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Live-cell and super-resolution imaging reveal that the distribution of wall-associated protein A is correlated with the cell chain integrity of *Streptococcus mutans*

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SUMMARY

***Streptococcus mutans* is a primary pathogen responsible for dental caries. It has an outstanding ability to form biofilm, which is vital for virulence. Previous studies have shown that knockout of Wall-associated protein A (WapA) affects cell chain and biofilm formation of *S. mutans*. As a surface protein, the distribution of WapA remains unknown, but it is important to understand the mechanism underlying the function of WapA. This study applied the fluorescence protein mCherry as a reporter gene to characterize the dynamic distribution of WapA in *S. mutans* via time-lapse and super-resolution fluorescence imaging. The results revealed interesting subcellular distribution patterns of WapA in single, dividing and long chains of *S. mutans* cells. It appears at the middle of the cell and moves to the poles as the cell grows and divides. In a cell chain, after each round of cell division, such dynamic relocation results in WapA distribution at the previous cell division sites, resulting in a pattern where WapA is located at the boundary of two adjacent cell pairs. This WapA distribution pattern corresponds to the breaking segmentation of *wapA* deletion cell chains. The dynamic relocation of WapA through the cell cycle increases our understanding of the mechanism of WapA in maintaining cell chain integrity and biofilm formation.**

INTRODUCTION

Streptococcus mutans, a Gram-positive bacterium, is a common conditioned pathogen in the human oral cavity. By attaching to the tooth surface and forming biofilm, *S. mutans* changes the local environmental conditions, leading to a lower pH and the occurrence of caries (Loesche, 1996).

Many proteins are known to be involved in biofilm formation, including secreted proteins (Lynch *et al.*,

2007), membrane proteins (Danese *et al*., 2000), and cell-wall-anchored (CWA) proteins (Foster *et al*., 2014). At this time, a number of studies have revealed unique patterns of CWA protein distribution in various bacteria using immunofluorescence imaging (Carlsson *et al*., 2006; Raz *et al*., 2012; Yu & Gotz, 2012). However, few studies have explored the distribution of CWA proteins in *S. mutans*.

Wall-associated protein A (WapA) is one of six CWA proteins in *S. mutans* UA159 based on the analysis of its genome sequence (Ajdic *et al*., 2002). Several studies have reported the function of *wapA* in biofilm formation. It has been reported that a *wapA* knockout strain derived from *S. mutans* UA159 shows impaired biofilm formation in the absence of sucrose (Levesque *et al*., 2005). In addition, Zhu *et al.* reported that the *wapA* knockout mutant of strain *S. mutans* UA140 forms shorter cell chains and biofilm of a different morphology compared with the wild-type (WT) in the absence of sucrose (Zhu *et al*., 2006). However, as a type of a surface protein, characterizing the mechanism of action of WapA is difficult without localization information.

To increase our understanding of the mechanism of action of *wapA*, we constructed an *S. mutans* UA159 strain with chromosomally mCherry-fused *wapA* and examined its dynamic distribution using live cell time-lapse imaging and super-resolution three-dimensional Structure Illumination Microscopy (3D-SIM). The unique dynamic distributions and patterns of WapA, as well as the fine 3D view of WapA organization, provide important information on the mechanism of action of WapA in cell division and cell-chain formation.

METHODS

Construction of bacterial strains

Two types of *S. mutans* mutation strains; *wapA* in-frame deletion (Δ*wapA*) and chromosomal *wapA* labeled strains, were constructed as described previously (Xie *et al*., 2011) (see Supplementary material, Table S1). mCherry was used as a reporter gene for fluorescence labeling of WapA in *S. mutans*; the strain was named *wapA*-sw-mCherry (W-sw-M) and was used for live cell imaging. To select antibiotic-resistant colonies, brain–heart infusion (BHI; Difco Laboratories) agar plates were supplemented with 12 μg/ml erythromycin (Sigma, St Louis, MO ,USA). Selection of the second transformation was performed on BHI plates supplemented with 4 mg/ml p-cl-Phe (Sigma). All strains were grown at 37°C aerobically (95% air, 5% CO_2) in Todd–Hewitt (TH; Difco Laboratories) medium without sucrose or on BHI agar plates. For time-lapse observations, *S. mutans* were cultured at room temperature in a sealed chamber.

Sample preparation for WapA imaging in *S. mutans* cells

Coverslips (Fisher 24 × 50, No. 1) were cleaned using Piranha solution (30% H_2O_2: 98% H_2SO_4 v : v, 1 : 3 at 90°C for 30 min) and were used for conventional fluorescence imaging. Generally, strain W-sw-M grown overnight in 3 ml of TH was diluted 1 : 100 into TH medium without sucrose for further applications.

For imaging of WapA distribution in individual *S. mutans* cells, the diluted bacteria were incubated to mid-log phase (5–6 h) and re-diluted in the same manner twice into TH medium. Mid-log phase bacterial culture (1 ml) was harvested by centrifugation at 2400 ***g*** for 5 min, washed three times with 1 × PBS, and then resuspended in 1 × PBS, after which Hoechst 33258 (Sigma) was added when required. Resuspended bacteria (3 μl) was loaded between a coverslip and a 1.5% agarose pad (15 mg Low Melting Agarose in 1 ml 1 × PBS) for conventional and SIM imaging.

To image WapA in long-chained bacteria, 20 μl of diluted W-sw-M overnight culture was injected into a chamber assembled with a coverslip and a glass slide using 3 M double-sided tape, and the chamber was sealed using nail enamel. Bacteria were incubated at 37°C for 16 h before imaging.

For time-lapse observations of *S. mutans* chain formation and the dynamic distribution of WapA, diluted overnight cultures of the WT, Δ*wapA* and W-sw-M strains were injected into a flow chamber, incubated at room temperature, and imaged for 16 h. Intermittent bright-field and 561-nm laser illumination (5-min intervals) were used to minimize photo-bleaching and photo-damage. Three frames were collected at each time point.

For 3D-SIM imaging of WapA in long cell chains, a diluted overnight culture of strain W-sw-M was transferred to a 35-mm dish with a cover-glass bottom (In Vitro Scientific, Sunnyvale, CA, USA) and incubated for 6 h, after which the sample was washed and imaged.

Image collection and analysis

Conventional images were collected in the Total Internal Reflected Fluorescence (TIRF) mode using a Nikon TiE inverted fluorescence microscope equipped with a 100 × oil objective (Nikon PLAN APO, 1.49 NA, Belfast, Northern Ireland) and Andor-897 EMCCD (Andor) and the perfect focus system (PFS, Nikon, Tokyo, Japan). Super-resolution 3D-SIM imaging was performed using N-SIM (Nikon). Deconvolution of conventional images was performed using MetaMorph (Molecular Devices, Sunnyvale, California). The ring diameter and fluorescence intensity analysis were performed with the Nis-Elements AR software (Nikon). Microbe Tracker suite (Sliusarenko *et al.*, 2011) and custom MATLAB scripts were used to analyze the relative positions of fluorescence peaks of WapA in a cell, the distance between fluorescence peaks, and the peaks to cell poles.

RESULTS

WapA is required for cell chain formation in *S. mutans*

Streptococcus mutans is known to form chains, which are important for biofilm formation. To understand the role of WapA in this process, we compared the cell chain formation of the Δ*wapA* and WT strains. Consistent with previous reports (Zhu *et al.*, 2006), our results showed that the deletion of *wapA* led to shorter cell chains compared with WT (Fig. 1). More specifically, the time-lapse observations indicated that in the Δ*wapA* strain the cell chains broke at around the seventh hour. Interestingly, the breaking occurred at every other cell–cell boundary and left didymous, dumbbell-shaped cells (Fig. 1A and see Supplementary material, Video S1). In contrast, the WT strain formed long chains. Although there were also breaks in the cell chain, their frequency was markedly lower than that of the Δ*wapA* strain (Fig. 1B).

Dynamic distribution of WapA in individual *S. mutans* cells

Here, we used mCherry as the reporter to label *wapA* chromosomally in *S. mutans* and assess its subcellular localization using TIRF imaging; it showed a clear localized distribution. In newborn cells, WapA was localized at the middle of the cell. In elongated cells, both polar and mid-cell distributions were observed. In pairs of dividing cells, WapA was found to mostly localize at the far ends of the paired cells; almost no

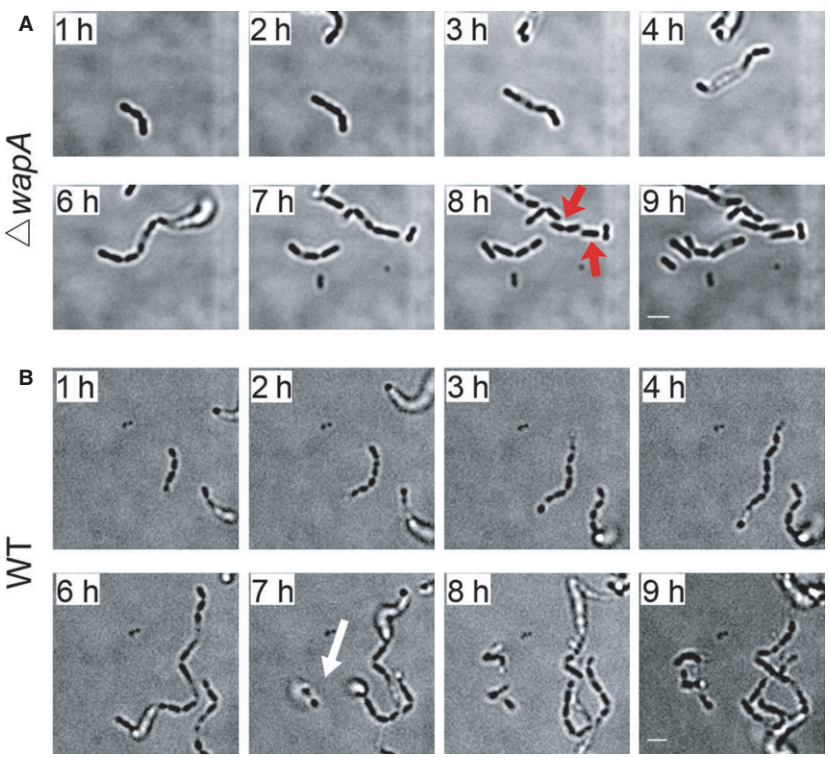

Figure 1 Wall-associated protein A (WapA) is required to maintain the stability of long cell chains. (A) Time-lapse imaging of cell chain formation by the Δ*wapA* strain. The strain formed cell chains of a normal morphology during the initial 6 h, and breaks occurred around the 7th hour of incubation. Red arrows indicate that the breaking segmentation unit was most frequently a pair of cells (dumbbell). (B) For the wild-type (WT) strain, breaks were occasionally observed (white arrows), but at a considerably lower frequency. Scale bar: 2 μm. The images represent the results of three independent experiments.

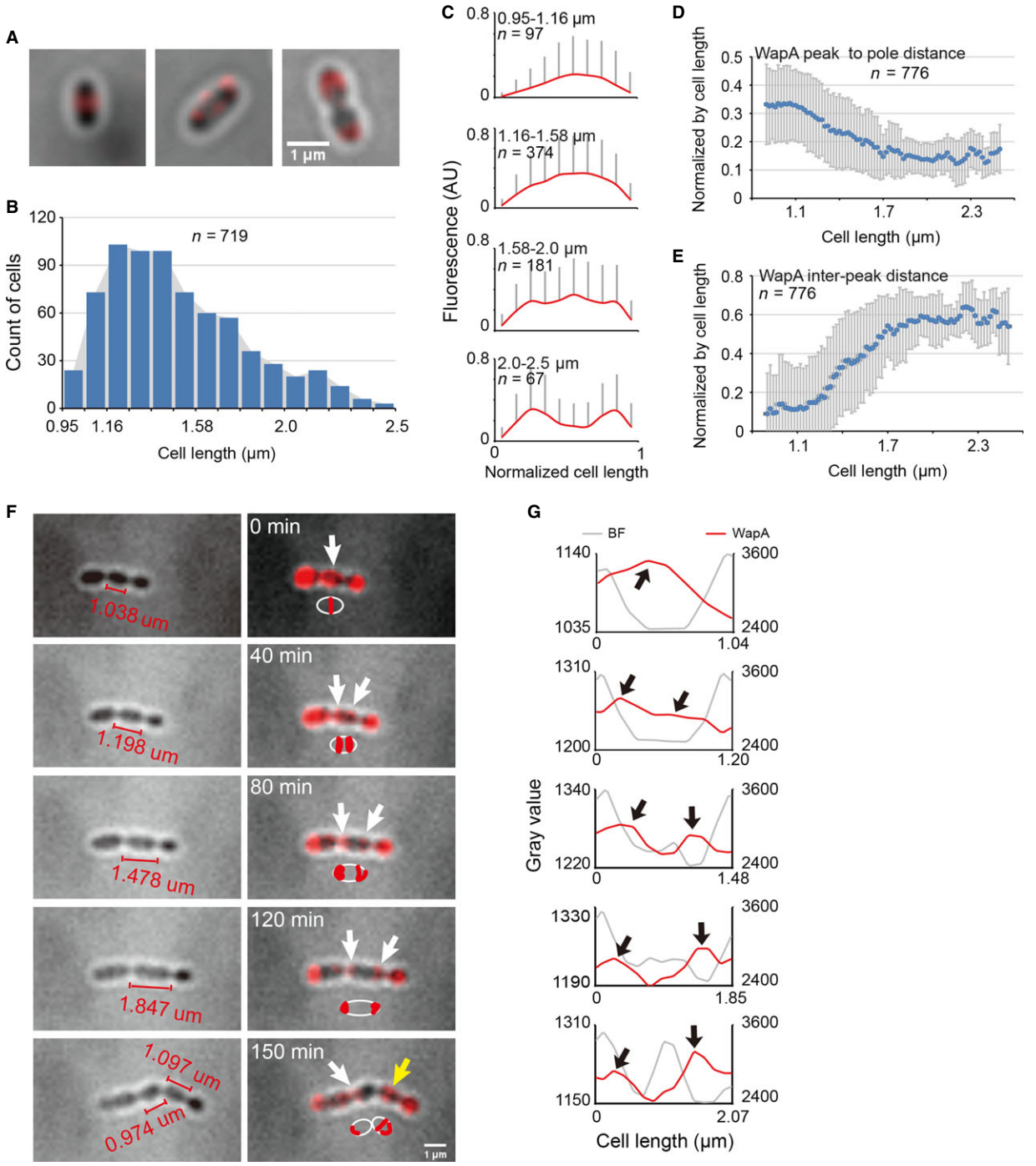

Figure 2 Dynamic distribution of wall-associated protein A (WapA) in *Streptococcus mutans*. (A) Merged image of bright-field (phase contrast) and fluorescence signal. Fluorescence (red) shows the distribution of WapA in short, long and dividing *S. mutans* cells. (B) The cell size distribution in the population (n = 719). The distribution is partitioned into four length categories to quantify the distribution of WapA as a function of cell length in (C). (C) Averaged fluorescence intensity profiles of cells of the four cell length categories. *x*-axis is normalized cell length. (D, E) Distance between outer fluorescence peaks and between fluorescence peaks and the closest pole. Each point is the average distance in cells of similar size (± 0.105 μm) (n = 776). (F) Time-lapse observation of a cell division; white arrows indicate the position of WapA, and the yellow arrow shows the newborn mid-cell distribution of WapA. (G) Intensity profiles corresponding to the cell in (F). Grey traces are the bright-field (BF) images, marking the boundary of the cell, and the red traces are the WapA fluorescence intensity. Black arrows show the fluorescence peaks. The time lapse images represent three independent experiments.

WapA was detected at the division sites (Fig. 2A). For a quantitative analysis, the bacterial cells (n = 719) were grouped based on their length as an indicator of the cell cycle (Fig. 2B). We then analyzed the relative peak positions of the fluorescence signal (Fig. 2C). The analysis of 776 bacterial cells showed that the distance between the fluorescence peaks increased as a function of the cell length, whereas the distance between the fluorescence peak and the cell pole decreased (Fig. 2D,E). Live cell time-lapse imaging (Fig. 2F,G) showed a dynamic distribution of WapA in the cell at the middle of the short chain. The dynamics of WapA distribution were consistent with the statistical results in Fig. 2(C,D), and it is worth noting that a new mid-cell distribution pattern reappeared (the yellow arrow) immediately after completion of cell division. In summary, WapA was secreted at the mid-cell and moved to the poles as the cell grew.

WapA is localized at the outer poles of dividing cells in *S. mutans* cell chains

The effect of *wapA* deletion (Fig. 1) suggests that WapA may play essential roles in cell chain formation and maintenance. A representative image of WapA localization in the cell chain is shown in Fig. 3(A), in which WapA is localized mainly near the cell–cell boundary. More interestingly, the intensity profiles of the bright field and fluorescence images (Fig. 3B), corresponding to the yellow line in Fig. 3(A), indicated that WapA was not localized at all cell–cell boundaries. Rather, it was distributed at the outer poles of a pair of dividing cells, which corresponded to the division sites of the previous round of cell divisions, in long cell chains.

Super-resolution 3D-SIM imaging of WapA

The size of cells of *S. mutans* or other streptococci (≈1 μm) is close to the diffraction limit of optical resolution (≈0.2 μm); therefore, obtaining precise WapA organizational and localization information in cells is difficult. Here, we used 3D-SIM to image WapA in *S. mutans*. Interestingly, 3D-SIM results revealed that WapA at the middle of the cell formed a ring-like structure (Fig. 4A and see Supplementary material, Video S2). The 69 rings had an average diameter of 0.43 ± 0.07 μm, compared with 0.53 ± 0.05 μm for bacterial cells (Fig. 4B). We imaged 5-h cultured W-sw-M cells to visualize the super-resolution structures of WapA in long cell chains. In this cell chain, WapA was localized mostly at the boundary of dumbbell cell pairs, such as cells 1, 2, 3 and 4 (Fig. 4C,D and see Supplementary material, Video S3), consistent with observations by conventional fluorescence imaging (Fig. 3). There were also cases in which WapA formed a sandglass structure, such as the boxed region in Fig. 4C,E, where WapA seemed to undergo transitions and converge at the cell–cell boundary.

DISCUSSION

Many studies of WapA in *S. mutans* have focused on its functions (Harrington & Russell, 1993; Qian & Dao, 1993; Zhu *et al.*, 2006; Li & Wang, 2014), but the localization has not been well characterized. In 2012, Berk *et al.* found that four secreted proteins in living *Vibrio cholerae* are organized in a complementary architecture during biofilm formation (Berk *et al.*, 2012), indicating that the functions of surface proteins

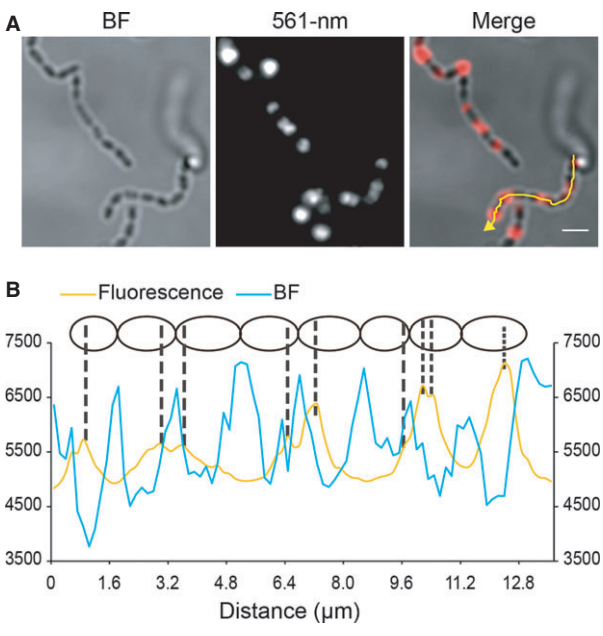

Figure 3 Localization of wall-associated protein A (WapA) in long cell chains. (A) Separate and merged images of bright-field and fluorescence signal. Scale bar: 2 μm. (B) Corresponding intensity profiles along the hand-drawn trace marked yellow in (A). Blue traces are the bright-field (BF) images, marking the boundary of the cells, which are shown as connected ovals. The yellow trace is the WapA fluorescence intensity. Dotted lines show the local fluorescence peaks.

are tightly associated with their spatial distribution. In this report, we imaged the subcellular localization of WapA in the cell chain to investigate its function–localization relationship. Examination of chain breaking showed that the *S. mutans* cells broke as a pair (Fig. 1A and see Supplementary material, Video S1). In a cell chain, each round of cell division generates new cell pairs, so the observation in Fig. 1(A) suggests that WapA may be localized to the older division sites in the cell chain and maintain the connection between two adjacent cells during their division.

This assumption was supported by fluorescence imaging of chromosomally labeled WapA in *S. mutans*. The subcellular localization of WapA showed clear patterns in individual cells (Fig. 2A), short cell chains (Fig. 2F), and long cell chains (Figs 3 and 4). The results of single cells and dividing pairs (Fig. 2) suggest that WapA is first secreted to the septum at the middle of the cell, where new cell wall synthesis occurs. Then, with elongation of the cell, WapA gradually redistributes to the poles and eventually localizes at the outer poles of the dividing cell pair (Fig. 2). This is also true for long cell chains and nearly resulted in an evenly spaced distribution of WapA, with a mean separation of two cells (Fig. 3). This WapA distribution is consistent with the observation in Fig. 1, in which long cell chains formed by *wapA*-knockout *S. mutans* strain broke in pairs, suggesting that WapA may be associated with holding adjacent cells together and so maintaining cell chain integrity.

As cells do not necessarily synchronize their cell cycles, we also observed in long cell chains several transition patterns of WapA, which are readily visualized using super-resolution SIM imaging (Fig. 4). In addition to the time-lapse imaging in Fig. 2(F), the transition patterns further suggest that WapA is secreted at the middle of the cell and moves to the poles as the cell grows. Super-resolution SIM imaging revealed that secreted WapA forms a ring at the septum. The diameters of the WapA rings were ~100 nm

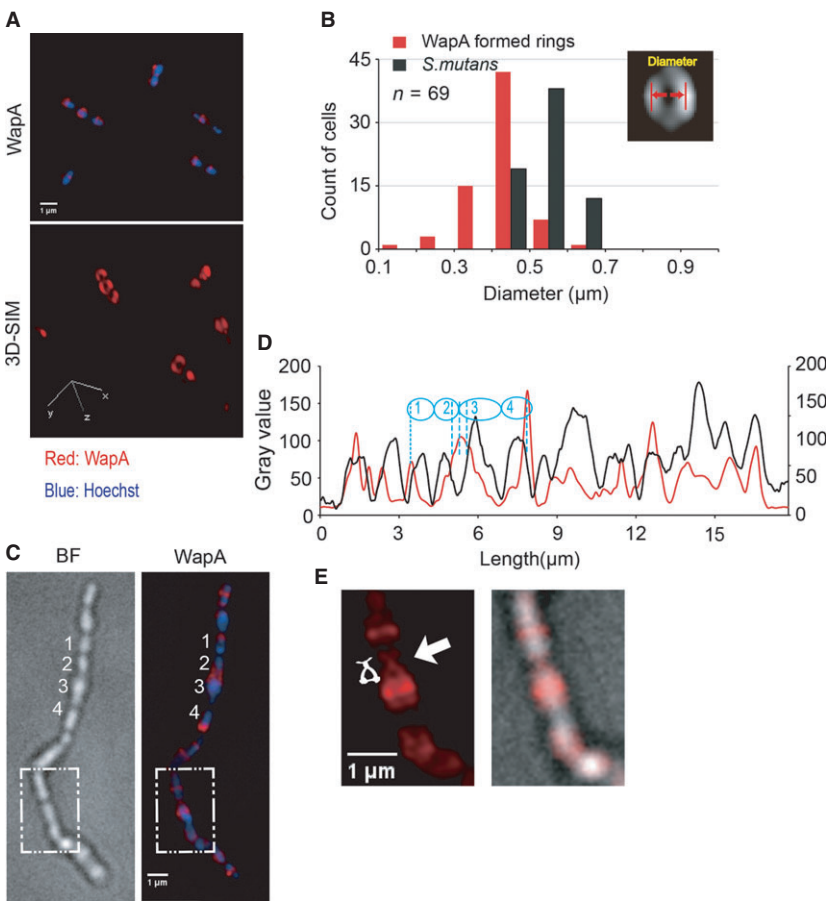

Figure 4 Super-resolution three-dimensional Structure Illumination Microscopy (3D-SIM) imaging of wall-associated protein A (WapA) in cell chains of different lengths. (A) WapA formed rings in the middle of the cell; DNA was stained with Hoechst 33258. (B) Histogram of WapA ring diameters (red) and that of a *Streptococcus mutans* cell (black). Upper right, measurement of the ring diameter (n = 69). (C, D) 3D-SIM of WapA in a long cell chain and the corresponding intensity profiles. (E) Boxed region in (C) reveals that the organization of WapA exhibited a sandglass shape at the boundary of two adjacent cells.

less than that of *S. mutans* cells. This suggests that WapA may be anchored to the newly synthesized cell wall, which invaginates about 50 nm from the middle of the cell (Wheeler *et al.*, 2011). Regarding the relocation of WapA from the middle to the poles of the cell, we think it may be simply because the peptidoglycan molecules that are initially anchored by WapA are pushed toward the poles as new cell wall is synthesized.

Although the current study provides information on WapA distribution and illustrates the function–localization relationship of WapA in the maintenance of long cell chains, further studies are required to explore the mechanism of action of WapA. There are two possibilities regarding the mechanism by which WapA at the cell–cell boundary holds two adjacent cells together. One is that as a cell wall anchoring protein, WapA molecules may bind to each other either directly or indirectly and create connections between two bacterial cells, similar to the role of cadherin at the adherens junctions between epithelial cells (Guo *et al.*, 2007). It is also possible that WapA competes or suppresses autolysins. Formation of long cell chains is based on uncompleted hydrolysis of cell wall peptidoglycan, which is regulated by autolysins (Smith *et al.*, 2000) in *S. mutans* (Ajdic *et al.*, 2002). The accumulation of WapA at the cell–cell boundary may attenuate the function or accumulation of autolysins.

In summary, this work describes the distribution of WapA, a CWA protein of *S. mutans*, and increases our understanding of its function and mechanism of action. WapA forms clear distribution patterns that show interesting dynamic relocations as a function of the cell cycle stage (see Supplementary material, Fig. S1). In a newborn cell, WapA is distributed at the middle of the cell and relocates to the poles. The cycle leads to a pattern in which WapA is distributed at the boundary of two adjacent cell pairs in a long cell chain; this distribution may be correlated with the integrity of the cell chain. To confirm this hypothesis, further studies on the interactions of WapA with both other WapA molecules and autolysins are required.

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