase in the quantity of dead cells when 3.125 µg/mL of MVs was used, compared to the other concentrations. The use of 31.25 µg/mL of MVs derived from *S. mutans* resulted in a significant decrease in the quantity of dead cells compared to 3.125 µg/mL of MVs derived from *S. mutans*. Alternatively, the quantity of live cells were poorly observed at each concentration (Fig. 1b). Based on these results, the appropriate concentration of MVs derived from *S. mutans* for the experiment was 3.125 µg/mL.

*Relationship between* MVs *and fimbriae in INAC formation in A. oris*

We investigated the relationship between *S. mutans*-derived MVs and the fimbriae of *A. oris* in the promotion of INAC formation in *A. oris*. Three points one hundred twenty five µg/mL of *S. mutans* MVs were applied to INAC formation in *A. oris* WT. In CLSM images, the INAC formation of *A. oris* WT with MVs tended to increase the amount of dead cells compared to *A. oris* WT without MVs (Fig. 2a; B, E). Quantification of CLSM images results showed a significant induction of dead cells in INAC formation of *A. oris* WT with MVs compared to *A. oris* WT without MVs (Fig. 2b). In CLSM images, INAC formation of *A. oris* Δ*fimA* with MVs increased dead cell quantities compared to *A. oris* WT without MVs (Fig. 2a; H, K). However, the dead cell quantities in INAC formation of *A. oris* Δ*fimA* with *S. mutans* MVs were lower than that of *A. oris* WT with *S. mutans* MVs (Fig. 2b). CLSM image quantification indicated a significant decrease in dead cells for INAC formation of *A. oris* Δ*fimA* with MVs compared to *A. oris* without MVs (Fig. 2b). MVs derived from *S. mutans* were also applied to INAC formation of *A. oris*Δ*fimP* and Δ*fimQ*, showing similar results to INAC formation of *A. oris*Δ*fimA* with MVs of *S. mutans* (Fig. 2c, d). Among all *A. oris* strains, no increase in live cells was observed with *S. mutans* MVs.

*Effect of insoluble glucan synthase from S. mutans MVs on INAC formation of A. oris*

We investigated the effect of insoluble glucan synthases from *S. mutans*-derived MVs on INAC formation in *A. oris*. To exclude the effect of insoluble glucan synthase, we performed INAC formation of *A. oris* using MVs derived from *S. mutans* Δ*gtfBC*, which lacks insoluble glucan synthase. In CLSM images, the INAC formation of *A. oris* WT with MVs from *S. mutans* ΔgtfBC showed no significant difference in the quantity of live and dead cells compared to the non-activated case (Fig. 3a; A to F). Similarly, image quantification results showed no

significant difference in the quantity of live and dead cells during INAC formation of *A. oris* WT when MVs of *S. mutans* Δ*gtfBC* were applied, compared to the non-activated case (Fig. 3b).

*Role of eDNA in INAC formation of A. oris promoted by MVs derived from S. mutans*

eDNA is thought to promote biofilm formation. MVs contain various substances (18). MVs derived from *S. mutans* also contain eDNA (23). Therefore, we investigated the role of eDNA on INAC formation of *A. oris* promoted by MVs of *S. mutans*. To exclude the influence of eDNA, DNase I was employed in the *A. oris* INAC formation assay. When DNase I was added to the INAC formation of *A. oris* WT, no significant difference in the quantities of live and dead cells was observed compared to the absence of DNase I in CLSM images (Fig. 4a; A to F), and the quantitative results yielded similar outcomes (Fig. 4b). Alternatively, in CLSM images, the quantities of dead cells in the INAC formation of *A. oris* WT was significantly reduced by the introduction of DNase I when *S. mutans* MVs were added (Fig. 4a; G to L), and the quantification corroborated a significant inhibition of dead cells (Fig. 4b). Moreover, DNase I significantly inhibited the INAC formation of *A. oris* Δ*fimA* (Fig. 4c, d). When DNase I was added to the INAC formation of *A. oris* Δ*fimP*, there was no significant difference in the quantities of live and dead cells of *A. oris* Δ*fimP* compared to *S. mutans* MVs without DNase I in CLSM images (Fig. 5a; A to F), and the quantitative results echoed this observation (Fig. 5b). When MVs of *S. mutans* and DNase I were introduced, there was no significant decrease in the quantities of dead cells of *A. oris* Δ*fimP* in INAC formation compared to *S. mutans* MVs without DNase I (Fig. 5a; G to L), and the quantitative results showed no significant inhibition of dead cells (Fig. 5b). Similar results were obtained for *A. oris* Δ*fimQ* when DNase I was added to the INAC formation*,* as evidenced by CLSM images and quantification (Figs. 5c, d).

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**Discussion**

MVs derived from *S. mutans*, a cariogenic bacterium, promote biofilm formation in early adherent populations (23). MVs derived from *P. gingivalis*, a periodontal pathogenic bacterium, aggregate with oral streptococci, *A. naeslundii*, *Actinomyces viscosus*, and *F. nucleatum*. In addition, oral streptococci treated with MVs produced by *P. gingivalis* can co-aggregate with *Staphylococus aureus* (34). Considering the interaction between oral bacteria and MVs derived from other oral bacteria is highly significant. Initial attachment bacteria, including *A. oris*, co-aggregate with various oral bacteria to form mature oral biofilms (5). In this study, we investigated the effect of *S. mutans*-produced MVs on INAC formation of *A . oris*.

INAC formation of *A. oris* increased in a concentration-dependent manner with *S. mutans*-produced MVs, primarily attributed to the augmentation of dead cells. In other words, *S. mutans*-produced MVs promote INAC formation by inducing cell death in *A. oris*, suggesting the induction of stress on *A. oris*. Notably, the MVs-promoted INAC formation by cell death of *A. oris* at final concentrations from 0.03125 to 3.125 μg/mL; however, at a final concentration of 31.25 μg/mL, INAC formation of *A. oris* was significantly reduced. *S. mutans*-produced MVs inhibited biofilm formation by decreasing gene expression involved in soluble and insoluble glucan production in the *S. gordonii* DL-1 and *S. sanguinis* ATCC10556 strains (35). MVs derived from the gram-negative bacterium *Burkholderia thailandensis* inhibited biofilm formation in *S. mutans* (36), influenced by broad-spectrum antimicrobial substances 4-hyoxy-3-methyl-2-(2-nonenyl)-quinoline and rhamnolipids present in MVs produced by *B. thailandensis* (37). The present results suggested that there is an optimal concentration for the promotion of INAC formation in *A. oris* by *S. mutans*-produced MVs. Moreover, this result indicated that at high concentrations, an unidentified factor in *S. mutans*-produced MVs inhibits INAC formation of *A. oris*. *A. oris* utilizes fructosyltransferase (Ftf) to produce levan-type

fructans from sucrose, which contributes to oral biofilm formation (38). High concentrations of *S. mutans*-produced MVs may contribute to the reduced fructan-forming capacity of *A. oris* and may act as an antimicrobial agent. However, the effect of *S. mutans*-produced MVs in fructan synthesis gene expression and their possession of antimicrobial substances remains unexplored. Furthermore, the actual concentration of MVs in the oral cavity has not been reported to our knowledge.

*A. oris* has two types of fimbriae on the bacterial surface that contribute to initial attachment, aggregation, and biofilm formation (9-12). *A. oris*Δ*fimA* increased cell death and promoted INAC formation when *S. mutans*-produced MVs were used. However, the quantities dead cells were reduced compared to that of *A. oris* WT. When *S. mutans*-produced MVs were treated, INAC formation of *A. oris* Δ*fimP* and *A. oris* Δ*fimQ* was similar to that of *A. oris* Δ*fimA*. Type 1 fimbriae contribute to initial attachment to the tooth surface. Wu et al. reported that adhesion experiments on PRP-coated latex beads were performed in 1- to 2-minute cultures (10). In this experiment, the enhancement of INAC formation by *S. mutans*-produced MVs by increasing cell death, independent of FimA, FimP, and FimQ of *A. oris*.

*S. mutans*-produced MVs have various components. One is an insoluble glucan synthase (GtfBC) and adheres to the surface of *S. mutans*-produced MVs (23-25). Therefore, we hypothesized that GtfBC is responsible for the enhancement of INAC formation in *A. oris* by *S. mutans*-produced MVs. In addition of *S. mutans* Δ*gtfBC*-produced MVs, there was no significant increase in the amount of live and dead cells of *A. oris*. In the presence of sucrose, GtfBC primarily synthesizes insoluble glucans (21, 22). It also binds to the salivary pellicle covering the tooth surface and helps *S. mutans* and *S. sanguinis* attach (39). GtfB adheres to *A. viscosus* in large numbers and contributes to the synthesis of insoluble glucans (40). These synthesized insoluble glucans have a substantial impact on caries (41,42). In summary, our results suggested that the insoluble glucans synthesized by GtfBC may be responsible for the

promotion of INAC formation of *A. oris* by *S. mutans*-produced MVs. Furthermore, it was suggested that the insoluble glucan may act in a fimbriae-independent manner in *A. oris*.

INAC formation of *A. oris* WT, Δ*fimA*, Δ*fimP*, and Δ*fimQ* were not significantly inhibited by DNase I when MVs was not added. In other words, these results suggested that INAC formation of *A. oris* is not dependent on eDNA. eDNA is an important component for the structural stability of young biofilms (18). INAC formation of *A. oris* WT and Δ*fimA*, the promotion of cell death-dependent INAC formation by MVs was significantly inhibited by DNase I. Notably, in *A. oris* Δ*fimP* and Δ*fimQ*, the promotion of cell death-dependent INAC formation by MVs was not inhibited by DNase I. Acid-base interaction of eDNA contribute the aggregation of bacteria with each other and their attachment to the surface (43). eDNA also facilitates the attachment of insoluble glucans formed by GtfB to saliva-coated tooth surfaces (44). In other words, our results suggested that eDNA derived from *S. mutans*-produced MVs may act in FimP and FimQ of *A. oris*. Moreover, it is suggested that MVs-derived insoluble glucan may contribute to the structural stability of INAC formation in *A. oris*.

In summary, MVs derived from *S. mutans* promoted INAC formation of *A. oris* in a fimbriae-independent and DNA-dependent manner. GtfBC of *S. mutans*-produced MVs synthesized insoluble glucan, which promoted INAC formation by *A. oris* in an insoluble glucan-dependent manner. In this insoluble glucan-dependent INAC of *A. oris*, type I fimbriae of *A. oris* may be involved in stabilizing the INAC structure by interacting with eDNA. This contributes to the elucidation of novel mechanisms involved in the onset and progression of oral infectious diseases such as dental caries and periodontal disease associated with *A. oris* and *S. mutans*, respectively.

**Ethical considerations**

This study was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo（EC23-008）.

## Conflict of Interest

The authors declare that they have no conflicts of interest associated with this manuscript.

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**Figure legends**

Fig. 1.

INAC formation of *A. oris* treatment with *S. mutans*-produced MVs in a concentration-dependent manner. (a) INAC formation of *A. oris* treatment with final concentrations ranging from 0.03125 to 31.25 µg/mL observed via CLSM. The left column shows live cells, the center column dead cells, and the right column shows merging. The bar indicates 200 µm. (b) Quantification of CLSM images using ImageJ software. The vertical axis indicates the ratio of initial attachment cells to all areas, and the horizontal axis indicates live and dead cells. The data indicate the mean ± standard deviation (SD) of three independent experiments. The results were analyzed using one-way ANOVA, *: $p < 0.05$, **: $p < 0.01$.

Fig. 2.

INAC formation of *A. oris* WT and fimbriae constitutive protein gene deletion strains after treatment with *S. mutans* WT-produced MVs. (a) CLSM images of INAC formation of *A. oris* WT and Δ*fimA* when *S. mutans* WT-produced MVs were added at a final concentration of 3.125 µg/mL. A to F: *A. oris* WT; G to L: *A. oris* Δ*fimA*. The left column shows live cells, the middle column shows dead cells, and the right column shows merging. The bar indicates 200 µm. (b) Quantification of CLSM images using ImageJ software. The vertical axis indicates the ratio of initial attachment cells to all areas, and the horizontal axis indicates live and dead cells. The results were analyzed using one-way ANOVA, **: $p < 0.01$. (c) CLSM images of INAC formation of *A. oris* Δ*fimP* and Δ*fimQ* when *S. mutans* WT-produced MVs were added at a final concentration of 3.125 µg/mL. A to F: *A. oris* Δ*fimP* ; G to L: *A. oris* Δ*fimQ*. The left column shows live cells, the middle column shows dead cells, and the right column shows merging. The bar indicates 200 µm. (d) Quantification of CLSM images using ImageJ software. The vertical axis indicates the ratio of initial attachment cells to all areas, and the horizontal axis

indicates live and dead cells. The data indicate the mean ± standard deviation (SD) of three independent experiments. The results were analyzed using one-way ANOVA, **: p <0.01.


Fig. 3.

INAC formation of *A. oris* WT and fimbriae constitutive protein gene deletion strains of *S. mutans* Δ*gtfBC*-produced MVs. (a) CLSM images of INAC formation of *A. oris* WT and Δ*fimA* when *S. mutans* Δ*gtfBC*-producing MVs were added at a final concentration of 3.125 μg/mL. A to F: *A. oris* WT; G to L: *A. oris* Δ*fimA*. The left column shows live cells, the middle column shows dead cells, and the right column shows merging. The bar indicates 200 μm. (b) Quantification of CLSM images using ImageJ software. The vertical axis indicates the ratio of initial attachment cells to all area, and the horizontal axis indicates live and dead cells. The data indicate the mean ± standard deviation (SD) of three independent experiments. The results were analyzed using one-way ANOVA, *: p < 0.05, **: p < 0.01. (c) CLSM images of INAC formation *A. oris* Δ*fimP* and Δ*fimQ* when *S. mutans* Δ*gtfBC*-producing MVs at a final concentration of 3.125 μg/ml were added. A to F: *A. oris* Δ*fimP*; G to L: *A. oris* Δ*fimQ*. The left column shows live cells, the middle column shows dead cells, and the right column shows merge. The bar indicates 200 μm. (d) Quantification of CLSM images using ImageJ. The vertical axis indicates the ratio of initial attachment cells to all area, and the horizontal axis indicates live and dead cells. The data indicate the mean ± standard deviation (SD) of three independent experiments.


Fig. 4

Effect of DNase I on INAC formation of *A. oris* WT and Δ*fimA* treatment with MVs produced by *S. mutans* WT (a) CLSM images of INAC formation of *A. oris* WT when *S. mutans* WT-produced MVs added. A to C: no MVs and no DNase I; D to F: no MVs and DNase I; G to I:

MVs and no DNase I; J to L: MVs and DNase I. MVs produced by *S. mutans* WT was 3.125 µg/ml and 100 units of DNase I was used. The left column shows live cells, the middle column shows dead cells, and the right column shows merge. The bar indicates 200 µm. (b) Quantification of CLSM images using ImageJ software. The vertical axis indicates the ratio of initial attachment cells to all areas, and the horizontal axis indicates live and dead cells. The data indicate the mean ± standard deviation (SD) of three independent experiments. The results were analyzed using one-way ANOVA, **: $p < 0.01$. (c) CLSM images of INAC formation of *A. oris* Δ*fimA* when *S. mutans* WT-produced MVs were added. A to C: no MVs and no DNase I; D to F: no MVs and DNase I; G to I: MVs and no DNase I; J to L: MVs and DNase I. *S. mutans* WT MV concentration was 3.125 µg/mL, and 100 units of DNase I were used. The left column shows live cells, the center column shows dead cells, and the right column shows merging. The bar indicates 200 µm. (d) Quantification of CLSM images using ImageJ software. The vertical axis indicates the ratio of initial attachment cells to all areas, and the horizontal axis indicates live and dead cells. The data indicate the mean ± standard deviation (SD) of three independent experiments. The results were analyzed using one-way ANOVA, **: $p < 0.01$.

Fig. 5

Effect of DNase I on INAC formation of *A. oris* Δ*fimP* and Δ*fimQ* treatment with MVs produced by *S. mutans* WT (a) CLSM images of INAC formation of *A. oris* Δ*fimP* when *S. mutans* WT-produced MVs were added. A to C: no MVs and no DNase I; D to F: no MVs and DNase I; G to I: MVs and no DNase I; J to L: MVs and DNase I. *S. mutans* WT MV concentration was 3.125 µg/mL, and 100 units of DNase I were used. The left column shows live cells, the middle column shows dead cells, and the right column shows merging. The bar indicates 200 µm. (b) Quantification of CLSM images using ImageJ software. The vertical axis indicates the ratio of initial attachment cells to all areas, and the horizontal axis indicates live

and dead cells. The data indicate the mean ± standard deviation (SD) of three independent experiments. The results were analyzed using one-way ANOVA, *: $p < 0.05$, **: $p < 0.01$. (c) CLSM images of INAC formation of *A. oris* Δ*fimQ* when *S. mutans* WT-produced MVs were added. A to C: no MVs and no DNase I; D to F: no MVs and DNase I; G to I: MVs and no DNase I; J to L: MVs and DNase I. *S. mutans* WT MV concentration was 3.125 µg/mL, and 100 units of DNase I were used. The left column shows live cells, the center column shows dead cells, and the right column shows merging. The bar indicates 200 µm. (d) Quantification of CLSM images using ImageJ software. The vertical axis indicates the ratio of initial attachment cells to all areas, and the horizontal axis indicates live and dead cells. The data indicate the mean ± standard deviation (SD) of three independent experiments. The results were analyzed using one-way ANOVA, *: $p < 0.05$, **: $p < 0.01$.