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BINDING OF ALBUMIN PROMOTES BACTERIAL SURVIVAL AT THE EPITHELIAL SURFACE

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Running head: Albumin protects bacteria
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Albumin (HSA) is the dominating protein in human plasma. Many bacterial species, especially streptococci, express surface proteins that bind HSA with high specificity and affinity, but the biological consequences of these protein-protein interactions are poorly understood. Group G streptococci (GGS), carrying the HSA-binding protein G, colonize the skin and the mucosa of the upper respiratory tract, mostly without causing disease. In case of bacterial invasion, pro-inflammatory cytokines are released that activate the epithelium to produce antibacterial peptides, in particular the chemokine MIG/CXCL9. In addition, the inflammation causes capillary leakage and extravasation of HSA and other plasma proteins; environmental changes at the epithelial surface to which the bacteria need to respond. In this study, we find that GGS adsorb HSA from both saliva and plasma via binding to protein G, and that HSA bound to protein G binds and inactivates the antibacterial MIG/CXCL9 peptide. Another surface protein of GGS, FOG, was found to mediate adherence of the bacteria to pharyngeal epithelial cells through interaction with glycosaminoglycans. This adherence was not affected by the activation of the epithelium with a combination of IFN-γ and TNF-α, leading to the production of MIG/CXCL9. However, at the activated epithelial surface, adherent GGS were protected against killing by MIG/CXCL9 through protein G dependent HSA-coating. The findings identify a previously unknown bacterial survival strategy that help to explain the evolution of HSA-binding proteins among bacterial species of the normal human microbiota.

Several streptococcal species, among them Group G Streptococci (GGS; *Streptococcus dysgalactiae,* subspecies *Equisimilis*), colonize the human dermis and the mucosa of the upper airways. In addition to being part of the normal microbiota GGS are opportunistic pathogens, causing both superficial infections, such as pharyngitis and erysipelas, as well as deep and severe infections (1, 2). In the event of bacterial invasion, an inflammatory host response is initiated where dendritic, NK, and T cells residing in subepithelial tissues, recognize bacterial products by pattern recognition receptors (3). This results in the production of proinflammatory cytokines, among them IFN-γ and TNF-α, rendering an activated phenotype to adjacent cells, including the epithelial lining (4-6). As a consequence, epithelial cells start producing host defense molecules, e.g. the antibacterial chemokine MIG/CXCL9 (7, 8). In addition, production of chemokines causes recruitment and activation of neutrophils (3). Release of neutrophil granule proteins, in particular heparin-binding protein (HBP), together with neurogenic mechanisms, contribute to extravasation of plasma constituents, e.g. albumin (9-11). These changes of the habitat at the epithelial surface, force the bacteria to use adaptive countermeasures to survive, including the release of proteases and proteins that attenuate the activity of antibacterial peptides (12-15).

More than thirty years ago it was reported that different streptococcal species bind human serum albumin (HSA) with high affinity (16). In GGS (and group C streptococci), protein G, an IgG-binding surface protein (17, 18), was found to bind also HSA (19). In protein G so-called GA modules, located in the NH_2-terminal region, bind HSA (20-22), whereas the IgG-binding C domains are found in the COOH-terminal half of the molecule (23-24). The interactions with HSA and IgG occur independently, and the organization of protein G allows GGS to coat their surface with an inner layer of IgG and an outer layer of HSA, the two most abundant proteins of human plasma. Surface proteins containing HSA-binding GA modules are also present in strains of the anaerobic bacterium *Finegoldia magna* (22) and in a biofilm-

generating protein (Embp) of *Staphylococcus epidermidis* (25, 26). The bacterial species that express proteins containing GA modules, are all part of the normal human bacterial flora. However, in addition they are opportunistic pathogens, and it is noteworthy that the GA-containing proteins increase the virulence of *F. magna* and *S. epidermidis* (26, 27). A more recently discovered surface protein in GGS, FOG, has also been ascribed a role in virulence by interfering with neutrophil function and binding to collagen (28, 29).

The central question addressed in this paper is whether FOG and the HSA-binding property of protein G affect the survival of GGS at the epithelial surface, when the conditions dramatically change under the influence of proinflammatory chemokines. The results show that FOG is essential for the adherence of GGS to both activated and non-activated epithelial cells, and that the adsorption of HSA to the surface of adherent GGS via the GA modules of protein G, provide the bacteria with a protective shield of HSA against antibacterial MIG/CXCL9 released by the activated epithelium.

Experimental Procedures

Chemicals and reagents- Recombinant human MIG/CXCL9, CCL3, IFN–γ, TNF–α, and rabbit antibodies against MIG/CXCL9 were from Peprotech, Rocky Hill, NJ. Novicidin (KNLRRIIRKGIHIIKKYF), a synthetic peptide derived from the ovine cathelicidin SMAP-29 (30), was generously provided by Dr. Hans-Henrik Kristensen, Novozymes, Copenhagen, Denmark.

Human serum albumin (HSA) was from Sigma (cat. no. A3782). The GA module protein (residues 213 – 265 of protein PAB) was produced as described (31). Dermatan-sulfate 36 (DS36) and heparan-sulfate 6 (HS6) were provided by Lars-Åke Fransson (Lund, Sweden).

Bacterial strains and growth conditions- The *Streptococcus dysgalactaie* subsp. *equisimilis* (GGS) strain G148 and the *Streptococcus mutans* strain α3201 were collected at the Department of Clinical Microbiology, Lund University Hospital, Sweden. The GGS strain G45 was collected at the Royal Brisbane Hospital, Australia. G45 carry protein FOG on their surface while G148 naturally lacks protein FOG. Streptococci were routinely grown in Todd-Hewitt (TH) Broth (Difco) containing Yeast Extract, (2g/L; Oxoid Ltd) in 5% CO_2 at 37°C. The isogenic mutants G45ΔFOG and G45ΔG, lacking protein FOG and protein G, respectively, were grown in the presence of erythromycin (μg/mL) to maintain the knocked out phenotype (32).

Bactericidal assays- The GGS strains G148, G45, and isogenic mutants of the latter, were grown to mid-log phase in TH broth, washed, and diluted in incubation buffer. 50 μl of bacteria ($2x10^6$ cfu/mL) were incubated with MIG/CXCL9 at various concentrations, or buffer alone, for one hour at 37°C. To quantify bactericidal activity, serial dilutions of the incubation mixtures were plated on TH agar and the number of cfu was determined after incubation overnight. To determine bactericidal activity on the surface of the pharyngeal epithelial cell line Detroit 562 (ATCC, Rockville, MD), cells were grown in 24-well tissue culture plates (Costar) to near confluence in Minimal Essential Medium (MEM; GIBCO) with heat-inactivated FBS (10%). Thereafter, cells were washed three times with MEM without FBS and incubated with serum-free MEM in the absence or presence of the indicated stimuli. This was followed by addition of 25 μL of bacteria (AP1; $2x10^6$/mL in PBS), layered on top of the epithelial cells. Centrifugation at 300 x *g* for 10 minutes was performed to promote cell-bacteria interaction and was followed by incubation for 30 minutes at 37°C. Trypsin (2.5 mg/mL in PBS) was used to detach the cells from the wells and Triton X-100 (0.025% in PBS) was added to lyse the cells and release internalized bacteria. The number of cfu was determined by plating appropriate dilutions on culture plates. In some experiments, the mixture of cells and bacteria were detached using a rubber policeman, fixed, and embedded in epon.

Surface plasmon resonance- HSA and GA-module were diluted in sodium acetate (10 mM, pH 4) at a concentrations of 10 μg/mL followed by immobilization via amine coupling to flow cells of a CM5 sensorchip (BIAcore, Uppsala, Sweden). Binding and dissociation phases were monitored using a BIAcore 2000 instrument. HSA and MIG/CXCL9 were injected at different concentrations (typically 31 – 500 nM) at a flow rate of 35 μL/min and a temperature of

25˚C over the flow cells using a running buffer (10 mM Hepes, 150 mM NaCl, 0.005% surfactant P20, and 3.4 mM EDTA; pH 7.5). To determine the association of MIG/CXCL9 with HSA in complex with immobilized GA domain, an equal concentration of HSA (250 nM) was repetitively injected before injecting various concentrations (31 – 500 nM) of MIG/CXCL9 at a later time-point. The association (Ka) and dissociation (Kd) rate constants were determined simultaneously using the equation for 1:1 Langmuir binding in the BIA Evaluation 4.1 software (BIAcore). The binding curves were fitted locally and the equilibration dissociation constants (K_D) were calculated from mean values of the obtained rate constants.

HSA adsorption experiments- Bacteria (strain G148; 2 x 10^8 cfu) were incubated in 100% saliva (collected and pooled from 12 healthy donors after more than one hour of fasting using a sponge device (Salivette™; Sarstedt, Nümbrecht, Germany), 10% citrate-treated plasma or HSA (4 mg/mL in PBS) at room temperature for 20 min. The bacteria were washed extensively in PBS, and bound proteins eluted using glycine-HCl (0.2 M, pH 2). Eluates were separated by SDS-PAGE under reducing conditions and visualized by Coomassie brilliant blue staining.

Preparation of cytoplasts and electron microscopy- Cytoplasts (cell-derived vesicles free of nuclei) mimic the epithelial cell-surface but are smaller and more stable than intact cells, making them suitable for negative staining and electron microscopy. To prepare cytoplasts, a suspension (7.5 x 10^7/mL) of washed Detroit 562 cells was pressurized with nitrogen for 5 min at 350 psi in a custom made nitrogen cavitation bomb. The cavitate was collected and a sub-cellular fraction of membranes was prepared by sequential centrifugation, first at 1,000 × *g* for 10 min followed by 10,000 × *g* for 20 min, whereafter the resulting pellet was collected. MIG/CXCL9 and albumin were labeled with 4 and 15 nm colloidal gold respectively, as described (8). GGS in TBS, (G148 strain, 2×10^6 cfu/mL) were incubated for 20 min with 15 nm gold-labeled HSA. Thereafter, a suspension of the membrane fraction, dissolved in TBS, was incubated with gold-labeled MIG/CXCL9. An equimolar amount of gold-labeled HSA was added and the incubation was allowed to proceed for an additional 20 min. The specimens were subjected to negative staining and examined in a JEOL 1200EX transmission electron microscope. In the case of epon-embedded specimens, ultrathin sections were incubated with rabbit anti-MIG/CXCL9 antibodies (5 μg/mL) and visualized by a secondary step using goat ant-rabbit antibodies labeled with colloidal gold as described (33).

Labeling of bacteria and binding assay- Bacteria were labeled with ^{125}I using lactoperoxidase (Sigma). In brief, one mL of a 2% bacterial suspension (4 x 10^9 cfu/mL in PBS) was heat-killed by incubating the bacteria at 80°C for 10 min. The bacteria were pelleted by centrifugation and resuspended in 100 μl PBS, followed by addition of 0.2 mCi ^{125}I. Two μL lactoperoxidase (2 mg/mL) was added together with 2 μL freshly made 0.015% H_2O_2 in PBS and the suspension was incubated for 10 min at room temperature. The bacteria were washed four times in cold PBS to remove unincorporated ^{125}I, and finally dissolved in 1 mL PBS. Radio-labeling of DS36 and HS6 with ^{125}I and binding to bacteria was performed and analyzed as described (17).

Statistical analysis- Statistical signficance was determined using the Student's *t*-test for paired observations.

RESULTS

Killing of GGS by MIG/CXCL9 is blocked by HAS. MIG/CXCL9 is produced by IFN-γ activated pharyngeal epithelial cells and plays a key role in mediating bactericidal activity at the epithelial cell surface (8). Since GGS can cause pharyngitis, these bacteria will encounter MIG/CXCL9 in this context. Using a viable count assay, GGS (strains G45wt and G148) were dose-dependently killed upon exposure to this antibacterial protein. The CC chemokine CCL3 exerted low or no antibacterial activity at similar concentrations (Fig. 1A). Preincubation of MIG/CXCL9 with HSA for 20 minutes caused an efficient and dose-dependent inhibition of the antibacterial activity at HSA concentrations corresponding to that present in 1%, 10%, and 100% plasma (0.4, 4, and 40 mg/mL), respectively (Fig. 1B). **In these experiments, the two GGS isolates mentioned above were used; strain G148 expresses HSA-binding protein G (19, 20) but lacks FOG, whereas strain G45wt**

expresses both protein G and FOG (19, 20, 28). In contrast to MIG/CXCL9, the antibacterial activity of the ovine cathelicidin-derived synthetic peptide Novicidin, was not significantly affected by HSA.

Analysis of the interactions between GGS, HSA and MIG/CXCL9. The binding between MIG/CXCL9 and HSA was investigated using surface plasmon resonance. MIG/CXCL9 showed a dose-dependent binding to immobilized HSA with a K_D of 28 nM (Fig.1C). To investigate the HSA-binding properties of GGS in physiological contexts, bacteria of the G148 strain were incubated with saliva (undiluted), plasma (10%), or HSA (4 mg/mL in PBS). After washings in PBS, bound proteins were dissociated from the bacterial surface by incubation with acid glycine, separated by SDS-PAGE, and stained with Coomassie (Fig. 1D). The results show that the bacteria adsorb HSA when incubated with plasma or saliva from healthy donors. As judged from the SDS-PAGE bands, the amount of HSA in saliva under non-inflamed conditions is lower than in the diluted plasma sample and the HSA solution. The two bands around 25 and 56 kDa following incubation in plasma, are explained by binding of IgG to the IgG-binding modules of protein G. They correspond to IgG light and heavy chains, respectively (the SDS-PAGE was run under reducing conditions). An α-streptococcus, *Streptococcus mutans*, did not bind any plasma proteins as judged by SDS-PAGE, thus serving as a negative control. In addition, several other gram-positive coccal species, such as *Staphylococcus aureus* and *Staphylococcus epidermidis,* have been reported not to bind albumin (34).

Next, surface plasmon resonance was used to determine whether HSA in complex with the GA modules still binds MIG/CXCL9. GA modules were immobilized on a sensor chip followed by injection of HSA (Fig. 1E), and a strong and stable binding of HSA to the chip was recorded (K_D= 0.5 nM). In a subsequent step, increasing concentrations of MIG/CXCL9 were injected over the chip, and MIG/CXCL9 bound strongly to the GA/HSA complexes with a K_D of 5 nM. No binding was obtained when MIG/CXCL9 was injected over GA alone (not shown).

Similar to most chemokines, MIG/CXCL9 has glycosaminoglycan (GAG)-binding properties (8, 35). To visualize a possible simultaneous interaction between GAGs, MIG/CXCL9, HSA, and GGS, electron microscopy with negative staining was applied (Fig. 1F). Intact cells cannot be used for these studies, whereas cytoplasts (cell-derived vesicles free of nuclei) mimic the epithelial cell-surface but are smaller and more stable. Cytoplasts were prepared from pharyngeal epithelial cells by nitrogen cavitation. GGS (strain G148) were washed and incubated with HSA labeled with 15nm colloidal gold particles and MIG/CXCL9 labeled with smaller (4 nm) gold particles. At the interface between bacteria and cytoplasts, colloidal gold particles of different sizes co-localized, showing the presence of both MIG/CXCL9 and HSA. The large number of the smaller particles, corresponding to MIG/CXCL9, suggests a high local concentration of the chemokine at the interface.

Surface protein FOG of GGS promotes adherence to pharyngeal epithelial cells. A question raised by the results described above, is whether GGS colonizing an epithelial surface are protected by protein G-bound HSA when antibacterial MIG/CXCL9 is produced in response to an inflammatory stimulus. Previous work has demonstrated that the adherence of another streptococcal species, *Streptococcus pyogenes*, to epithelial cells, is promoted by interactions between M protein (for references, see 36) at the bacterial surface and GAGs of the epithelium (37), whereas the mechanisms promoting adherence of GGS to epithelial cells are poorly understood. Initial experiments demonstrated that the G148 strain used in the experiments described above, in contrast to strain G45 (another clinical isolate of GGS), adhered less to epithelial cells and exhibited low or no interaction with highly sulfated GAGs (see below). For these reasons, the G45 strain was used in the subsequent experiments.

Apart from protein G, G45 bacteria express a fibrous M protein-like surface protein called FOG (28) that is not found in the G148 strain, suggesting that this protein could contribute to GAG-binding and adherence. Heat-killed and ^{125}I-labeled wild type (wt) G45 bacteria and the isogenic mutants G45ΔFOG and G45ΔG, lacking protein FOG and protein G, respectively, were pre-incubated in PBS, citrated human plasma, saliva, or HSA solution, followed by washing in PBS. Thereafter, the bacteria were incubated with

epithelial cells that were either non-activated or activated overnight with IFN-γ and TNF-α. The cell culture medium was discarded and the bacteria were incubated with epithelial cells for three hours. After washing, the radioactivity of remaining attached bacteria was determined (Fig. 2 A and B). The adherence was consistently and significantly lower for bacteria lacking protein FOG (G45ΔFOG), both during non-activated and activated conditions. The binding of radio-labeled soluble GAGs (heparan- and dermatan-sulfate; HS and DS) to G148, G45 and G45 mutant bacteria (Fig. 2C), showed that FOG is mainly responsible for the interaction with the GAGs. HS is the dominating GAG at epithelial surfaces, found in proteins such as CD44 and syndecan (38, 39**)**, and the interaction was more pronounced with HS than DS. A decrease in the binding of both GAGs, although to a much lesser degree, was also observed comparing wild type G45 bacteria with the isogenic mutant lacking protein G, whereas strain G148, naturally lacking FOG, bound HS and DS in the order of G45ΔFOG. Taken together, the results demonstrate that FOG interacts with GAGs and promotes adhesion of GGS to both non-activated and activated epithelium.

Binding of HSA protects GGS against killing also at the surface of cytokine-activated pharyngeal epithelial cells. As shown above, the adherence of GGS to the epithelial cells was not influenced by cytokine activation, but these experiments did not address the question whether the survival of the bacteria was affected. GGS were therefore incubated with resting or cytokine-activated pharyngeal epithelial cells. To investigate the role of HSA, the experiments were performed in the absence or presence of HSA and included both wild type and isogenic GGS mutant bacteria lacking the HSA-binding protein G (G45ΔG). Wild type and mutant bacteria were preincubated with HSA in PBS or in PBS alone, washed, and added to epithelial cells grown to confluence. After incubation for three hours, non-adherent bacteria were discarded by washing with PBS, and the viability of the remaining adherent bacteria was determined by viable counts. Wild-type bacteria (G45wt) displayed decreased survival on cytokine-activated pharyngeal epithelial cells compared with non-activated epithelium. However, pretreatment with HSA significantly increased bacterial survival on the surface of activated cells. In contrast, the presence of HSA during the first incubation step, did not influence the survival of the non-HSA-binding G45ΔG mutant on cytokine-activated epithelial cells (Fig 3A, B). Bovine serum albumin (BSA) has a 2-3 orders of magnitude lower binding affinity for the GA-module compared with HSA and was therefore included as an irrelevant control (30). Pretreatment of bacteria (G45wt) with BSA did not increse the survival on cytokine-activated epithelial cells. Taken together, these results suggest that the binding of HSA to the surface of GGS via protein G, protects GGS from antibacterial activity on the activated epithelium.

Previous work has shown that the bactericidal activity of activated pharyngeal epithelial cells against *S. pyogenes* is mainly attributable to MIG/CXCL9 (8). To investigate if this is the case also with GGS, electron microscopy was used to visualize bacteria interacting with epithelial cells and MIG/CXCL9. Bacteria (G45wt) were incubated with either non-activated or cytokine-activated pharyngeal epithelial cells (Fig. 3C-H). Bacteria adhering to the non-activated cells displayed a preserved integrity with a visible cell wall and plasma membrane (Fig. 3A and F), and immunogold staining to detect MIG/CXCL9, indicating low or no presence of the chemokine. In contrast, GGS associated with the surface of cytokine-activated pharyngeal epithelial cells displayed a loss of integrity; cell walls and plasma membrane were no longer apparent (Fig. 3D and G). MIG/CXCL9 was found both at bacterial surfaces as well as intracellularly, indicating uptake of the bactericidal protein. Bacteria incubated with HSA prior to exposure to activated pharyngeal epithelial cells had a preserved morphology with intact plasma membrane and cell wall (Fig. 3E and H). In the latter case, MIG/CXCL9 was seen accumulating at the peripheral parts of the cell wall corresponding to the location of protein G.

DISCUSSION

With regards to structure and protein binding properties, protein G is one of the most well-characterized surface proteins of Gram-positive bacteria. Especially the IgG-binding modules have been studied by several research

groups, and from a functional point of view the binding of IgG via the Fc region was shown to add selective advantage to the bacteria by blocking effector functions of IgG antibodies (for references, see 40). The biological function of the HSA-binding so-called GA modules of protein G is less well understood, and the starting point for this investigation was an early experiment showing that GGS efficiently adsorbed HSA from the saliva of healthy humans, despite a low concentration of HSA. This finding suggested that GGS in the normal bacterial flora of the upper airways under physiological and non-inflammatory conditions, bind HSA to their surface via the GA modules of protein G. It also indicated that the HSA-coating of colonizing GGS should be more pronounced during inflammation and vascular leakage, leading to a high local concentration of HSA at the mucosa. However, the biological consequences of the interaction with HSA remained unclear; was bacterial adherence and colonization affected and/or could HSA at the surface of GGS influence innate immune mechanisms?

A series of experiments showed that protein G and HSA had no or very small impact on the adherence of GGS to epithelial cells, despite some loss of HS- and DS-binding to bacteria lacking protein G. Instead another surface protein, FOG, was found to play a crucial role in the adherence through interactions with highly sulfated GAGs such as HS. During inflammation, a plethora of GAG-binding proteins, among them MIG/CXCL9 and other AMPs, are produced. Interestingly, FOG-mediated adhesion was not changed during inflammation, suggesting that the protein does not compete with chemokines for binding sites on GAGs. This implies that AMPs (e.g. MIG/CXCL9) and FOG bind to different epitopes on GAGs, or that FOG has a higher affinity for shared epitopes on GAGs, thus possibly displacing GAG-attached AMPs. In both cases, a mechanism neutralizing the AMPs close to the bacterial surface should be beneficial to the colonizing GGS.

The central finding of this study is that HSA bound to the surface of GGS via protein G protects the bacteria against MIG/CXCL9, a major and important bactericidal peptide released at the epithelial surface in response to an inflammatory stimulus (8). To utilize a pre-made host protein present in large quantities in inflammatory exudates to form a protective shield against a powerful antibacterial polypeptide, represents a novel bacterial survival strategy which is schematically depicted in Figure 4. From a bacterial point of view, this mechanism appears as a rational and economic way to handle the harsh environment during inflammation. The observation that GGS adsorb HSA from saliva, could indicate that these bacteria as members of the indigenous flora of the oropharynx, also under normal and non-inflammatory condition use HSA-binding for nutrition (see below) and as a defense against constitutively expressed antibacterial peptides/proteins, such as CCL28, histatins, and lysozyme (41, 42).

The HSA-binding GA module, repeats of 45 amino acid residues each, were originally discovered in protein G (19, 20), but was given their name (GA stands for protein G-related Albumin-binding module) when homologous modules were identified in an HSA-binding protein of *Finegoldia magna* (formerly *Peptostreptococcus magnus*), called PAB (22). *F. magna* is an anaerobic Gram-positve *coccus* found in the normal human bacterial flora at all non-sterile body surfaces, including the skin and the oropharynx, and analogous to GGS *F. magna* is also an opportunistic pathogen (for references see 43). So-called exon or module shuffling resulting in functional hybrid proteins has played a major role in the evolution of eukaryotic genes, and in this process introns have been crucial by increasing the probability of favorable duplication and recombination events (44, 45). The known cases of module shuffling took place millions of years ago and there were no contemporary examples (46) until the discovery of PAB revealed that this protein is the product of a transfer of the GA module from the protein G gene of GGS into the PAB gene of *F. magna*, and that the conjugative plasmid pCF10 from *Enterococcus faecalis* participated in the transfer and recombination events resulting in the mosaic organization of PAB (22). The high degree of homology between the GA modules of proteins G and PAB and the short generation times in bacteria, suggested that the shuffling of GA had occurred recently. Since prokaryotic genes as a role lack introns, these results raised the question how multidomain bacterial proteins like PAB evolve. This was explained by the identification of *recer* sequences, a new kind of genetic element promoting interdomain in frame recombination

at the gene level, and acting as structure-less spacers in the corresponding protein (27). Another question relates to the evolutionary pressure behind the shuffling of the GA module between various bacterial species. *F. magna* strains expressing PAB are tetracycline resistant (27), and the pCF10 plasmid mentioned above carries TetM, a common and important tetracycline resistance determinant (47), suggesting that the exposure to antibiotics has stimulated the rapid evolution of GA-containing proteins in bacterial species of the normal flora.

The binding of HSA to GA was shown to promote bacterial growth, presumably by making the free fatty acids transported by HSA (in plasma 99% of these are bound to HSA) available for the bacteria (27), and the present work presents another selective advantage of the GA module, especially for bacteria of the normal microbiota. Apart from GGS (and the closely related group C streptococci) and *F. magna,* a GA-containing protein (Embp) has also been identified in *S. epidermidis* (25). Embp is a giant protein (1 MDa) which is necessary for biofilm formation and it contains 38 GA modules (26). GGS, *F. magna,* and *S. epidermidis* are all members of the normal flora and they are often found in the same ecological niches (this is also true for *E. faecalis* that contributes to the shuffling of GA with the pCF10 plasmid). When an inflammatory stimulus, for instance a pathogen trying to establish itself on an epithelial surface inhabited by these commensals, results in the exudation of HSA and the production of antibacterial MIG/CXCL9, the binding of HSA to GA will provide both nutrients and protection against MIG/CXCL9. This efficient usage of the dominating human plasma protein emphasizes the power of bacterial adaptation and sheds further light on the rapid and ongoing evolution of GA-containing proteins among bacterial species of the normal human flora. A final aspect of the properties and spread of the GA module and the selective advantages it offers, is related to virulence. Commensals expressing GA-containing proteins are also opportunistic pathogens, and there are numerous reports suggesting that they represent a growing medical problem. Thus, under antibiotic pressure novel proteins are formed that tilt the delicate balance between bacteria of the normal flora and the human host towards virulence.

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FOOTNOTES

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The abbreviations used are: MIG/CXCL9, Monokine Induced by Gamma-interferon/CXC-Ligand 9; TH, Todd-Hewitt broth; cfu, colony forming unit; HSA, human serum albumin; GGS, group G streptococci.

FIGURE LEGENDS

Fig. 1. Characterization of the interactions between the antibacterial chemokine MIG/CXCL9, GGS, and HSA. (*A*) Bactericidal activity of MIG/CXCL9 against GGS. Bacteria (strains G45wild and G148) were incubated with MIG/CXCL9 or CCL3 at the indicated concentrations, or with buffer alone. To calculate % killing, the cfu present after exposure to the polypeptides was compared with the cfu obtained after incubation in buffer alone. The data shown represent mean ± SEM from three separate experiments. (*B*) HSA attenuates the bactericidal activity of MIG/CXCL9. Bacteria were incubated with recombinant MIG/CXCL9 (0.3 μM) for one hour at 37°C, or with MIG/CXCL9 that had been preincubated with HSA at 0.4, 4, or 40 mg/mL (corresponding to the HSA-content of 1, 10, and 100% plasma), respectively, for 20 min, prior to exposure to GGS (strains G45wild or G148) for one hour at 37°C. The number of cfu after incubation in buffer alone was set to 100%. The antibacterial activity of the peptide Novicidin (NC; 0.3 μM) was not significantly affected (p=0.25) by the presence of albumin (40 mg/mL). The data shown represent mean ± SEM from three separate experiments. (*C*) HSA binds MIG/CXCL9 in surface plasmon resonance experiments. HSA was immobilized on a sensor chip and increasing concentrations of MIG/CXCL9 were injected over the surface. The start and stop of injection is denoted by an arrowhead and arrow, respectively. (*D*) GGS adsorb HSA from saliva and plasma. Bacteria were incubated with undiluted saliva, 10% plasma, or HSA (4 mg/mL) for 20 minutes. After washings in PBS, bound proteins were eluted from bacteria with glycin-HCl. These eluted proteins were separated by SDS-PAGE and visualized by Coomassie staining. Bands corresponding to albumin (arrow) are seen in all lanes, except the PBS control. The bands of approx. 25 and 56 kDa in the lane with material eluted from bacteria incubated with plasma (arrowheads), correspond to light and heavy chains of IgG, respectively. The *Streptococcus mutan* (strain α3201) was incubated with plasma but no bound proteins were visible after elution. (*E*) Binding of albumin to immobilized GA modules followed by a dose dependent increase in binding of MIG/CXCL9 as determined by surface plasmon resonance. GA modules were immobilized on a sensor chip, followed by injection (between the two arrowheads) and binding of HSA to the chip. Thereafter, increasing concentrations of MIG/CXCL9 were injected over the surface (indicated by the arrow), displaying a stable binding to the surface-bound GA/HSA complexes. (*F*) Electron micrograph showing the interaction between cytoplasts derived from pharyngeal epithelial cells, MIG/CXCL9 (labeled with 4 nm colloidal gold particles; arrowheads), HSA (labeled with 15 nm colloidal gold particles; arrow), and bacteria.

Fig. 2. Binding of GGS to pharyngeal epithelial cells. (*A, B*) GGS strain G45; wild type (wt) and the isogenic mutants G45ΔFOG and G45ΔG, lacking protein FOG and protein G, respectively, were killed by heat and labeled with ^{125}I. Pharyngeal epithelial cells were cultured in the absence (non-activated) or presence (activated) of a combination of IFN-γ (100 U/mL) and TNF-α (10 ng/mL). ^{125}I-labelled G45wt, G45ΔFOG, and G45ΔG bacteria were incubated for 15 min in PBS, 10% human plasma, whole saliva (*A* panel) or HSA (0.4 mg/mL) (*B* panel), washed, and incubated with epithelial cells for three hours at 37°C. After washing, the radioactivity of the remaining pellets was calculated, and the radioactivity in percent of total added radioactivity was determined. Values are mean ±SEM of

at least three separate experiments, and Student's *t*-test for paired observations was used to calculate *p*-values. (*C*) GGS bind highly sulfated glycosaminoglycans. G45wt, G45ΔFOG, G45ΔG, and G148 bacteria were incubated with ^{125}I-labeled heparan-sulfate (HS) or dermatan-sulfate (DS) for one hour at room temperature. After washing the radioactivity of the bacterial pellets was determined and compared with the total radioactivity added. Data represent mean ±SEM of four separate experiments. *P*-values were calculated using Student's *t*-test for paired observations.

Fig. 3. Bacterial survival at the surface of non-activated and activated pharyngeal epithelial cells. (A) GGS (strain G45wt) were incubated with human serum albumin (4 mg/mL) or PBS, washed, and added to non-activated or IFN-γ and TNF-α activated epithelial cells. Thereafter, bacteria and epithelial cells were co-incubated for three hours. Following washing, the epithelial cells were detached and lysed, and the resulting debris was plated on agar plates. The number of cfu was calculated and related to the control; non-activated epithelial cells and G45 wt pretreated with HSA. Values are mean ±SEM from three separate experiments, and *p*-values were calculated using Student's *t*-test for paired observations. (*B*) The same experiment as in (*A*) performed with the isogenic mutant G45ΔG devoid of protein G. Values represent mean ±SEM of three separate experiments.

(*C)* Electron microscopy of G45wt bacteria (GGS) incubated with non-activated pharyngeal epithelial cells (Ep). Ultrathin sections of cells and bacteria were incubated with anti-MIG/CXCL9 antibodies followed by visualization of bound antibodies using secondary antibodies conjugated with 10 nm colloidal gold particles. The bacteria have a preserved integrity with a visible cell wall/plasma membrane. A few scattered gold particles are seen, indicating low or no presence of MIG/CXCL9. (*D*) G45wt bacteria visualized at the surface of pharyngeal epithelial cells activated with a combination of IFN-γ and TNF-α. The integrity of bacteria is lost and cell wall/plasma membrane is no longer apparent. MIG/CXCL9 is seen associated both with the bacterial surface (arrows) and intracellularly (arrowheads). (*E*) G45wt bacteria coated with HSA prior to exposure to activated pharyngeal epithelial cells. Bacterial integrity is preserved, and large amounts of MIG/CXCL9 is detected at the bacterial surface but not intracellularly (arrows). Bar = 1 μm. *(F)* Higher magnification (10x) from *(C)*, showing bacterial cell wall (W; indicated with a zigzag-line) and the lipid bilayer of the plasma membrane (M) of a *streptococcus* in contact with non-activated epithelial cell. *(G)* Disintegrated plasma membrane and cell wall with dispersed presence of MIG/CXCL9 in a *streptococcus* in contact with cytokine-activated epithelium. *(H)* Higher magnification (10x) of (*A*) of a *streptococcus* coated with HSA. MIG/CXCL9, visualized as colloidal gold particles, accumulates at the cell wall (W; indicated with a zigzag-line) and the plasma membrane (M) is intact**.**

Fig. 4. Schematic and hypothetical representation of GGS at the epithelium of the oropharynx. (A) During normal, non-activated conditions, GGS adhere to the epithelium via binding of FOG to highly sulfated GAGs. It is likely that the GA modules of protein G adsorb HSA from saliva, coating the bacteria also in the absence of an inflammatory stimulus causing vascular leakage and extravasation of HSA. (*B*) In response to pro-inflammatory stimuli, epithelial cells produce antibacterial peptides, including MIG/CXCL9. This chemokine is associated with GAGs at the epithelial surface but is also present in a soluble form. HSA bound to protein G binds and attenuates the bactericidal activity of MIG/CXCL9. Also at the activated epithelial surface, protein FOG remains an important mediator of bacterial adhesion.

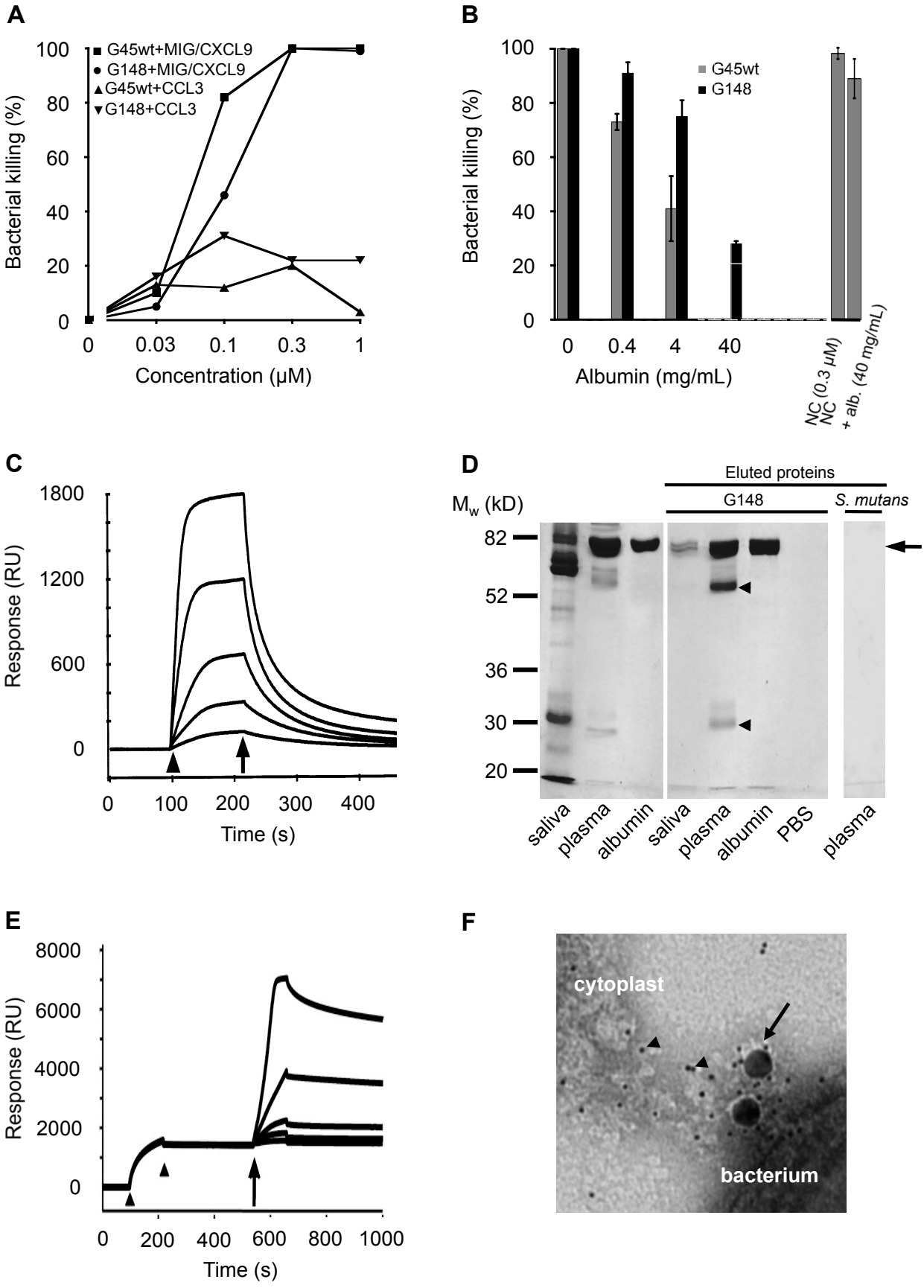
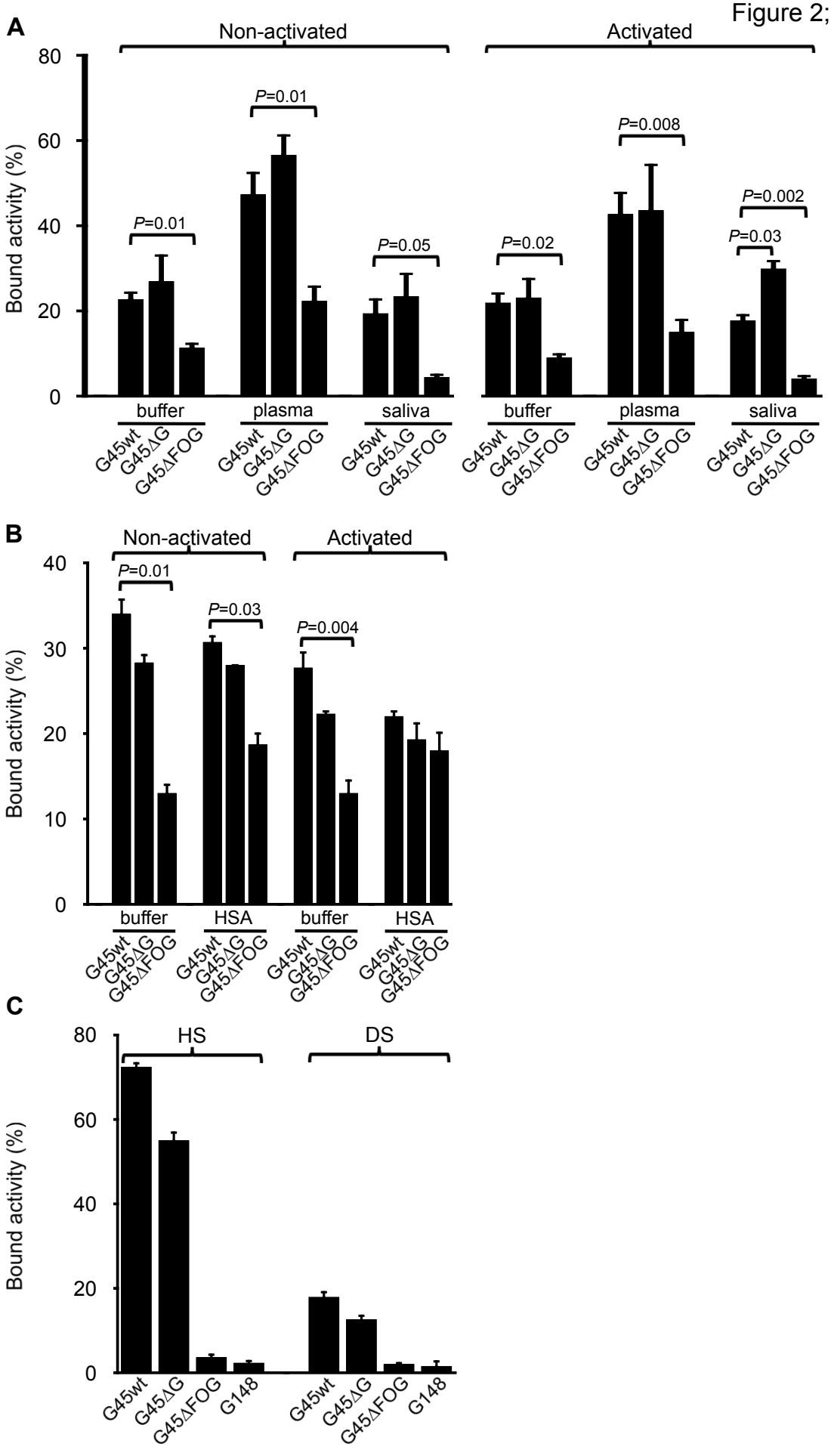

A
Bacterial killing (%)
G45wt+MIG/CXCL9
G148+MIG/CXCL9
G45wt+CCL3
G148+CCL3
Concentration (μM)
B
Bacterial killing (%)
G45wt
G148
Albumin (mg/mL)
NC (0.3 μM)
NC + alb. (40 mg/mL)
C
Response (RU)
Time (s)
D
Eluted proteins
G148
S. mutans
Mw (kD)
saliva
plasma
albumin
PBS
E
Response (RU)
Time (s)
F
cytoplast
bacterium

Figure 2; Egesten *et al.*



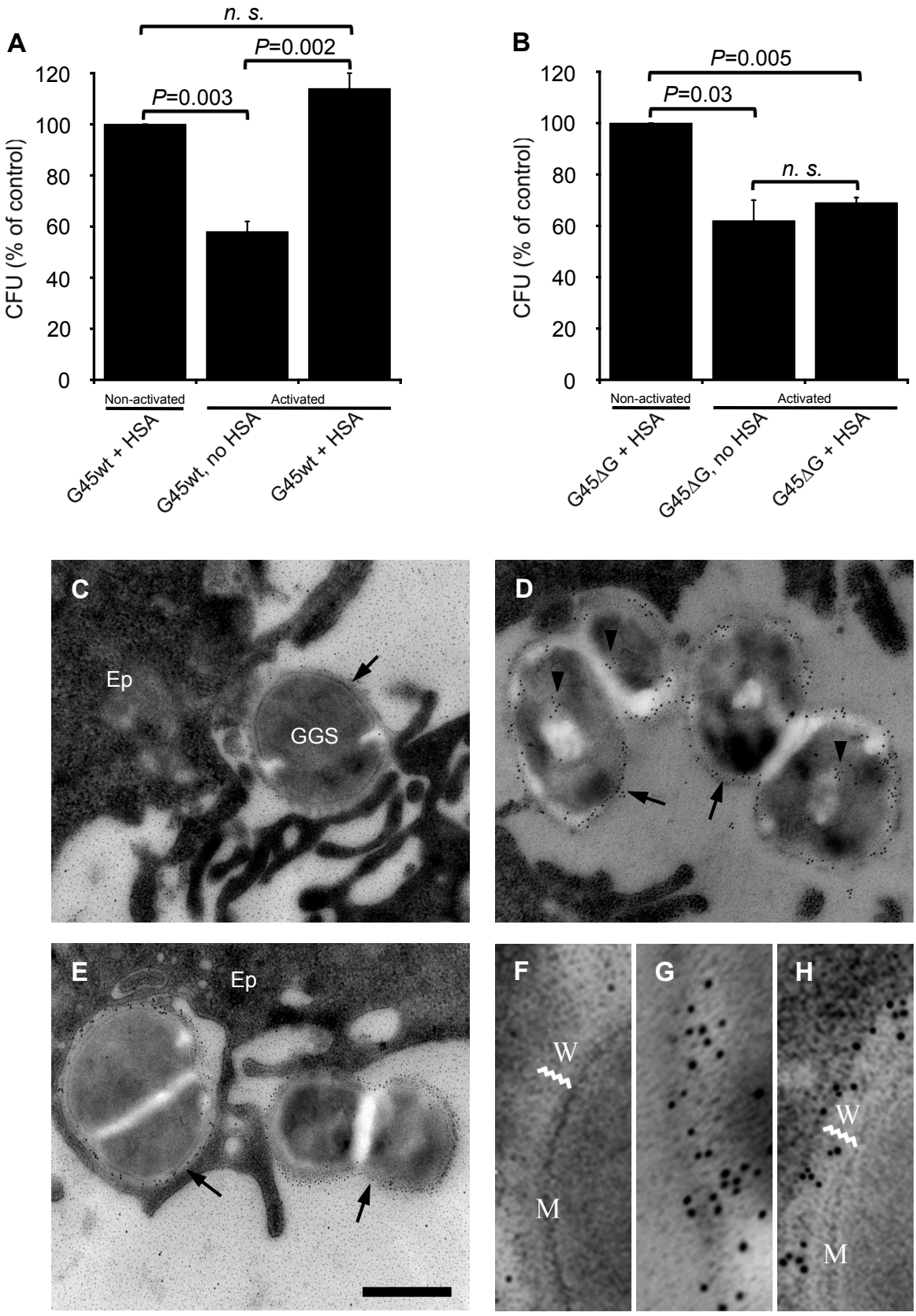

A
n. s.
P=0.002
P=0.003
CFU (% of control)
120
100
80
60
40
20
0
Non-activated
Activated
G45wt + HSA
G45wt, no HSA
G45wt + HSA
B
P=0.005
P=0.03
n. s.
CFU (% of control)
Non-activated
Activated
G45ΔG + HSA
G45ΔG, no HSA
G45ΔG + HSA
C
Ep
GGS
D
E
Ep
F
W
M
G
H
W
M

Figure 4; Egesten *et al.*