Cellular aspects of M protein and SfbI anchoring to *Streptococcus pyogenes* wall

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Summary

Wall-anchored surface proteins are critical for the *in vivo* survival of *Streptococcus pyogenes*. Cues in the signal sequence direct the membrane translocation of surface proteins: M protein to the septum, and SfbI to the poles. Both proteins are subsequently anchored to the wall by the membrane bound enzyme sortase A. However, the cellular features of these pathways are not fully understood. Here we show that M protein and SfbI are anchored simultaneously throughout the cell cycle. M protein is rapidly anchored at the septum, and in part of the cell cycle, is anchored simultaneously at the mother and daughter septa. Conversely, SfbI accumulates gradually on peripheral peptidoglycan, resulting in a polar distribution. Sortase is not required for translocation of M protein or SfbI at their respective locations. Methicillin-induced unbalanced peptidoglycan synthesis diminishes surface M protein but not SfbI. Furthermore, overexpression of the division regulator DivIVA also diminishes surface M protein but increases SfbI. These results demonstrate a close connection between the regulation of cell division and protein anchoring. Better understanding of the spatial regulation of surface anchoring may lead to the identification of novel targets for the development of anti-infective agents, given the importance of surface molecules for pathogenesis.

Introduction

Streptococcus pyogenes is an important human pathogen, responsible for 500,000 deaths per year worldwide (Carapetis *et al.*, 2005). Infection ranges from a mild strep throat or skin infection, to severe invasive conditions, including toxic shock syndrome, septicemia, and necrotizing fasciitis or "flesh-eating disease". Untreated infection may lead to sequela including rheumatic heart disease and glomerulonephritis (Cunningham, 2000). *S. pyogenes* employs an impressive array of wall-anchored virulence factors that are critical for its *in vivo* survival (Bisno *et al.*, 2003, Marraffini *et al.*, 2006, Nobbs *et al.*, 2009). Wall-anchored surface proteins possess a conserved C-terminal anchor domain comprised of an LPXTG motif followed by a hydrophobic region and a few positively charged amino acids (Fischetti *et al.*, 1990, Schneewind *et al.*, 1992). The anchoring domain is recognized by sortase, a membranal transpeptidase, which cleaves the LPXTG motif between the threonine and glycine residues (Mazmanian *et al.*, 1999), and connects the freed threonine to the peptidoglycan precursor lipid II (Perry *et al.*, 2002, Marraffini *et al*., 2006).

While the biochemical aspects of the sorting reaction have been studied in detail, the spatial regulation of this process is not as well understood (Marraffini *et al*., 2006, Hendrickx *et al.*, 2011, Spirig *et al.*, 2011). Proteins are anchored to the wall of *S. pyogenes* at two distinct locations, the septum and the poles (Carlsson *et al.*, 2006). Anchoring of M protein to the cell wall takes place exclusively at the septum (Cole & Hahn, 1962, Swanson *et al.*, 1969), where newly anchored M protein localizes within areas of active lipid II export and wall synthesis (Raz & Fischetti, 2008). Since the

synthesis of new cell wall is restricted to the septum of *S. pyogenes*, septal anchoring leads to the coating of the entire cell surface with M protein. In contrast to M protein, SfbI (also known as protein F, or PrtF), which is a major fibronectin binding protein in certain streptococcal strains (Talay *et al.*, 1992, Hanski & Caparon, 1992), displays a polar distribution (Ozeri *et al.*, 2001).

The signal sequence directs surface proteins for translocation at their ultimate cellular location (Carlsson *et al.*, 2006, DeDent *et al.*, 2008). Signal sequences containing a YSIRK-G/S motif are targeted to the septum, while signal sequences lacking this motif are targeted to the poles. Although the YSIRK-G/S motif is required for efficient signal sequence processing (Bae & Schneewind, 2003), mutations in this motif do not affect targeting to the septum (Carlsson *et al.*, 2006, DeDent *et al.*, 2008). Surface proteins possessing a YSIRK-G/S motif are found in *S. pyogenes* (Carlsson *et al*., 2006), *Staphylococcus aureus* (DeDent *et al*., 2008) and *Streptococcus pneumoniae* (Tsui *et al.*, 2011), are rare in *Enterococcus faecalis* (Kline *et al.*, 2009), and are absent from *Listeria monocytogenes* (Bruck *et al.*, 2011).

S. pyogenes sortase A (SrtA), which is involved in the anchoring of both M protein and SfbI (Barnett & Scott, 2002), localizes to a number of membranal foci (Raz & Fischetti, 2008). These foci are preferentially associated with the division septum, near sites of active M protein anchoring, and are recruited to daughter septa at an early stage of the division cycle. The distribution pattern of *S. pyogenes* pilus-specific sortases, SrtB and SrtC (Barnett & Scott, 2002, Barnett *et al.*, 2004, Mora *et al.*, 2005), is unknown at present.

In this study we use deconvolution immunofluorescence microscopy to study the two anchoring pathways of *S. pyogenes*. We show that M protein and SfbI are anchored simultaneously throughout the cell cycle. The anchoring of M protein is restricted to the septum, and occurs simultaneously at the closing mother septum and the forming daughter septa at certain stages of the cell cycle. SfbI on the other hand, is anchored at large peripheral areas, and its gradual accumulation on peptidoglycan results in polar distribution. Sortase is not required for the correct localization of M protein and SfbI translocation. Methicillin-induced unbalanced peptidoglycan synthesis, disrupts the proper assembly of the septum, and results in a marked reduction in surface M protein, but not SfbI. Overexpression of DivIVA also disrupts the septum, and results in a decrease in surface M protein, but an increase in SfbI.

Results

Localization of M protein and SfbI anchoring sites

In an attempt to better understand the two anchoring pathways in *S. pyogenes*, we used 3D structured illumination microscopy (3D-SIM) to follow the anchoring of M protein and SfbI (Fig. 1A). As expected, M protein (red) covers the entire surface of log phase D471 cells, although at this high resolution the distribution appears somewhat irregular, while SfbI (green) is present at the poles. It is of note however, that while the majority of cells displayed a similar level of M protein fluorescence, the SfbI fluorescence varied greatly between different poles.

The cellular location of active M protein anchoring has previously been studied by digesting existing surface proteins with trypsin, and following the regeneration of

new M protein in medium without trypsin (Cole & Hahn, 1962, Swanson *et al*., 1969). We found that complete removal of SfbI required the use of pronase in addition to trypsin, both of which were included in the growth medium (Fig. 1B, no regeneration). These proteases cannot cross the *S. pyogenes* cell wall, and their effect is therefore limited to the outer surface of the bacterium. Following a wash and 2 minutes regeneration in medium without proteases, M protein was detected strictly at the septum, while SfbI was detected in patches over a relatively large peripheral area (Fig. 1B, 2 minutes regeneration). A Z-stack view detailing the distribution of M protein and SfbI through the different layers of a representative streptococcal chain is presented in Figure 1C.

M protein and SfbI are anchored simultaneously throughout the cell cycle

To gain further insight into the anchoring of M protein and SfbI, we followed the localization patterns of these proteins as a function of the cell cycle stage. For this purpose D471 cells were treated with proteases as described above, washed, and resuspended in medium without proteases for two minute, allowing the regeneration of surface proteins. Numerous DeltaVision images were processed as average-intensity 3D-projections. A population of 781 cells was obtained by analyzing all the cells whose growth axis was parallel to the slide (and thus the septum was perpendicular to the slide), and that had no signal interference from other cells. Each cell was assigned to one of 6 groups based on cell length. While this division is arbitrary, it provides useful information about cell populations in different stages of the division cycle. Since *S. pyogenes* grows in chains, a clear-cut distinction of the

116 end of one cell cycle and the beginning of another is not immediately apparent. The
117 first division stage in our analysis was chosen in a manner roughly analogous to that
118 described by Higgins and Shockman (Higgins & Shockman, 1976), in which most
119 cells reveal only preliminary peptidoglycan assembly at the forming septum, and
120 where the mother septum is often not completely closed. A plot profile was generated
121 for each cell, displaying the fluorescent signal intensity relative to the cellular
122 position along the growth axis (see experimental procedures section). Whenever the
123 chain orientation made it possible to determine which pole represented the previous
124 division site (often for stages 1-4, but less often for stages 5-6), that pole was aligned
125 to the left. A representative cell for each division stage, as well as its individual plot
126 profile, are presented (Fig. 2A, left and middle columns). The mean intensity and
127 standard deviation values for each cellular position among all the cells in the group
128 were calculated, and these values are presented as population plots (Fig. 2A, right
129 column). The experiment was repeated two more times, with smaller cell populations,
130 yielding comparable results (not shown). The major cellular regions referred to in this
131 study are presented in Figure 2B.

132 The population plots show that M protein and SfbI are anchored throughout the
133 cell cycle. At the earliest stage (stage 1), M protein is observed primarily at the
134 closing mother septum (here presented on the left). As the cell cycle progresses
135 (stages 2-4), M protein anchoring activity is progressively redistributed to the forming
136 daughter septum, and newly anchored M protein can often be observed
137 simultaneously at both mother and daughter septa. Eventually, M protein is primarily
138 anchored at the daughter septum (stage 5-6). SfbI on the other hand is anchored at a

broad peripheral region. In early division (stage 1), SfbI is preferentially anchored at the pole distal to the previous division site. As division progresses (stages 2-4) SfbI is anchored at the old pole as well as the inter-septal region. At the late stages of division (stages 5-6) SfbI is anchored at both poles. Active division septa consistently show the least amount of SfbI anchoring.

To test in a more direct manner whether M protein and SfbI are anchored simultaneously to the surface of individual cells, the total M protein fluorescence of each cell in the population was plotted against the total SfbI fluorescence of the same cell (Fig. 2C). Only relatively few cells were found on the upper-left portion of the plot (representing high M protein fluorescence but no SfbI fluorescence), and the lower right portion (representing high SfbI fluorescence but no M protein fluoresce). Although the cells presented a wide range of fluorescence intensities, most fell in the middle region, indicating that both proteins were anchored. While these data represent the outcome of 2 minutes of protein anchoring activity rather than real-time anchoring, the prevalence of cells displaying both proteins, as well as the anchoring of the two proteins in all stages of the division cycle, indicate that the two proteins are anchored in parallel throughout the cell cycle.

M protein is anchored simultaneously at the closing mother septum, and forming daughter septa

Ovococci are a group of bacteria with slightly elongated coccus morphology, which divide in a single plain, and often form chains of organisms (Zapun *et al.*, 2008). This situation makes the overlap of two successive division cycles possible,

with the initiation of daughter septa assembly before the mother septum is completely closed (Higgins & Shockman, 1976, Gibson *et al.*, 1983). Early examinations of the anchoring of M protein to the cell wall of *S. pyogenes* following trypsinization and 15 minutes of protein regeneration suggested that simultaneous anchoring at the mother and daughter septa is possible (Cole & Hahn, 1962). To study the possibility of simultaneous anchoring at these locations in more detail, we used population-level analysis. While our initial population-level analysis is in agreement with simultaneous anchoring (Fig. 2A), this method averages the fluorescence signal across the cells in the group, and is therefore inadequate for this purpose.

We therefore developed a method for automatically analyzing the distribution of M protein in individual cells. To minimize the possibility that sequential anchoring at the mother and then daughter septa would be interpreted as simultaneous anchoring, protease treated cells were suspended directly in medium without proteases and allowed the regeneration of surface proteins for only 30 seconds before fixation (omitting the one-minute wash step, in which some protein anchoring does occur). Each cell in a population of 1,039 was assigned to one of six groups according to length, and its M protein distribution plot was generated as described above. The resulting plots were analyzed individually using MATLAB (see experimental procedures section), and assigned to one of four categories: M protein anchored at the mother septum alone, the daughter septum alone, both septa, or no septal anchoring (Fig. 3A). To supplement the automated analysis, the entire population was also classified into similar categories by direct observation of the cells (Fig. 3B). While the manual method classified slightly more cells as presenting simultaneous

anchoring at both septa, the two methods were generally in good agreement. The majority of cells in the youngest cell population (stage 1) displayed M protein only at the mother septum. With the progression of the cell cycle (stages 2-4) anchoring activity was gradually redistributed to the daughter septum, with roughly 20-30% of stage 2 cells, 30-40% of stage 3 cells, and 20-30% of stage 4 cells displaying M protein at both mother and daughter septa. At the later division stages (stages 5-6) M protein was found almost exclusively at the daughter septum. The analysis of M protein anchoring was repeated with an additional population of 890 cells, resulting from four different cultures, yielding comparable results (Fig. S1A). Additionally, this same analysis was applied to the cell population described in Figure 2, which was allowed two minutes of protein regeneration (Fig. S1B). As expected, this population showed a higher occurrence of M protein detected at both mother and daughter septa. This increase may be attributed in part to the brighter fluorescent signal, yet with longer regeneration time, some sequential anchoring at the mother followed by the daughter septa, cannot be ruled out.

M protein anchoring following 30 seconds regeneration was also visualized using 3D Structured Illumination Microscopy (3D-SIM). For this purpose the cells were labeled with M protein specific antibodies (green), and a vancomycin Rhodamine red conjugate (red), which preferentially labels sites of active peptidoglycan synthesis (Daniel & Errington, 2003). Sites of M protein anchoring localized to regions strongly labeled with vancomycin Rhodamine red. In cells displaying both mother and daughter septa, M protein was often observed at both locations (Fig. 3C). These results suggest that in rapidly dividing *S. pyogenes* cells, simultaneous anchoring of

M protein to the mother and daughter septa during the initial stages of daughter septum formation is a common occurrence.

Polar distribution results from gradual accumulation of SfbI on peripheral peptidoglycan

As discussed above, although SfbI was anchored throughout the peripheral region of the cell, the final distribution was distinctly polar, and displayed great variations in fluorescence intensity between different poles. One hypothesis that may explain how diffuse SfbI anchoring could lead to the observed distinctly polar distribution is that SfbI is anchored at a relatively constant pace, and accumulation of more SfbI on older poles leads to the observed difference in fluorescence intensity. To test whether there is a correlation between the amount of SfbI found on the pole and its age we used a fusion protein between Green Fluorescent Protein (GFP) and the binding domain of the phage lysin PlyC (GFP-PlyC/BD) in a "pulse-chase" type experiment. The binding domain of the PlyC lysin binds tightly to the cell wall carbohydrate (Nelson *et al.*, 2006), making it recognizable by its GFP fluorescence. D471 cells were grown in TH+Y media to OD_{600} 0.15, at which stage purified GFP-PlyC/BD was added to the medium for 30 minutes ('pulse'). Cells examined at this time point were brightly fluorescent in the green channel (Fig. 4A,'before chase'). The cells were then washed and incubated for one hour in medium lacking this fusion protein ('chase'), at which time the green fluorescence was limited to poles that were already formed during the 'pulse' (Fig. 4A, '1 hour chase'). These poles, which are at least two generations old, showed the most SfbI labeling, while younger poles showed considerably less SfbI

231 labeling. The prevalence of SfbI on old poles labeled with GFP-PlyC/BD is apparent
232 when the two fluorescent signals are examined side by side (Fig. 4B).
233 To quantify the relations between the age of the pole and the amount of SfbI
234 fluorescence, the signal distributions of SfbI and GFP-PlyC/BD were analyzed in a
235 population of 714 cells, derived from two separate experiments (each experiment
236 supported the final result, when analyzed individually). Each cell was divided into
237 two regions: a "young pole" consisting of the "mother septum" and "inter-septal"
238 regions (Fig. 2B), and an "old pole" equivalent to the "pole" region (Fig. 2B). The
239 signal at the "daughter septum" region at the middle of the cell (Fig. 2B) was not
240 analyzed, and this region served as buffer between the two poles. For each pole, the
241 average SfbI and GFP-PlyC/BD fluorescence were determined, and the two values
242 were plotted against each other (Fig. 4C). Young poles (blue) were not yet formed at
243 the time of the GFP-PlyC/BD "pulse" and thus did not display substantial GFP-
244 PlyC/BD fluorescence. The majority of these poles displayed only a modest amount
245 of anchored SfbI. Old poles (red) represent a heterogeneous group with ages ranging
246 from one to several generations. The majority of one-generation-old poles was
247 created during the "chase" period, and thus displayed only modest GFP-PlyC/BD
248 fluorescence. Poles that are two or more generations old were already formed during
249 the "pulse" period, and thus displayed a high GFP-PlyC/BD fluorescence. Since the
250 growth of *S. pyogenes* is not synchronized, a third group of poles are those that were
251 only partly formed during the "pulse", and thus displayed an intermediate level of
252 GFP-PlyC/BD fluorescence. The average fluorescence values for the poles in each of
253 these four groups are presented in Figure 4D. Both direct examination (Fig. 4C), and

comparison of the average SfbI fluorescence associated with each age group (Fig. 4D), reveal a direct correlation between the level of SfbI fluorescence and the age of the pole.

To supplement the "pulse-chase" results, D471 cells were grown in medium containing trypsin and pronase as described above, washed, and incubated in medium without proteases for 50 minutes to regenerate surface proteins. Since M protein is only anchored to newly synthesized peptidoglycan at the septum, areas devoid of M protein represent poles that were already fully formed at the time of protease treatment, and are therefore at least two generations old (Fig. 4E). When the labeling intensity of SfbI in these cells was determined, the "oldest" poles (with the least M protein labeling) displayed the most SfbI labeling, poles that were one generation old displayed weaker labeling, and newly formed poles displayed very weak SfbI labeling (see Fig. 7 for a model representation). The combined results of the two approaches suggest that gradual accumulation of SfbI on peripheral peptidoglycan, leads to the observed polar distribution, with older poles displaying a greater amount of SfbI. These observations also suggest that poles remain active sites of SfbI anchoring for at least two generations following their formation.

Translocation of M protein and SfbI at distinct locations is maintained in the sortase mutant AR01

Our next aim was to better understand the mechanisms underlying the differences between M protein and SfbI anchoring patterns. We first tested whether deletion of sortase A, which anchors both proteins to the cell wall (Barnett & Scott, 2002), could

affect their translocation pattern. The lack of M protein and SfbI anchoring to the cell wall of the sortase mutant strain AR01 was validated by fractionation and Western blot analysis (Fig. S2A). Although no anchoring took place, both proteins were translocated across the plasma membrane, and remained trapped to some extent in the cell wall. M protein and SfbI accumulated at distinct locations on the surface of untreated AR01 cells, and did not substantially colocalize (Fig. S2B). Regeneration of surface proteins following protease treatment revealed that M protein and SfbI first became visible on the surface of AR01 in cellular locations resembling that of wild type D471, namely M protein was translocated at the septum and SfbI at the cell periphery. A Z-stack view of a representative streptococcal chain is presented in Figure S2C. These results indicate that sortase is not required for spatially correct translocation of surface proteins.

Unbalanced peptidoglycan synthesis induced by methicillin results in a marked reduction in the cellular amount of M protein but not SfbI

At the initial stages of protein sorting, a nascent surface protein is cleaved near the C-terminus and covalently attached to lipid II through a sortase-mediated transpeptidation reaction. The ensuing complex serves as substrate for penicillin binding proteins (PBPs), resulting in covalent attachment of the surface protein to the cell wall. PBPs assemble peptidoglycan through two distinct activities, namely the polymerization of glycan chains through transglycosylation, and the cross-linking of these chains through transpeptidation. β-lactam antibiotics, which inhibit the transpeptidation activity but not the transglycosylation activity of PBPs, do not

300 directly affect the sorting reaction (Ton-That & Schneewind, 1999). An interesting
301 feature of β-lactam antibiotics is their ability to bind various PBPs with different
302 affinities, resulting in unbalanced peptidoglycan synthesis (Williamson *et al.*, 1980,
303 Gutmann *et al.*, 1981, Pucci *et al.*, 1986, Lleo *et al.*, 1990). In particular, methicillin
304 was shown to specifically inhibit septal peptidoglycan synthesis in several ovococci,
305 resulting in the formation of rod shaped cells (Lleo *et al*., 1990, Perez-Nunez *et al.*,
306 2011). We reasoned that if similar morphological effects could be induced in *S.*
307 *pyogenes*, the manner in which M protein and SfbI are anchored during unbalanced
308 peptidoglycan synthesis could shed light on the differences between the two
309 anchoring pathways.

310 *S. pyogenes* D471 cells were grown in the presence of trypsin and pronase to
311 digest existing surface proteins until OD_{600} 0.5 was reached, at which time the culture
312 was diluted 1:4 into a similar medium containing ascending concentrations of
313 methicillin. Following one-hour growth, during which time the phenotypic effects of
314 methicillin on peptidoglycan synthesis became apparent, the cells were washed and
315 resuspended in medium containing a similar concentration of methicillin, but lacking
316 proteases. Following 10 minutes of surface protein regeneration, the cells were fixed
317 and the anchoring patterns of M protein and SfbI were determined by
318 immunofluorescence (Fig. 5A). Morphological defects, resulting from unbalanced
319 peptidoglycan synthesis, became apparent at 0.2 μg/ml methicillin, as many cells
320 displayed an elongated or rod-shaped morphology with multiple septa. At higher
321 methicillin concentrations the cells became bulbous with no obvious septa. In rod-
322 shaped cells, M protein was typically anchored at several septa per cell, albeit at a

323 reduced quantity compared to untreated cells, while SfbI was anchored both at the
324 poles and at the inter-septal regions. At higher methicillin concentrations the amount
325 of M protein on the cells was diminished substantially, while significant anchoring of
326 SfbI was still observed. Western blot analysis of fractionated cells revealed a similar
327 pattern, where the amount of M protein was greatly reduced at methicillin
328 concentrations above 0.2 μg/ml, while the amount of SfbI remained substantial (Fig.
329 5B). At 3 μg/ml methicillin, the level of SfbI anchoring dropped as well, however this
330 reduction may be due to a general decline in cellular functions. To verify that
331 methicillin did not directly interfere with the sorting reaction, cell cultures treated in a
332 similar manner were harvested, and boiled in 2% SDS. The SDS supernatant was
333 collected (Fig. 5C, "SDS-soluble"), and the cells were lysed using the phage lysin
334 PlyC, thereby releasing covalently bound surface proteins (Fig. 5C, "SDS-
335 insoluble"). Treatment with methicillin did not result in the release of substantial
336 quantities of M protein or SfbI following boiling in SDS, indicating that the covalent
337 attachment of surface proteins by sortase was not affected. The anchoring pattern of
338 M protein and SfbI on methicillin-induced rod-shaped cells was further studied by
339 3D-SIM (Fig. 5D). These images show in greater detail the anchoring of M protein at
340 multiple septa per cell, and the anchoring of SfbI, not only at polar regions, but also at
341 inter-septal regions.
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Overexpression of the cell division protein DivIVA results in aberrant cellular morphology, a reduction in the level of M protein, and an increase in SfbI

DivIVA plays a major role in the regulation of septum placement in Gram-positive organisms. *Bacillus subtilis* DivIVA is targeted to areas of negatively curved membrane at the poles and the septum, recruits MinC and MinD to the poles, and thus prevents the assembly of polar division rings. In the absence of DivIVA, division is severely inhibited and septa are misplaced (Cha & Stewart, 1997, Edwards & Errington, 1997, Marston & Errington, 1999, Lenarcic *et al.*, 2009, Ramamurthi & Losick, 2009). *S. pneumoniae* and *E. faecalis* do not possess a MinCD system, nevertheless, DivIVA is important for the correct placement of the septum through a mechanism that is not completely understood (Fadda *et al.*, 2003, Fadda *et al.*, 2007, Ramirez-Arcos *et al.*, 2005). We postulated that overexpression of *S. pyogenes* DivIVA could similarly interfere with septum placement, and thus provide an additional way to test the dependence of protein anchoring on a functioning septum.

To test the effects of DivIVA overexpression, three plasmids carrying a spectinomycin (spec) selectable marker were constructed: pAR291_DivIVA, which expresses DivIVA under the control of the highly active M protein promoter, pAR287_GFP-HT, which expresses a GFP-HaloTag fusion protein under the control of the same promoter (unrelated protein control), and pAR161, an empty vector. For microscopy studies, overnight cultures of D471 harboring pAR291_DivIVA and pAR161 were diluted 1:50 into TH+Y+spec medium containing trypsin, and pronase. At OD_{600} 0.5, the cells were washed, incubated in medium without proteases for 5 minutes, and the anchoring of M protein and SfbI was examined. Cells harboring

368 pAR291_DivIVA had a reduced growth rate, and displayed aberrant morphology,
369 including enlarged volume and irregular shape (Fig. 6A). The amount of M protein
370 associated with these cells was greatly reduced compared to control samples. When
371 observed on cells, M protein was still associated with the septa despite the aberrant
372 morphology. SfbI was found in wall-associated patches, and displayed no clear
373 cellular localization preference, although the exact distribution pattern was difficult to
374 ascertain due to the irregular shape of these cells. For Western blot analysis,
375 overnight D471 cultures harboring pAR161, pAR287_GFP-HT, or pAR291_DivIVA,
376 were diluted 1:50 into TH+Y+spec medium, and were fractionated upon reaching
377 OD_{600} 0.5. The amount of M protein in cells harboring pAR291_DivIVA was greatly
378 reduced compared to cells containing pAR161, or pAR287_GFP-HT (Fig. 6B).
379 Conversely, the amount of SfbI was increased in cells containing pAR291_DivIVA.
380 The reduction in the level of M protein was not due to the introduction of the M
381 protein promoter on the DivIVA expression plasmid, since cells harboring
382 pAR287_GFP-HT, which possess a similar promoter, did not display altered M
383 protein expression level. When examined by fluorescent microscopy, cells harboring
384 pAR287_GFP-HT were brightly fluorescent in the green channel, confirming the
385 functionality of the M protein promoter used in this study (not shown). It is of note
386 that disruption of proper septum assembly by methicillin or the overexpression of
387 DivIVA, both resulted in a marked reduction in the level of surface M protein.
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Discussion

Anchoring of surface proteins to the cell wall of streptococci and staphylococci is divided into septal and peripheral anchoring pathways (Carlsson *et al*., 2006, DeDent *et al*., 2008). We used *S. pyogenes* M protein and SfbI as a model system to better understand the relations between the division cycle and protein anchoring through the two pathways, in an attempt to reach a more unified view of these processes. We took two general approaches: the first was largely descriptive and followed the two anchoring pathways throughout the cell cycle using population-level analysis, while the second tested how interference with the proper assembly of the division septum affects protein anchoring. We found that M protein and SfbI are anchored simultaneously throughout the cell cycle. M protein anchoring is strictly limited to the septum and occurs simultaneously at the mother and daughter septa at certain stages of the cell cycle. Conversely, SfbI is anchored in patches throughout the peripheral peptidoglycan, with some preference for the poles, and its accumulation over time results in polar distribution. Figure 7 summarizes these observations in a model form. We also found that sortase A is not required for localized translocation of the two proteins. Methicillin-induced unbalanced peptidoglycan synthesis, disrupts the proper assembly of the septum, and results in a marked reduction in surface M protein, but not SfbI. Overexpression of DivIVA also disrupts the septum, and results in a decrease in surface M protein, but an increase in SfbI.

The relations between the anchoring of surface proteins and the division cycle

The observed overlap in the anchoring of M protein at the mother and daughter septa of *S. pyogenes* is in agreement with a model proposing the simultaneous synthesis of peptidoglycan at these locations. Electron microscopy studies of cell division in enterococci revealed that initiation of peptidoglycan synthesis at future division sites begins before the mother septum is completely closed, and is independent of the completion of chromosome replication (Higgins & Shockman, 1976, Gibson *et al.*, 1983). The localization pattern of the *S. pneumoniae* division factors FtsZ (Morlot *et al.*, 2003), FtsA (Lara *et al.*, 2005), and FtsW (Morlot *et al.*, 2004), often shows simultaneous labeling at the mother and daughter septa. The labeling pattern of fluorescent vancomycin, which preferentially labels regions of lipid II export and cell wall synthesis, also shows simultaneous localization at the mother and daughter septa of both *S. pneumoniae*, and *S. pyogenes* (Daniel & Errington, 2003, Ng *et al.*, 2004, Raz & Fischetti, 2008). The penicillin binding proteins PBP1b, PBP2a, PBP2b, and PBP2x, all show simultaneous localization at the mother and daughter septa of *S. pneumoniae* at certain stages of the cell cycle (Morlot *et al*., 2003, Morlot *et al*., 2004, Zapun *et al*., 2008). Examination of the locations where M protein is regenerated following protease digestion, has previously been used to study septal peptidoglycan synthesis (Cole & Hahn, 1962). While this method does not provide real-time results, in this work we have reduced the regeneration time to the bare minimum of 30 seconds, and analyzed the anchoring pattern of this protein in large populations of cells. Our finding that M protein is anchored simultaneously at

436 the mother and daughter septa, strongly supports the model of simultaneous
437 peptidoglycan synthesis at these locations in *S. pyogenes*.

438 In contrast to M protein, SfbI is anchored in patches to large peripheral regions of
439 the peptidoglycan, with preference for the poles. The polar distribution of SfbI on *S.*
440 *pyogenes* cells is the result of two processes: preference for anchoring at non-septal
441 regions, and gradual accumulation on preassembled peptidoglycan, which results in a
442 correlation between the amount of SfbI anchored and the pole's age. Our results also
443 demonstrate that SfbI is actively anchored at the poles for at least two generations
444 following their formation. Expanding on this basic model, it should be noted that the
445 expression level of non-YSIRK-G/S proteins might also affect their observed
446 distribution. A low expression level may result in substantial labeling only on poles
447 that are far apart, while a high expression level may result in the anchoring of a
448 substantial quantity of proteins even on relatively young poles.

449

450 **Protein anchoring and the localization of sortase**

451 M protein and SfbI are both anchored to the cell wall by sortase A (Barnett &
452 Scott, 2002). We previously studied the distribution pattern of sortase in *S. pyogenes*
453 and found that it localizes to a number of membrane-bound foci in each cell.
454 Consistent with the anchoring pattern of M protein described here, sortase foci are
455 preferentially associated with division septa, are recruited to the forming septa at an
456 early stage, and are often present at both mother and daughter septa simultaneously
457 (Raz & Fischetti, 2008). Despite their relative abundance at the division septa, sortase
458 foci are not strictly confined there, and some foci are regularly seen at other cellular

459 locations. One way of explaining the apparent bias in sortase distribution towards
460 septal anchoring is that the streptococcal septum is constantly being split to form
461 peripheral peptidoglycan, which can no longer facilitate the anchoring of YSIRK-G/S
462 proteins, and therefore the time-window available for septal anchoring is very limited.
463 The need for efficient anchoring at the septum is met through the coupling of protein
464 anchoring to septal peptidoglycan synthesis, which ensures a high level of lipid II,
465 PBPs, and possibly other septal factors. Localized translocation of M protein at the
466 septum is also likely to play an important role in ensuring efficient anchoring
467 (Carlsson *et al*., 2006). The higher prevalence of sortase foci at the septum is
468 therefore likely to play a role in mediating efficient anchoring at this location. SfbI on
469 the other hand, is anchored gradually over a relatively large cellular area, and its
470 anchoring may therefore require a lower concentration of sortase.

471 The model presented here for the anchoring of surface proteins to the cell wall of
472 *S. pyogenes* differs substantially from the model proposed for *Streptococcus mutans*
473 (Hu *et al.*, 2008) and *E. faecalis* (Kline *et al*., 2009). These studies showed that
474 sortase A colocalizes with SecA in a single membranal microdomain termed
475 ExPortal. The ExPortal, first described in *S. pyogenes*, is a membranal microdomain
476 enriched in anionic lipids, in which the secretion related ATPase SecA and the
477 membranal protease/chaperone HtrA localize, and facilitates the secretion and
478 maturation of the streptococcal secreted protease SpeB (Rosch & Caparon, 2004,
479 Rosch & Caparon, 2005, Rosch *et al.*, 2007). A different study however, found that
480 SecA is distributed throughout the streptococcal membrane (Carlsson *et al*., 2006),
481 and the reason for this difference is not clear. Recently, *S. pneumoniae* SecA, and

SecY were found to be dynamically localized to both the septum and periphery suggesting the absence of an ExPortal in this organism (Tsui *et al.*, 2011). *S. pneumoniae* sortase A displays a punctate pattern, but does not show preferential distribution to the septum (Tsui *et al.*, 2011). Further study is needed to fully understand the relation between protein translocation and anchoring in *S. pyogenes*, as well as the molecular mechanisms underlying the distinct anchoring patterns observed in different ovococci.

The effects of septum disruption on protein anchoring

To better understand the mechanisms governing the anchoring of M protein and SfbI to the wall of *S. pyogenes*, we interfered with two processes: protein sorting, and septum assembly. The deletion of sortase did not alter the cellular locations where M protein and SfbI were translocated, indicating that sortase does not play a role in determining the site of translocation. Conversely, interference with the placement of the septum through the use of methicillin or the overexpression of DivIVA resulted in an increase in cell size, a reduction in the number of septa, and a reduction in the amount of M protein found at the cell surface. The level of SfbI was only little changed following methicillin treatment, and was increased when DivIVA was overexpressed. These data suggest that a functioning septum is critical for the anchoring of YSIRK-G/S-type proteins. When septum formation is prevented, the anchoring of YSIRK-G/S-type proteins does not become delocalized but rather, these proteins are absent from the bacterial surface. The mechanism underlying this phenomenon is currently under investigation. While inhibition of M protein

505 anchoring by the overexpression of DivIVA was linked to severe deformities in
506 cellular morphology, the possibility that DivIVA is involved in the regulation of
507 surface protein anchoring in a more direct manner should not be completely ruled out.
508 Of particular note is that *S. pneumoniae* DivIVA is localized to both the division
509 septum, and to a certain extent, the poles (Fadda *et al*., 2007).

510 One interesting phenomenon was the formation of rod-shaped cells displaying
511 multiple septa, following exposure to an intermediate concentration of methicillin,
512 similar to the effect observed in *Lactococcus lactis* (Perez-Nunez *et al*., 2011). In
513 contrast to *L. lactis* and most other ovococci however, *S. pyogenes* lacks PBP2b,
514 RodA, MreC and MreD homologues, which are important for peripheral
515 peptidoglycan synthesis in ovococci (Zapun *et al*., 2008). This distinct peptidoglycan
516 synthesis mechanism adds cell wall material at the splitting septum, and is
517 responsible for the slightly elongated shape of many ovococci. Formation of *S.*
518 *pyogenes* rod-shaped cells suggests therefore that the septal mechanism for
519 peptidoglycan synthesis may be sufficient to facilitate the coccus-to-rod
520 transformation. The specific molecular mechanisms involved in this alteration of *S.*
521 *pyogenes* shape however, are not fully understood at present.

522 The formation of rod-shaped *S. pyogenes* cells provided us with an interesting
523 model, in which to test the relations between surface protein anchoring and cell
524 shape. We found that M protein was regularly anchored simultaneously at multiple
525 septa along the rod, which were analogous to mother and daughter septa in untreated
526 cells. Inhibition of septum closure therefore intensified the propensity to
527 simultaneously anchor M protein at these locations. We also found that SfbI was not

anchored solely at the poles of the rod, but was also anchored at the cylindrical inter-septal regions. This suggests that SfbI is not necessarily targeted to the poles as such, but rather, its anchoring pattern may best be described as exclusion from the septum. Interestingly, a distribution pattern of exclusion from the septum was also observed for the *S. pneumoniae* D,D-carboxypeptidase PBP3 (Morlot *et al*., 2004). Studying the manner in which such a distribution pattern is achieved may provide clues for the regulation of SfbI translocation. Further insight into the manner by which non-YSIRK-G/S-type proteins are anchored may come from studies dealing with rod-shaped bacteria such as *L. monocytogenes* (Bierne *et al.*, 2004, Rafelski & Theriot, 2006, Bruck *et al*., 2011). A direct comparison however, may be complicated by the different manner peptidoglycan synthesis is regulated in these organisms.

Recently, a screen of *S. aureus* transposon integration library revealed that disruption of proteins containing an abortive infectivity (ABI) domain results in reduced expression level of YSIRK-G/S type surface proteins (analogous to *S. pyogenes* M protein), but does not affect the expression of proteins that do not contain this motif. Interestingly, these mutants also display thicker septal peptidoglycan, and it is not clear whether the reduction in septal protein expression is a direct effect or the result of defects in septal peptidoglycan synthesis (Frankel *et al.*, 2010).

Conclusions

When considering the two distinct anchoring pathways, each appears to have its own unique characteristics and advantages. Septal anchoring allows efficient coating of the entire surface of the cell from the moment the peptidoglycan is formed.

551 Immediate and extensive coating is likely to be important for the proper function of
552 M protein and possibly other septum-anchored proteins. Consider that anchoring of M
553 protein through the peripheral anchoring pathway may result in large areas of the
554 streptococcal cell wall being devoid of this molecule at any given time. In the absence
555 of M protein and other such molecules, these areas may be subject to opsonization,
556 leading to the elimination of these bacteria through phagocytosis (Perez-Casal *et al.*,
557 1992). Peripheral anchoring of SfbI on the other hand has the unique characteristic of
558 creating cellular polarity. The extent to which the polar localization of virulence
559 factors is important for the pathogenic process and survival of *S. pyogenes* is not clear
560 at present. It was shown however, that polar distribution of SfbI resulted in reciprocal
561 clustering of integrins on the surface of host cells (Ozeri *et al.*, 2001), and future
562 studies are likely to uncover additional examples. The ends of a streptococcal chain
563 may have a better opportunity for contact with the host, and thus a higher
564 concentration of certain virulence factors at these locations may be advantageous.
565 Polar localization of proteins is a recurring theme in Gram-negative and Gram-
566 positive bacteria, and has been shown to play a role in the general functions of the
567 bacteria, as well as in pathogenesis (Shapiro *et al.*, 2002, Shapiro *et al.*, 2009, Rudner
568 & Losick, 2010).

569 The data presented here suggest that factors related to the division ring are likely
570 to play a role in promoting the anchoring of M protein. Over the recent years, the
571 function of many of these division factors has been elucidated (Zapun *et al*., 2008,
572 Adams & Errington, 2009, Shapiro *et al*., 2009, Rudner & Losick, 2010), and this
573 knowledge may facilitate direct examination of their importance for surface protein

anchoring. Defining the factors critical for the proper regulation of protein sorting could yield novel targets for the development of anti-infective agent, since pathogens lacking surface proteins are greatly impaired in their ability to cause disease (Marraffini *et al*., 2006, Maresso & Schneewind, 2008).

Experimental procedures

Bacterial strains and culture conditions. *Escherichia coli* strain DH5α was used for molecular cloning and recombinant protein expression. *S. pyogenes* strain D471 (an M6 serotype) was from the Rockefeller University collection. AR01 is a *srtA* knockout strain derived from D471 (Raz & Fischetti, 2008). *E. coli* was grown in Luria-Bertani (LB) medium, supplemented with 100 μg/ml ampicillin, when needed. *S. pyogenes* strains were grown in Todd-Hewitt medium (Difco) supplemented with 1% yeast extract (Fisher Scientific) at 37°C. Erythromycin was used at 15 μg/ml for *S. pyogenes*, and spectinomycin was used at 20 μg/ml for *E. coli* and 120 μg/ml for *S. pyogenes* when appropriate.

Reagents and antibodies. The M protein specific 10B6 monoclonal antibody (Jones *et al.*, 1985) was used at a 1:10,000 dilution for Western blot analysis, and 1:1000 for immunofluorescence. SfbI-specific rabbit serum and pre-immune serum (Molinari *et al.*, 1997), were used at a 1:1000 dilution. Goat anti-mouse IgG conjugated to either Rhodamine red (Jackson ImmunoResearch), or FITC (Sigma), were used at 1:1000. Goat anti-rabbit IgG, conjugated to either FITC (Sigma) or Alexa Fluor 647 (Invitrogen) were used at 1:1000. Wheat germ agglutinin (WGA)

597 Marina Blue conjugate (Invitrogen) was used at 5 μg/ml. DAPI (Sigma) was used at 1
598 μg/ml. Vancomycin was conjugated to NHS Rhodamine red according to
599 manufacturer's instructions (Thermo Scientific), and separated from unbound dye by
600 thin layer chromatography. GFP-PlyC/BD (see below) was used at a final
601 concentration of 50 μg/ml. All other reagents were purchased from Sigma unless
602 otherwise noted.
603
604 **DNA manipulation.** Standard procedures were used for DNA manipulation and
605 for *E. coli* transformations (Sambrook *et al.*, 1989). Transformation of *S. pyogenes*
606 was performed according to Perez-Casal (Perez-Casal *et al.*, 1991). Plasmid DNA
607 was isolated using QIAprep spin miniprep kit (Qiagen). PCR amplification
608 procedures were performed using either Vent or Phusion DNA polymerase (New
609 England Biolabs). Oligonucleotides were from Eurofins. Restriction enzymes were
610 from New England Biolabs. T4 DNA ligase was from Invitrogen.
611
612 **Construction of the plasmids.** For the construction of the GFP-PlyC/BD
613 expression vector, pAR159, the binding domain of the PlyC phage lysin was
614 amplified using the following primers: 5_PlyC-BD_XbaI (5'-
615 GGCTCTAGAATGAGCAAGATTAATGTAAACGTAGAAAATG-3') 3_PlyC-BD_PstI (5'-
616 CGCCTGCAGTTACTTTTTCATAGCCTTTCTGATAGCC-3'), and inserted into the *Xba*I and
617 *Pst*I sites of pBAD24 (Guzman *et al.*, 1995). Primers 5_H6_GFP_EcoRI (5'-
618 CGCGAATTCATGAGTAAAGGAGAACTTCATCATCATCATCATCATTCCTCCGCCATGAGTAAAG
619 GAGAAGAACTTTTC-3') and 3_GFP_KpnI (5'-GAGGGTACCTTTGTATAGTTCATCCATGCC-

620 3') were used to amplify the GFP_mut2 gene (Cormack *et al.*, 1996). An N-terminal
621 hexahistidine tag is encoded on the upstream primer. This PCR product was inserted
622 into the *Eco*RI and *Kpn*I sites of the above plasmid, yielding pAR159.
623 A modified version of the *S. pyogenes* shuttle vector pLZ12-spec (Husmann *et al.*,
624 1995) was constructed by replacing the original multiple cloning site (MCS) between
625 the *Eco*RI and *Sph*I sites, with a DNA fragment formed by aligning primers
626 5_new_plzMCS (5'-AATTCCCCAAGCTTCCCAGATCTAAACCGCGGAAACAGCTGAAAC-
627 CATGGAAAGCATG-3') and 3_new_plzMCS (5'-CTTTCCATGGTTTCAGCTGTTTCCG-
628 CGGTTTAGATCTGGGAAGCTTGGGG-3'), which contain the new MCS (*Eco*RI-
629 *HinD*III-*Bgl*II-*Sac*II-*Pvu*II-*Nco*I-*Sph*I). The resulting plasmid, termed pAR161, was
630 used for the construction of pAR287_GFP-HT and pAR291_DivIVA.
631 For the construction of pAR287_GFP-HT, The HaloTag gene was amplified from
632 pFN18A HaloTag T7 Flexi Vector (Promega) using primers 5_HaloL1_SacII (5'-
633 CCCCCGCGGGGTGCATCTGCCGGCATGGCAGAAATCGGTACTGGC-3') and
634 3_Halo_NcoI (5'-CCCCCATGGCTATCAGCCGGAAATCTCGAGCGTC-3'), and inserted
635 into the *Sac*II and *Nco*I sites of pAR161. The GFP_mut2 gene (Cormack *et al.*, 1996)
636 was amplified using primers 5_GFP_BglII (5'-
637 GAGAGATCTATGAGTAAAGGAGAAGAACTTTTC-3') and 3_GFP_SacII (5'-
638 GAGCCGCGGTTTGTATAGTTCATCCATGCC-3'), and inserted into the *Bgl*II and *Sac*II
639 sites of the resulting plasmid. Finally, the M protein upstream region was amplified
640 from genomic D471 DNA using primers 5_Mp_UTR_EcoRI (5'-
641 CGCGAATTCACAGCCTAGCCGCAGAAACTC-3') and 3_Mp_UTR_BglII (5'-

642 CCCAGATCTGCTCCTTATGTTATCATTTTTTAGG-3'), and inserted into the *Eco*RI and
643 *Bgl*II sites, yielding pAR287_GFP-HT.

644 The construction of pAR291_DivIVA begun with a derivate of pAR161
645 containing a myc-tag (that is not expressed in the final construct), formed by aligning
646 primers 5_myc_tag (5'-GGGAACAAAAACTTATTTCTGAAGAAGACCTGTAGC-3') and
647 3_myc_tag (5'-CATGGCTACAGGTCTTCTTCAGAAATAAGTTTTTGTTCCCGC-3'), and
648 inserting the resulting double stranded DNA fragment into the *SacII* and *NcoI* sites
649 pAR161. The M protein upstream region was inserted into the *Eco*RI and *Bgl*II sites
650 of this plasmid as described for pAR287_GFP-HT. The DivIVA gene, including a
651 stop codon, was amplified from the genome of D471 using primers
652 5_DivIVA_BamHI (5'-CCCGGATCCATGGCACTTACAACGCTAGAAATTAAAG-3') and
653 3_DivIVA_SacII (5'-CCCCCGCGGTTAGATATTTAATTTAAACGTTTGTGTTTCACTGAG-
654 3'), and inserted into the *Bam*HI and *Sac*II sites of the resulting plasmid, yielding
655 pAR291_DivIVA.

656

657 **Purification of GFP-PlyC/BD.** An overnight culture of *E. coli* DH5α harboring
658 pAR159 was diluted 1:100 into one liter of LB supplemented with ampicillin, grown
659 to OD_{600} 0.5, and induced with 0.2% L-arabinose at room temperature for 5 hours.
660 The construct was purified on a NiNTA column as previously described (Raz &
661 Fischetti, 2008). The eluted fraction was concentrated using an Amicon Ultra
662 centrifugal filter device with a cutoff limit of 5 kDa, and the buffer was changed to
663 PBS by repeated cycles of dilution in PBS and volume reduction. The final protein
664 concentration was 0.85 mg/ml.

665

666 **Fractionation of *S. pyogenes* cells and Western blot analysis.** Fractionation of
667 the cells, and Western blot analysis were carried out as previously described (Raz &
668 Fischetti, 2008). Samples were normalized to account for slight variations in OD.
669 Fractionation by boiling in SDS was carried out as follows: One milliliter of culture
670 at OD_{600} 0.5 was harvested and washed with 30 mM Tris, pH 6.3. The bacterial pellet
671 was suspended in 50 μl of 2% SDS, boiled for 10 minutes, and centrifuged for 2
672 minutes at 16000 rcf. The supernatant, containing non-covalently bound proteins, was
673 supplemented with 12 μl of 5× SDS loading buffer. The cell pellet, containing
674 covalently anchored proteins, was washed with 200 μl deionized water, suspended in
675 50 μl of 30 mM Tris, pH 6.3, containing 300 U/ml PlyC (Nelson *et al*., 2006) for 15
676 minutes at room temperature, and then supplemented with 12 μl 5× SDS loading
677 buffer.

678

679 **Regeneration of surface proteins following protease treatment.** For protein
680 regeneration studies, overnight cultures were diluted 1:100 in TH+Y containing 0.35
681 mg/ml trypsin (Sigma) and 0.04 mg/ml pronase (Sigma). Unless otherwise noted, the
682 cells were harvested upon reaching OD_{600} 0.5 by one-minute centrifugation at 16,000
683 rcf, resuspended in TH+Y without proteases, and immediately spun again. The cells
684 were immediately resuspended in medium without proteases, and incubated at 37°C
685 for the stated amount of time. Each experiment typically consisted of three to four
686 repeats, and was performed several times.

687

688 **Fluorescent microscopy.** Fluorescent microscopy procedures were carried out as
689 previously described (Raz & Fischetti, 2008); however, the membrane and cell wall
690 permeabilization steps were omitted since the antigens studied are exposed on the
691 bacterial surface. Immunofluorescence experiments involving the use of methicillin
692 or the overexpression of DivIVA were performed using polyclonal SfbI sera pre-
693 adsorbed with the M1 serotype *S. pyogenes* strain SF370, which lacks a *sfbI* gene
694 (Ferretti *et al.*, 2001). In those cases the cells were incubated sequentially with the M-
695 protein-specific mouse monoclonal 10B6, anti-mouse Rhodamine red, rabbit anti-
696 SfbI, and anti-rabbit FITC antibodies, for improved signal over background.
697 Structured Illumination microscopy was performed on a DeltaVision OMX Blaze 3D-
698 Structured Illumination Microscopy (3D-SIM) system (Applied Precision) fitted with
699 an Olympus 100x/1.40 NA UPLSAPO objective, Photometrics Evolve EMCCD
700 cameras, and 405, 488 and 568 lasers. 3D-SIM reconstruction and channel alignment
701 were performed using the SoftWoRx algorithms and reconstructed images were
702 exported as maximum projections.
703
704 **Analysis of protein anchoring using MATLAB.** For the purpose of signal
705 analysis, average intensity projections were produced containing the signal data from
706 all the Z-sections, using SoftWoRx (Applied Precision). These projections were
707 converted into tiff format using ImageJ (http://rsb.info.nih.gov/ij/), and the signal
708 distribution data were subsequently analyzed using MetaMorph offline (64-bit)
709 version 7.7.5.0 (Molecular Devices). In each image, all the cells whose growth axis
710 paralleled with the slide (and therefore the septum was perpendicular to the slide),

711 and that presented no signal interference from adjacent cells, were analyzed. Each cell
712 was confined in a rectangle whose long dimension parallels with the cell's growth
713 axis. To analyze the fluorescence intensity of the antigens in different cellular
714 regions, each rectangle was then sub-divided into pixel-wide cross-sections, and the
715 total fluorescence of each section was calculated. These raw fluorescence distribution
716 data were further analyzed using MATLAB version 7.6.0 R2008a (MathWorks). To
717 create cell-specific plots the fluorescence intensity of each section was plotted as a
718 function of its cellular position, represented as the distance from the younger of the
719 two poles, or mother septum. Population plots were obtained by aligning all the cells
720 in each group, and calculating the mean fluorescence signal and standard deviation
721 values for each cellular position.

722 For the analysis of M protein anchoring at the mother and daughter septa,
723 presented in Figure 3, each cell was divided into 4 regions: (1) mother septum,
724 defined as the 0.13 μm region (3 pixels wide) adjacent to the previous division site
725 (on the left); (2) daughter septum, defined as the 0.21 μm region (5 pixels wide) at the
726 middle of the cell; (3) inter-septal region, located between the mother and daughter
727 septa; and (4) old pole, defined as the remainder of the cell (see Figure 2B). A mother
728 septum region was defined as positive for the anchoring of M protein if it passed a
729 threshold of 200 fluorescence units, and displayed double the minimal signal of the
730 inter-septal region. A daughter septum region was defined as positive for M protein
731 anchoring if, in addition to the above conditions, it also displayed double the minimal
732 signal of the polar region. In the youngest cell population, resolution constraints
733 sometime resulted in protrusion of the mother septum fluorescent signal into the inter-

septal region, preventing the achievement of the two-fold fluorescence difference required for the recognition of mother septum anchoring. To address this situation, cells that were negative for M protein anchoring using the above definitions, but passed the fluorescence threshold and displayed 5-fold mother septum fluorescence compared to the old pole, were defined as positive for mother septum anchoring.

For the analysis of SfbI signal distribution following the GFP-PlyC/BD "pulse-chase" experiment, the signal distribution of the different fluorescent channels was acquired as described above. A 5-pixel-wide region in the middle of the cell was left unanalyzed, while the regions on both sides were defined as "young pole" and "old pole" according to the GFP-PlyC/BD distribution along the chain. The average fluorescence intensity of each channel was calculated for each pole. The code used for all the MATLAB operations is available upon request.

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Abbreviations

WGA – Wheat Germ Agglutinin.

PBP – Penicillin Binding Protein.

RFU – Relative Fluorescence Units.

3D-SIM – 3D Structured Illumination Microscopy.

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Figures

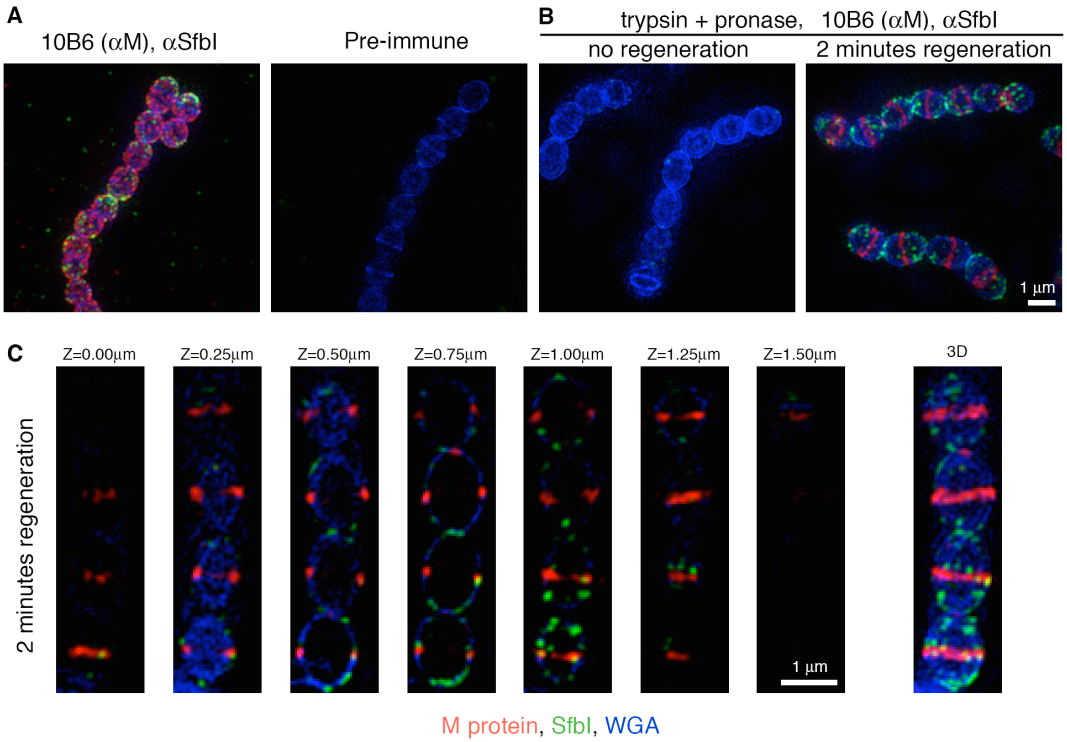
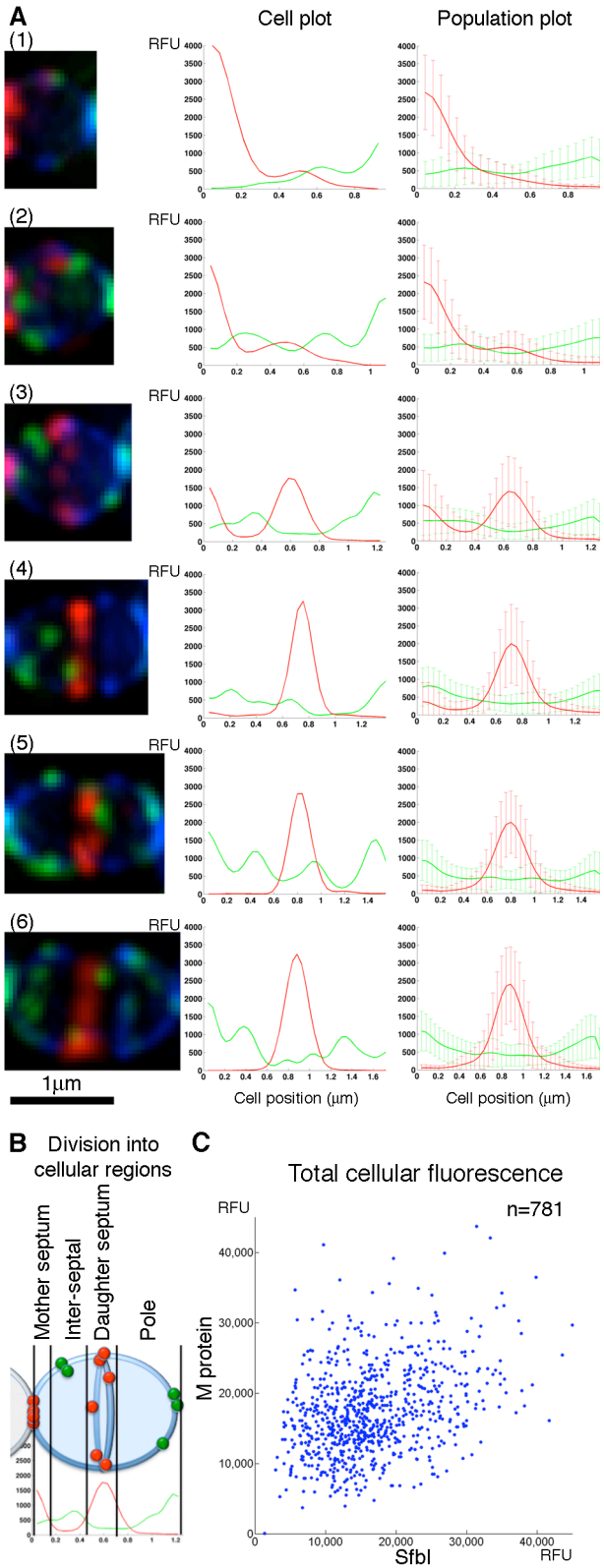


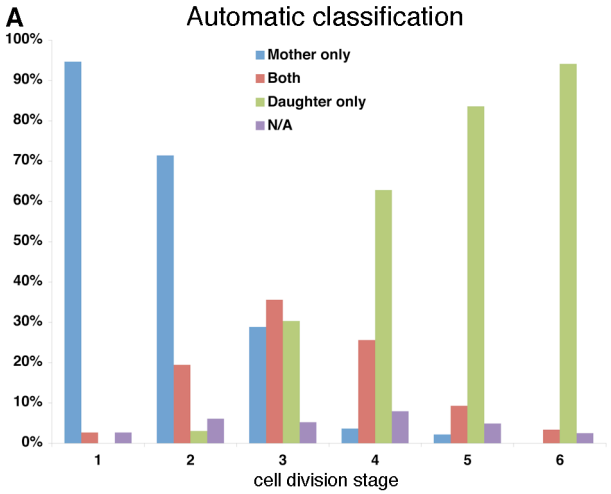
Figure 1. **The relative anchoring patterns of M protein and SfbI**. An overnight *S. pyogenes* D471 culture was diluted 1:100 into TH+Y (A), or TH+Y containing trypsin and pronase (B), and fixed upon reaching OD_{600} 0.5 (A, and B "no regeneration"). Protease treated cells were also harvested at OD_{600} 0.5, washed, and resuspended in TH+Y for 2 minutes at 37°C prior to fixation (B "2 minutes regeneration", and C). Specific antibodies were used to label M protein (red) and SfbI (green). The cell wall was stained with WGA marina blue (blue). 3D-SIM microscopy images are presented as maximum intensity projections composed of all the Z-sections (A and B), or as sequential Z-sections (C).

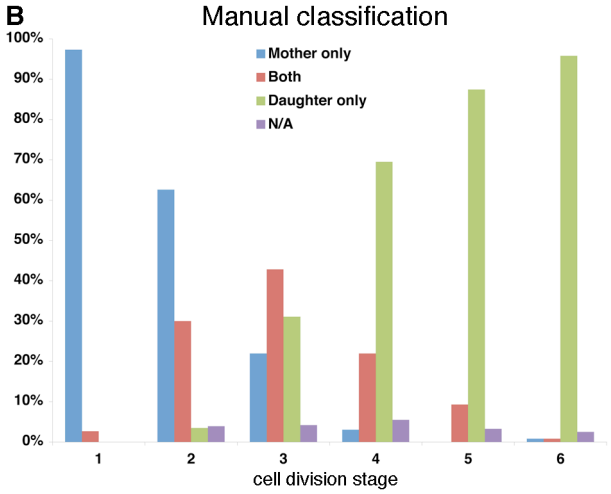

A
Cell plot
Population plot
(1)
(2)
(3)
(4)
(5)
(6)
RFU
1µm
Cell position (µm)
Cell position (µm)
B
Division into cellular regions
Mother septum
Inter-septal
Daughter septum
Pole
C
Total cellular fluorescence
n=781
RFU
M protein
SfbI
RFU

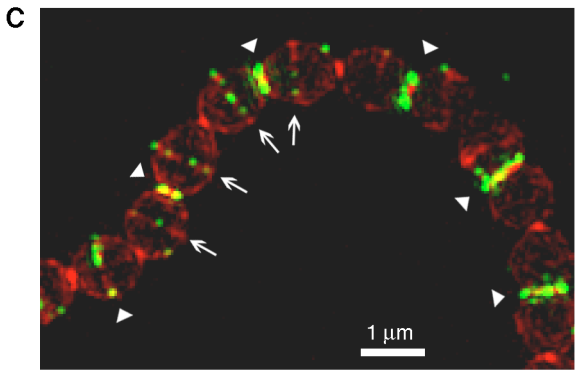
991

992

Figure 2. M protein and SfbI are anchored simultaneously throughout the cell cycle. (A) An overnight *S. pyogenes* D471 culture was diluted 1:100 into TH+Y containing trypsin and pronase and incubated at 37°C to OD_{600} 0.5, at which point the cells were washed, and resuspended in TH+Y without proteases for 2 minutes at 37°C prior to fixation. The cells were labeled for M protein (red), SfbI (green) and wall (WGA, blue). Average intensity 3D-projections were made for numerous DeltaVision images, resulting in a population of 781 cells. These cells were divided according to cell length to 6 groups, representing different stages of the cell cycle. Each cell was divided into pixel-wide strips, parallel to the division plane, and the total fluorescence intensity was calculated for each strip. These data, when plotted against the relative position in the cell, produced the cellular distribution plots. Representative cells for each division stage are presented on the left, and their respective plots are presented on the middle column. For each division stage, the group's average fluorescent signal distribution and standard deviation values are presented on the right. (B) A representation of the cellular regions of a streptococcal cell, as used in this study. (C) A plot displaying the total M protein fluorescence value for each cell in the population, plotted against its total SfbI fluorescence.







vancomycin Rhodamine red, M protein

Figure 3. M protein is anchored simultaneously at the mother and daughter septa. An overnight *S. pyogenes* D471 culture was diluted 1:100 into TH+Y containing trypsin and pronase and incubated at 37°C to OD_{600} 0.5, at which point the cells were harvested, and resuspended in TH+Y without proteases for 30 seconds at 37°C prior to fixation. M protein was labeled using specific antibodies and average intensity 3D-projections were made for numerous DeltaVision images, resulting in a population of 1,039 cells. The M protein distribution plots of these cells were analyzed using MATLAB (see experimental procedures section) to determine the presence of newly anchored M protein at the mother and daughter septa (A). The location of M protein anchoring on the same cells was determined by direct observation (B). (C) Cells treated in a similar manner were labeled with vancomycin Rhodamine red conjugate (red), and M protein specific antibodies (green), and then visualized by 3D-SIM. Arrowheads represent mother septa and arrows represent forming daughter septa.

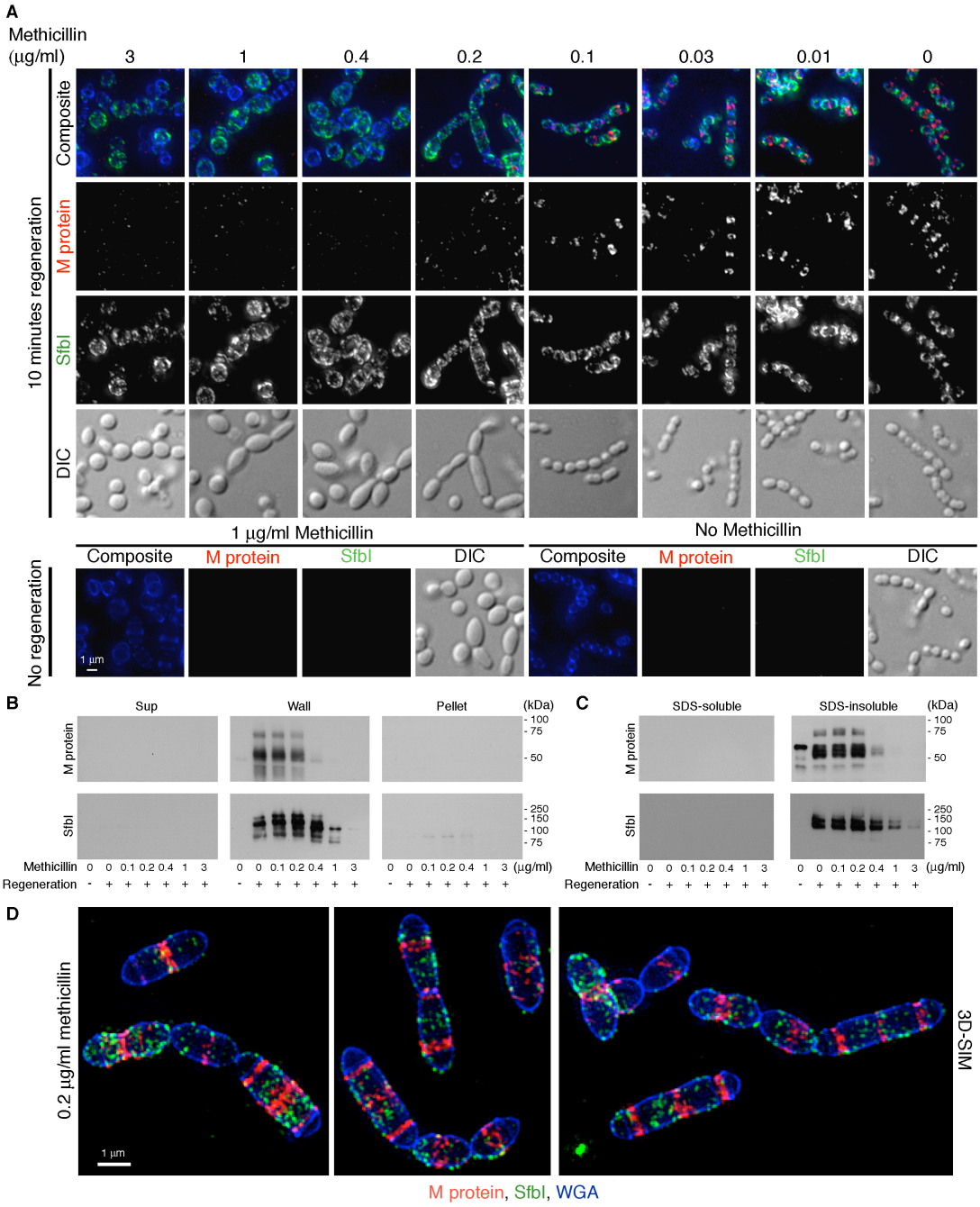


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Figure 4. Gradual accumulation of SfbI results in polar distribution. An overnight *S. pyogenes* D471 culture was diluted 1:100 into TH+Y and grown to OD_{600} 0.15, at which time GFP-PlyC/BD (green) was added to the medium for 30 minutes ("pulse"). The cells were then washed and suspended in TH+Y for one hour at 37°C ("chase") prior to fixation. SfbI was stained using a specific serum (red), and the cell wall was stained using WGA marina blue (blue). (A) Representative cells before and after the "chase" period. (B) Streptococcal chains following the "chase" period alongside the separate fluorescent channels. (C) Average intensity 3D-projections were made for numerous DeltaVision images, resulting in a population of 714 cells. The signal distribution plots of these cells were analyzed using MATLAB. Each cell was divided into "young pole" (blue) and "old pole" (red) regions. For each pole, the mean GFP-PlyC/BD and SfbI fluorescence values were plotted against each other. (D) The "old pole" group was sub-divided into 3 groups according to GFP-PlyC/BD fluorescence, representing the estimated age of the pole. The mean SfbI fluorescence the standard deviation values are presented for each group, as well as for the "young pole" group in its entirety. (E) An overnight *S. pyogenes* D471 culture was diluted 1:100 into TH+Y containing trypsin and pronase, and incubated at 37°C to OD_{600} 0.5, at which point the cells were washed, and resuspended in TH+Y without proteases for 50 minutes at 37°C prior to fixation. Specific antibodies were used to label M protein (red) and SfbI (green). The cell wall was stained with WGA marina blue (blue). Arrows denote wall regions that were already assembled at the time of protease treatment. DeltaVision images for (A), (B), and (E), are presented as maximum intensity 3D-projections.

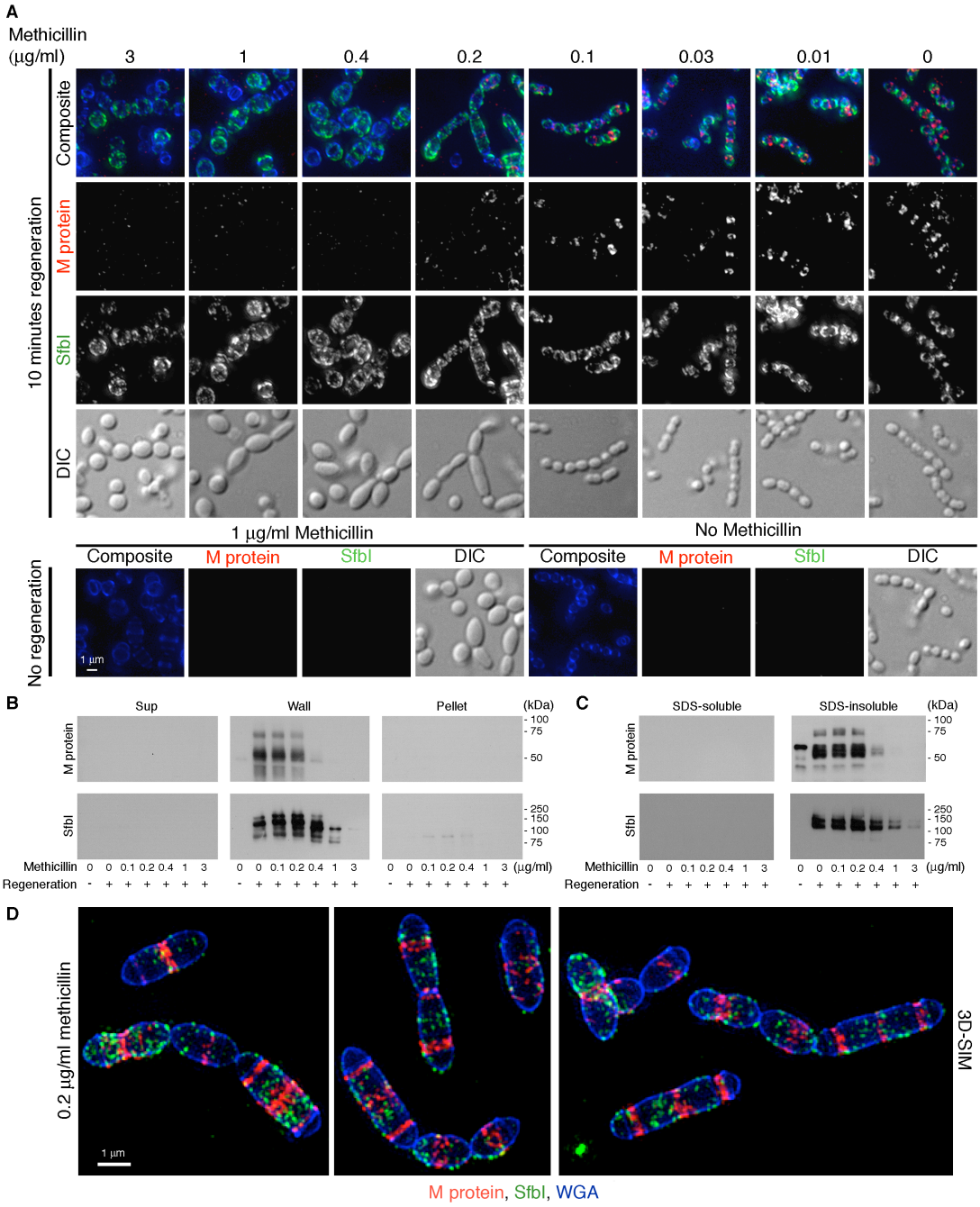
A
Methicillin (μg/ml)
3
1
0.4
0.2
0.1
0.03
0.01
0
Composite
M protein
SfbI
DIC
10 minutes regeneration
1 μg/ml Methicillin
No Methicillin
Composite
M protein
SfbI
DIC
No regeneration
1 μm
B
Sup
Wall
Pellet
(kDa)
M protein
SfbI
Methicillin
Regeneration
(μg/ml)
C
SDS-soluble
SDS-insoluble
D
0.2 μg/ml methicillin
1 μm
3D-SIM
M protein, SfbI, WGA

Figure 5. Methicillin-induced unbalanced peptidoglycan synthesis results in a substantial reduction in the cellular amount of M protein compared to SfbI. An overnight *S. pyogenes* D471 culture was diluted 1:100 into TH+Y containing trypsin and pronase, and grown at 37°C to OD_{600} 0.5. The cells were then diluted 1:4 into tubes containing TH+Y, trypsin, pronase, and ascending concentrations of methicillin. Following one hour, the cells were washed, and resuspended for 10 minutes in TH+Y containing a similar concentration of methicillin but lacking proteases, and then fixed. (A) M protein (red) and SfbI (green) were labeled using specific antibodies, and the cell wall was stained with WGA marina blue (blue). Deconvolution immunofluorescence images are presented as maximum intensity projections. (B) Similar cultures were fractionated into supernatant, wall, and spheroplast pellet, and processed by Western blot. (C) Additional cultures were harvested, boiled in 2% SDS, and separated into supernatant ("SDS Soluble" fraction), and cell pellet ("SDS insoluble" fraction), which was subsequently treated with the phage lysin PlyC to release wall-anchored proteins, prior to processing by Western blot. (D) Cells treated with 0.2 μg/ml methicillin, were visualized by 3D-SIM and are presented as maximum intensity projections.

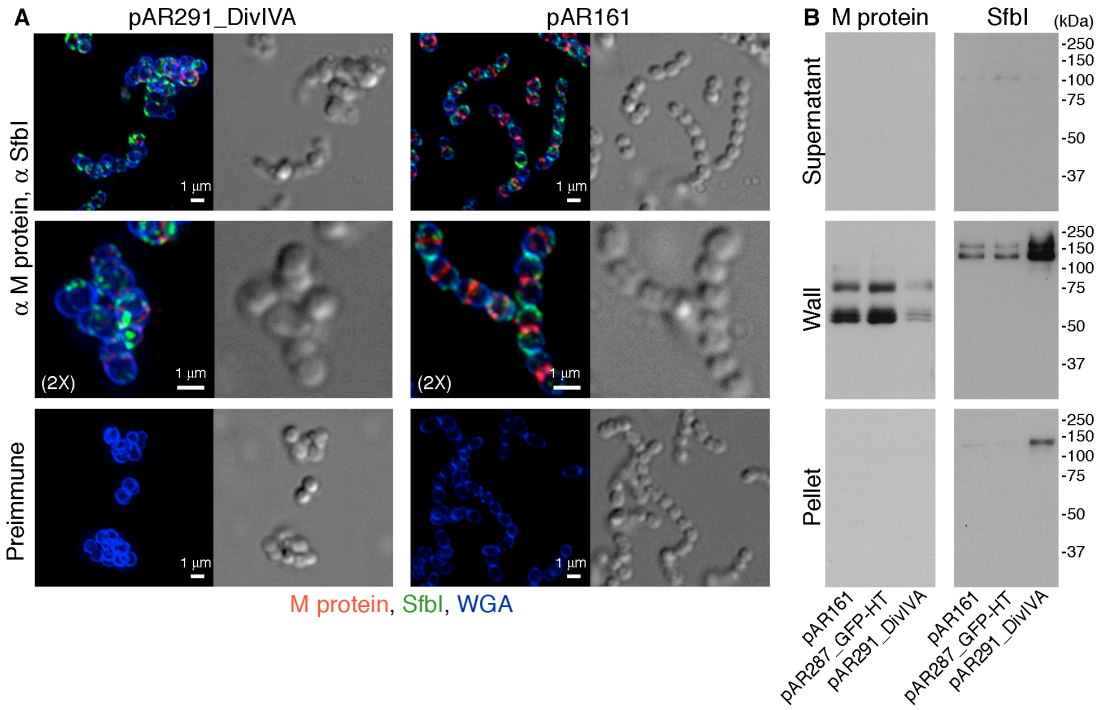

Figure 6. Overexpression of DivIVA results in a marked decrease in the cellular amount of M protein and an increase in SfbI. (A) Overnight cultures of D471 cells harboring pAR291_DivIVA, or the empty vector pAR161, were diluted 1:50 into TH+Y+spec containing trypsin and pronase and grown to OD_{600} 0.5. The cultures were then washed, resuspended in medium without proteases for 5 minutes, and then fixed. Specific antibodies were used to label M protein (red) and SfbI (green), and the cell wall was stained with WGA marina blue (blue). DeltaVision images are presented as maximum intensity projections. (B) D471 cells harboring pAR291_DivIVA, pAR287_GFP-HT (non-specific control), or the empty vector pAR161, were grown in TH+Y+spec to OD_{600} 0.5, and fractionated into supernatant, wall, and spheroplast pellet fractions. Samples were examined by Western blot using antibodies specific for M protein and SfbI.

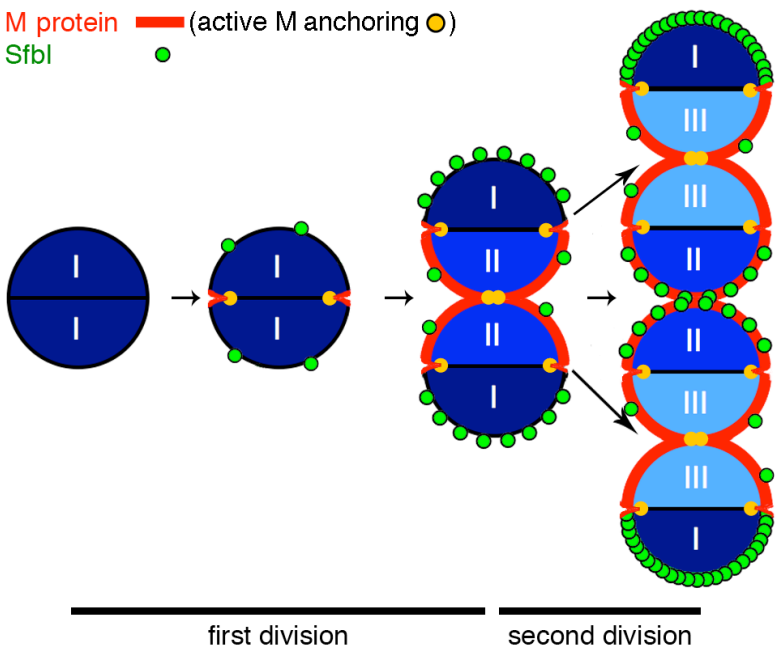

Figure 7. A model representation of the anchoring of M protein and SfbI. The regeneration of surface proteins during two division cycles is presented. M protein (red) is anchored exclusively to newly synthesized peptidoglycan at the septum (sites of active anchoring labeled yellow). Anchoring of M protein at daughter septa begins before the mother septum is completely closed, resulting in simultaneous anchoring at both locations. Following two generations, M protein is anchored to all newly synthesized, but not pre-existing, peptidoglycan. SfbI (green) is anchored over time in patches to peripheral peptidoglycan, with some preference to the poles. Following two generations, the oldest poles (I) display the most intense SfbI labeling, while one-generation-old poles show less labeling (II), and newly formed poles (III) show little SfbI labeling.

Supplementary figures

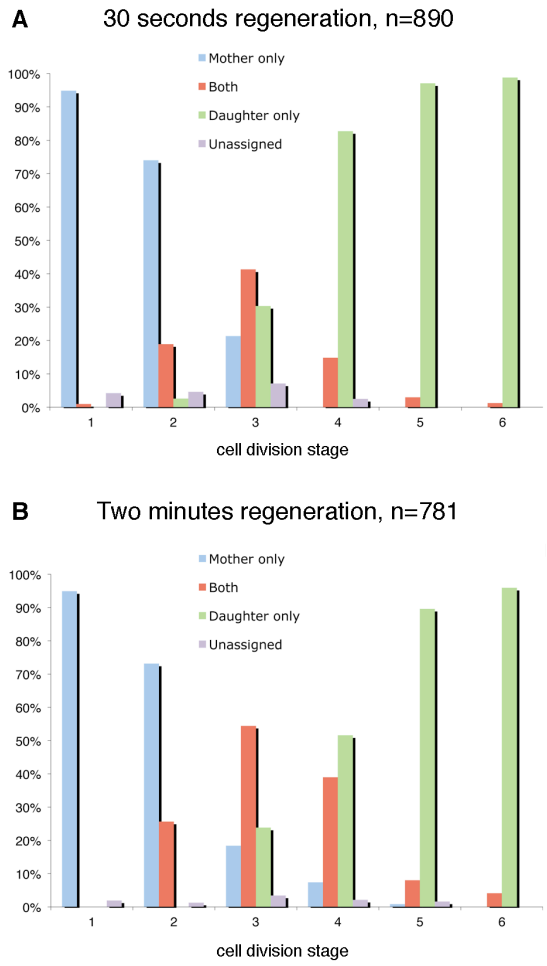

Figure S1. M protein is anchored simultaneously at the mother and daughter septa – analysis of additional populations. (A) Four overnight *S. pyogenes* D471 cultures were diluted 1:100 into TH+Y containing trypsin and pronase, harvested, and resuspended in TH+Y for 30 seconds at 37°C prior to fixation (each sample was processed separately). The fixed cells from the different cultures were mixed and processed together for microscopy. M protein was labeled using specific antibodies, and average intensity 3D-projections were made for numerous DeltaVision images, resulting in a population of 890 cells. The M protein distribution plots were analyzed using MATLAB to determine the presence of newly anchored M protein at the mother and daughter septa (see experimental procedures section). (B) The presence of newly anchored M protein at the mother and daughter septa, was analyzed in the cell population described in Figure 2 (following 2 minutes of protein regeneration), using MATLAB.

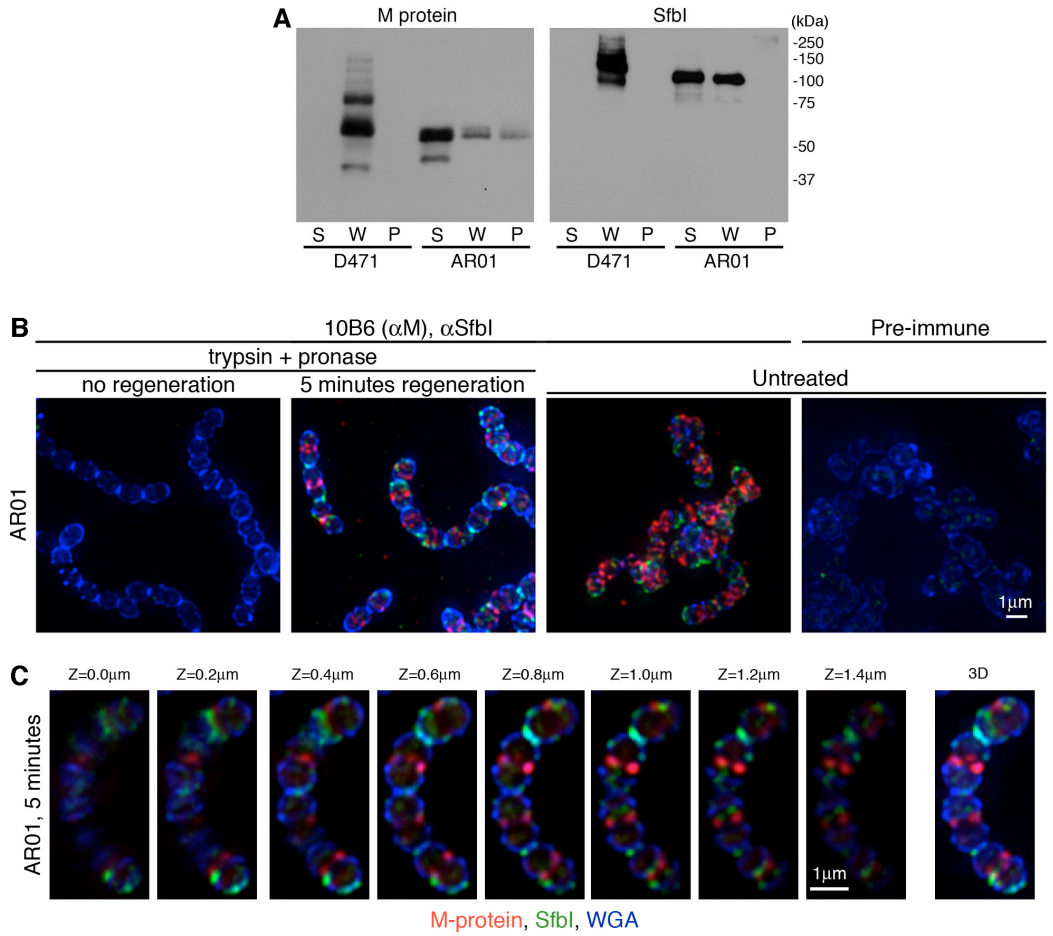

Figure S2. Distribution of M protein and SfbI in the sortase mutant, AR01. (A) Overnight cultures of the sortase deletion mutant AR01, and its parental strain D471, were diluted 1:100 into TH+Y, grown to OD_{600} 0.5, and fractionated into supernatant (S), wall (W), and spheroplast pellet (P). Samples were separated by 10% SDS-PAGE, and examined by Western blot using antibodies specific for M protein or SfbI. (B) Overnight cultures of AR01 were diluted 1:100 into TH+Y or TH+Y containing trypsin and pronase, and grown to OD_{600} 0.5. Protease treated cells were either fixed immediately upon reaching OD_{600} 0.5 (no regeneration), or washed and resuspended in TH+Y without proteases for 5 minutes at 37°C prior to fixation (5 minutes regeneration). Specific antibodies were used to label M protein (red) and SfbI (green), and the cell wall was stained with WGA marina blue (blue). Images are presented as maximum intensity projections. (C) Sequential Z-sections of a representative protease-treated chain following 5 minutes regeneration.