RESEARCH ARTICLE

A type VII secretion system of *Streptococcus gallolyticus* subsp. *gallolyticus* contributes to gut colonization and the development of colon tumors

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**Abstract**

*Streptococcus gallolyticus* subspecies *gallolyticus* (*Sgg*) has a strong clinical association with colorectal cancer (CRC) and actively promotes the development of colon tumors. However, the molecular determinants involved in *Sgg* pathogenicity in the gut are unknown. Bacterial type VII secretion systems (T7SS) mediate pathogen interactions with their host and are important for virulence in pathogenic mycobacteria and *Staphylococcus aureus*. Through genome analysis, we identified a locus in *Sgg* strain TX20005 that encodes a putative type VII secretion system (designated as *SggT7SS*). We showed that core genes within the *SggT7SS* locus are expressed *in vitro* and in the colon of mice. Western blot analysis showed that *Sgg*EsxA, a protein predicted to be a T7SS secretion substrate, is detected in the bacterial culture supernatant, indicating that this *SggT7SS* is functional. Deletion of *SggT7SS* (*TX20005Δesx*) resulted in impaired bacterial adherence to HT29 cells and abolished the ability of *Sgg* to stimulate HT29 cell proliferation. Analysis of bacterial culture supernatants suggest that *SggT7SS*-secreted factors are responsible for the pro-proliferative activity of *Sgg*, whereas *Sgg* adherence to host cells requires both *SggT7SS*-secreted and bacterial surface-associated factors. In a murine gut colonization model, TX20005Δesx showed significantly reduced colonization compared to the parent strain. Furthermore, in a mouse model of CRC, mice exposed to TX20005 had a significantly higher tumor burden compared to saline-treated mice, whereas those exposed to TX20005Δesx did not. Examination of the *Sgg* load in the colon in the CRC model suggests that *SggT7SS*-mediated activities are directly involved in the promotion of colon tumors.
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Taken together, these results reveal \( \text{SggT7SS}^{\text{T05}} \) as a previously unrecognized pathogenicity determinant for \( \text{Sgg} \) colonization of the colon and promotion of colon tumors.

Author summary

Colorectal cancer (CRC) is a leading cause of cancer-related death. The development of CRC can be strongly influenced by specific gut microbes. Understanding how gut microbes modulate CRC is critical to developing novel strategies to improve clinical diagnosis and treatment of this disease. \( S. \text{gallolyticus} \) subspp. \( g. \text{gallolyticus} \) (\( \text{Sgg} \)) has a strong clinical association with CRC and actively promotes the development of colon tumors. However, the specific \( \text{Sgg} \) molecules that mediate its pro-tumor activity are unknown. Here we report the first characterization of a type VII secretion system (T7SS) in \( \text{Sgg} \), designated as \( \text{SggT7SS}^{\text{T05}} \). We further demonstrate that \( \text{SggT7SS}^{\text{T05}} \)-mediated activities are important for \( \text{Sgg} \) to colonize the colon and to promote the development of colon tumors. These findings reveal \( \text{SggT7SS}^{\text{T05}} \) as a novel pathogenicity determinant of \( \text{Sgg} \) and provide a critical breakthrough in our efforts to understand how \( \text{Sgg} \) influences the development of CRC. Future investigations of the biological activities of specific effectors of \( \text{SggT7SS}^{\text{T05}} \) will likely lead to the discovery of \( \text{Sgg} \) molecules that can be used as diagnostic markers and intervention targets aimed at mitigating the harmful effect of \( \text{Sgg} \).

Introduction

Colorectal cancer (CRC) is the third most common cancer among men and women, and a leading cause of cancer-related deaths worldwide [1]. The ability of the gut microbiota to influence cancer risks and to modulate the development of CRC is well recognized [2–8]. The gut microbiota of CRC patients often displays distinct compositional differences and altered diversity compared to those from healthy individuals [8–11]. Furthermore, certain bacterial species are able to promote the development of colon tumors in pre-clinical models of CRC [2,3,5,6,12–14]. This has raised the possibility that by targeting specific CRC-promoting microbes, we can improve the way CRC is diagnosed, treated and managed. To achieve this goal, understanding the molecular mechanism underlying the cancer-promoting capability of specific microbes is critical.

\( \text{Streptococcus gallolyticus} \) subspp. \( g. \text{gallolyticus} \) (\( \text{Sgg} \)), previously known as \( \text{Streptococcus bovis} \) biotype I, is a member of the \( \text{Streptococcus bovis}/\text{Streptococcus equinus} \) complex [15]. \( \text{Sgg} \) is an opportunistic pathogen that causes bacteremia and infective endocarditis (IE). It is also known for its strong clinical association with CRC, as documented by numerous case reports and studies over the past several decades [12,16–28]. A study of case series and reports published from 1970 to 2010 found that on average, ~60% of patients with \( \text{Sgg} \) IE/bacteremia have concomitant colon adenoma or adenocarcinoma, a rate much higher than that in the general population [19]. In a prospective study, a higher percentage of patients with \( \text{Sgg} \) IE developed colonic neoplastic lesions in subsequent years compared to patients with IE caused by closely related enterococci (45% vs. 21%) [17]. A metagenomic study using datasets and samples from multiple countries across three continents further confirmed that \( \text{Sgg} \) is a biomarker for CRC [9]. Adding to this strong clinical association, functional studies have demonstrated that \( \text{Sgg} \) plays an active role in promoting the development of colon tumors. \( \text{Sgg} \) stimulates the proliferation of human colon cancer cells in a manner that is dependent on \( \beta \)-catenin signaling [12].
In vivo, exposure to Sgg resulted in larger tumors in a xenograft model [12], and higher tumor burden and dysplasia grade in an azoxymethane (AOM)-induced CRC model [12] and in a colitis-associated CRC model [27]. The mechanism underlying the pro-tumor activity of Sgg, however, remains elusive.

The type VII secretion system (T7SS), also called the Esx secretion system, is a specialized secretion system first discovered in pathogenic *Mycobacterium*. It has now been found widely distributed among Firmicutes and Actinobacteria [29,30]. T7SS plays an important role in bacterial virulence and persistent infection. In *M. tuberculosis*, ESX-1 is by far the most prominent virulence determinant and the principal distinction between virulent mycobacteria and the attenuated BCG vaccine strains [31–38]. Consequently, T7SS proteins have been actively pursued for the development of diagnostic markers, drugs and vaccines for tuberculosis [39]. In *Staphylococcus aureus*, T7SS is important for virulence and persistent infection in a variety of disease models including kidney abscesses, nasal colonization, pneumonia, skin and blood infection [40–43].

In this study, we report the characterization of a newly identified T7SS in Sgg strain TX20005 (SggT7SS\textsuperscript{T05}). We showed that SggT7SS\textsuperscript{T05} is active, as demonstrated by the expression of core T7SS genes \textit{in vitro} and \textit{in vivo}, and the secretion of a T7SS effector. Functional studies showed that SggT7SS\textsuperscript{T05} is important for Sgg adherence to CRC cells and stimulation of CRC cell proliferation. Animal studies further demonstrated that SggT7SS\textsuperscript{T05} is important for the colonization of the colon and is involved in the promotion of colon tumors by Sgg. These results reveal for the first time that T7SS is an important determinant of Sgg pathogenicity in the gut.

**Results**

**The SggT7SS\textsuperscript{T05} locus**

The genome of TX20005 was sequenced using the PacBio Sequel platform. The assembly produced one circular contig with a length of 2,258,025 bp (S1 Table). Genome annotation using the RAST [44] server identified 2,181 coding sequences, 18 rRNA operons, and 71 tRNA genes. Examination of the genome sequence revealed a putative T7SS locus that we designate as SggT7SS\textsuperscript{T05}. All T7SSs studied so far contain two conserved features: a protein of approximately 100 amino acids with a centrally positioned WXG motif (WXG100 proteins) that is secreted by T7SS, and EssC, a transmembrane protein of the FtsK-SpoIIIE-like ATPase family that is essential for the secretion machinery. These two features are present in the SggT7SS\textsuperscript{T05} locus (Fig 1A). The first gene in the SggT7SS\textsuperscript{T05} locus encodes a protein of 97 amino acids with a central WXG motif that is highly homologous to the EsxA protein of *S. aureus* (49% identity and 72% similarity). Hence this gene was named Sgg\_esxA. The Sgg\_esxA gene is followed by five protein-coding genes displaying strong sequence homology to core components of the T7SS secretion machinery in *S. aureus* (Fig 1A). The last of these encodes a transmembrane protein highly similar to EssC of *S. aureus* (42% identity and 61% similarity) and is predicted to contain two FtsK/ATP-binding domains in the large C-terminal cytosolic segment. Analysis of the membrane segments and the protein topology of these putative Sgg T7SS core components indicates that the Sgg proteins also show the same topology as their respective counterparts in *S. aureus* (Fig 1B).

The gene immediately downstream of Sgg\_essC (Sgg507) encodes a protein of 100 amino acids with ~32% similarity to EssC, a T7SS effector of *S. aureus* (Burts, 2008). The remaining five genes in the locus (Sgg508—Sgg512) encode hypothetical proteins. The first three of these are 129, 93 and 100 amino acids long, each of which contains an LXG motif in the middle. Analysis by HHpred [45] predicts that each of these adopts a four-helical bundle structure...
Fig 1. The SggT7SST05 locus. A. The genetic organization of SggT7SST05. The percentage beneath each gene indicates the % similarity in amino acid sequence to the corresponding protein in Staphylococcus aureus USA300 [90], analyzed using Global Align at NCBI. Dashed box indicates genes encoding core components of the secretion machinery.

B. Predicted protein topology of core components of SggT7SST05. Membrane protein topology was predicted using Phyre2 [91]. Secretion prediction was carried out using SecretomeP 2.0a [92].

C. The presence of a C-terminal consensus sequence motif in the 5 putative T7SS effectors within the locus. The 20 amino acid residues at the C-terminal end of SggEsxA, and Sgg507 to Sgg510 are shown.

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typical of WXG100 proteins, with a probability of 85.3%, 97.4%, and 98.8%, respectively. The C-terminal end of WXG100 proteins is known to contain a conserved sequence motif HxxxD/ExxIxxxH, where H denotes highly conserved and h less conserved hydrophobic residues [46]. This motif is considered to be important for the secretion of WXG100 proteins by T7SS [46]. Examination of the Sgg homologs of EssA (SggEsxA) and EsaC (Sgg507), as well as the three putative WXG100 proteins predicted by HHpred (Sgg508–Sgg510) indicates that all contain this motif (Fig 1C). Sgg511 is a protein with 439 amino acid residues. Its N-terminal region of ~100 residues contains a central LXG motif and is predicted to fold into a four-helical bundle structure, although with a relatively low probability (36.8%). The rest of the protein does not display significant sequence homology except for a low-level similarity to bacteriocin (PF10439) at the C-terminus. This raises the possibility that this protein may belong to the LXG polymorphic toxin family [47–49]. Based on these analyses, the five genes downstream of Sgg_essC appear to encode proteins that belong in the WXG superfamily and are likely secretion substrates of T7SS [46–48,50]. The last gene in the SggT7SS T05 locus (Sgg512) is predicted to contain six transmembrane segments with unknown function.

Genes within the SggT7SS T05 locus are located close to each other, with the exception of a sizeable gap of 74bp between Sgg_esaA and Sgg_esaA. Typical -35 and -10 regions were identified upstream of Sgg_esaA but not in the intergenic region between Sgg_esaA and Sgg_esaA using BPRoM [51]. There was also no transcriptional terminator identified in this intergenic region using the iTerm-PseKNC server [52], suggesting that genes within the locus are likely transcribed as one transcriptional unit. RT-PCR was performed to further confirm this by using primer pairs that span two adjacent genes all the way across the locus. The results showed that bands of the expected size from each of the adjacent pairs can be amplified from cDNA preparations from TX20005 (Fig 2), indicating that they are indeed transcribed.
together. We could not PCR amplify the region spanning Sgg512, the last gene in the locus, and the downstream gene Sgg513, suggesting that Sgg512 is the last gene in the mRNA transcript.

**SggT7SS<sup>T05</sup> encodes a functional T7SS**

We next sought to determine if core genes within the SggT7SS<sup>T05</sup> locus are expressed. We focused on the two conserved genes of T7SS, Sgg<sub>esxA</sub> and Sgg<sub>essC</sub>. Expression of these genes was analyzed by RT-PCR. We found that both Sgg<sub>esxA</sub> and Sgg<sub>essC</sub> were expressed in TX20005 grown in brain heart infusion (BHI) broth (Fig 3A, indicated by arrows) and when TX20005 was co-cultured with the CRC cell line HT29 (Fig 3B). To determine if these genes are expressed in vivo, we collected colon tissues from mice orally gavaged with TX20005 or saline and extracted tissue RNA. We found that both Sgg<sub>esxA</sub> and Sgg<sub>essC</sub> were expressed in the colonic tissues. We did not detect any PCR amplification products in saline-treated mice, indicating the specificity of the PCR method. In addition, bands of the expected size were not observed in PCR reactions using RNA preparations that had not been treated with reverse transcriptase, indicating that there is minimal DNA contamination in the RNA samples. Taken together, these results indicate that Sgg<sub>esxA</sub> and Sgg<sub>essC</sub> are expressed in vitro and in vivo.

We next sought to examine the secretion activity of SggT7SS<sup>T05</sup>. Tag-free recombinant SggEsxA protein (rEsxA) (S1 Fig) was obtained and used to generate rabbit antiserum. The anti-serum was used to determine if SggEsxA was secreted from TX20005 by western blot. We were able to detect a band with the expected molecular weight for SggEsxA (~10.9 kDa) in the culture supernatants (CS) from TX20005 but not in whole bacterial lysates (WBL) (Fig 4A). We generated a deletion mutant in which the DNA region encoding the core secretion machinery (Sgg<sub>esxA</sub> to an N-terminal portion of Sgg<sub>essC</sub>) was deleted. This resulted in a mutant (TX20005Δesx) missing the entire secretion machinery. TX20005Δesx did not have any growth defects compared to the parent strain TX20005 when grown in BHI broth (Fig 4B). The deletion also did not affect the transcription of the upstream (Sgg500) or the downstream (Sgg507) genes flanking the deleted region (S2 Fig). As expected, we did not detect a band of the expected molecular weight for SggEsxA in either the CS or WBL from the deletion mutant (Fig 4A and S3 Fig), indicating that the band we observed in the CS of TX20005 is...
specific for SggEsxA. An antibody against the cell division protein FtsZ was used as a cytosolic loading control. The antibody detected a band with the expected molecular weight in the WBL from both TX20005 and the deletion mutant, but not in the CS from either strain, suggesting
that the EsxA band we observed in the CS from TX20005 is secreted rather than released from bacterial lysis. Taken together, these results indicate that the $Sgg^{T7SS^{T05}}$ locus encodes a functional T7SS.

$Sgg^{T7SS^{T05}}$ is required for adherence of $Sgg$ to CRC cells

Interactions with colonic epithelial cells are important for $Sgg$ pathogenicity in the gut. Previous studies showed that $Sgg$ is capable of adhering to human CRC cell lines [12,53]. We sought to determine if $Sgg^{T7SS^{T05}}$ is important for $Sgg$ adherence to host cells. The results showed that TX20005Δesx adheres to HT29 cells at a significantly reduced level compared to that of TX20005 (~68% reduction) (Fig 5A). Immunofluorescence microscopy was also performed. The wild type and the mutant bacteria display similar morphology, appearing as single cells or in short chains as expected (Fig 5B). Again, the mutant adhered to HT29 at a significantly reduced level compared to TX20005 (Fig 5B and 5C), further confirming the result from using the plating method.

We next examined if $Sgg^{T7SS^{T05}}$-secreted factors are involved in $Sgg$ adherence to host cells. CS from TX20005 and TX20005Δesx were filter-sterilized and added to the WT or the mutant bacteria in the adherence assay. The results showed that the adherence of TX20005 to HT29 cells was significantly enhanced by WT CS, whereas CS from the mutant had no effect. Interestingly, the CS from neither TX20005 nor the mutant had any effect on the adherence of the mutant bacteria (Fig 5A), indicating that WT CS alone is insufficient to restore the adherence of TX20005Δesx to the WT level and that bacterial surface associated molecules are also required. Taken together, these results suggest that $Sgg^{T7SS^{T05}}$-secreted factors likely act as an adaptor that bridges $Sgg$ surface associated molecules with a host cell receptor(s). Furthermore, the results suggest that the bacterial surface molecules involved in adherence are also dependent on a functional T7SS.

$Sgg^{T7SS^{T05}}$ is required for $Sgg$-induced CRC cell proliferation

TX20005 is able to directly stimulate the proliferation of CRC cells in a β-catenin-dependent manner [12]. We tested the effect of disrupting $Sgg^{T7SS^{T05}}$ on the pro-proliferative capacity of $Sgg$. HT29 cells were cultured in the presence or absence of WT and mutant bacteria for 24 hours. TX20005 significantly increased cell proliferation as expected, whereas deletion of T7SS abrogated the ability of TX20005 to stimulate cell proliferation (Fig 6A). We confirmed that there is no difference in bacterial titers between TX20005 and TX20005Δesx during these cell proliferation assays (S4 Fig). We next tested the effect of CS on cell proliferation by incubating HT29 cells in media supplemented with filter-sterilized CS from TX20005 or TX20005Δesx. CS from TX20005 significantly increased cell proliferation compared to cells cultured in media only, whereas CS from TX20005Δesx had no effect (Fig 6A). To further validate this finding, we performed western blot analysis to probe for β-catenin and proliferating cell nuclear antigen (PCNA). The results showed that both β-catenin and PCNA were significantly increased in cells co-cultured with TX20005 bacteria or CS, whereas no increase was observed in either proteins in cells cultured with the mutant bacteria or CS (Fig 6B–6D). Taken together, these results indicate that $Sgg^{T7SS^{T05}}$-secreted factors are able to directly stimulate host cell proliferation.

$Sgg^{T7SS^{T05}}$ is important for the colonization of colon by $Sgg$

We investigated if $Sgg^{T7SS^{T05}}$ is important for the colonization of the colon by $Sgg$ in a mouse colonization model (Fig 7A) [26]. Fecal materials and colon tissues were collected at day 1, 3, and 7 post bacterial oral gavage, weighed, homogenized, and plated onto selective agar plates
Fig 5. Disruption of SggTSS3ß5 impairs Sgg adherence to host cells. A. HT29 cells were incubated with TX20005 or TX20005Δesx (MOI = 10), supplemented with or without CS from TX20005 or TX20005Δesx for 1hr. The cells were washed and attached bacteria were enumerated by dilution plating, as described in the Materials and Methods section. Adherence was calculated as the percentage of adhered bacteria vs. total bacteria added. Data shown are the mean ± SEM and combined from
three independent experiments. Ns, not significant; *: p < 0.05; **, p < 0.01; unpaired two-tailed t test. B. Immunofluorescence microscopy was performed as described in the Materials and Methods section. Representative images are shown. C. Fluorescence intensity at 488 nm was quantified using Image J on 20 randomly selected fields for each group. ****, p < 0.0001, unpaired two-tailed t test.

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Fig 6. SggT7SS^T05 is required for Sgg to stimulate host cell proliferation. A. Cell proliferation assay. HT29 cells were incubated with TX20005 or TX20005Δexs bacteria (MOI = 1), or in media supplemented with CS from TX20005 or TX20005Δexs for 24 hours. Viable cells were quantified using the CCK-8 assay. B-D. Western blot. HT29 cells were incubated with TX20005 or TX20005Δexs bacteria (MOI = 1), or in media supplemented with CS from TX20005 or TX20005Δexs for 9 hours. Whole cell lysates were analyzed by western blot, probed with antibodies against β-catenin, PCNA and β-actin. Representative images are shown (B). Band intensity was quantified using ImageJ, normalized to β-actin first, and then to cells only to calculate fold changes. Results shown are mean ± SEM, combined from at least three independent experiments (C and D). ns, not significant; *: p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; unpaired two-tailed t test, vs. cells only.

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We found that the Sgg load in the fecal materials from mice gavaged with TX20005Δesx was significantly lower than that in the TX20005 group at all three time points (Fig 7B). In the colonic tissues, the Sgg load from mice gavaged with the mutant was similar to that in mice gavaged with TX20005 at day 1, but significantly decreased at day 3 and 7 (Fig 7C). We further investigated the ability of WT and mutant Sgg to colonize the distal and proximal portion of the colon. We found no difference in the Sgg load between these two sites for either TX20005 or the mutant at day 3 or 7 (S5A and S5B Fig), suggesting that SggT7SS<sup>T05</sup> contributes to Sgg colonization of both the distal and proximal colon.

Taken together, the results described here indicate that SggT7SS<sup>T05</sup> is important for the colonization of the colon by Sgg.
SggT7SS\textsuperscript{T05} is important for the promotion of colon tumors

We next investigated if SggT7SS\textsuperscript{T05} plays a role in the development of colon tumors in an AOM-induced mouse model of CRC (Fig 8A). As expected, mice treated with WT TX20005

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**Fig 8.** Deletion of SggT7SS\textsuperscript{T05} impairs the ability of Sgg to promote the development of colon tumors. The effect of TX20005 and TX20005\textsuperscript{Δesx} on the development of colon tumors was examined in an AOM-induced mouse model of CRC, as shown in A. Macroscopic tumors were evaluated by blinded observers. Tumor burden (B) was calculated as the sum of tumor volumes from one mouse. Fecal materials were homogenized and dilution plated onto Enterococcus Selective Agar plates to enumerate Sgg bacteria (C). Results were combined from two independent experiments (n = 11–12 mice/group). The mean ± SEM are indicated in the graph. *, p < 0.05; ns, not significant; Mann-Whitney test.

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exhibited significantly increased tumor burden compared to mice treated with saline alone (Fig 8B). However, mice exposed to TX20005Δesx did not show a significant increase in the tumor burden compared to the saline group, suggesting that the mutant is attenuated in promoting the development of colon tumors. In addition, the mean tumor burden in TX20005Δesx-treated group is lower than that in TX20005-treated group, although the difference is not statistically significant (p = 0.1010, Mann-Whitney test). It is likely that SggT7SS\textsuperscript{T05} is not the only factor important for the promotion of colon tumors by Sgg, other factors are also involved. Interestingly, we did not observe any difference in the Sgg burden between TX20005-treated and the mutant-treated groups (Fig 8C). This could be due to the repeated oral gavage of bacteria during the animal procedure, which could have masked the difference in the colonization capacity between the WT and mutant strains, or that SggT7SS\textsuperscript{T05} is not involved in colonizing tumor-bearing colons. These results, combined with the pro-proliferative effect of CS from TX20005 shown in Fig 5, suggest that SggT7SS\textsuperscript{T05}-secreted factors are directly involved in promoting the development of colon tumors.

Discussion

Sgg has a long-standing clinical association with CRC [18–25,54–67]. Recent functional studies using pre-clinical models of CRC further demonstrate that Sgg actively promotes the development of colon tumors [12,27]. The molecular determinants responsible for Sgg pathogenicity in the colon, however, were unknown. Here we report the characterization of a newly identified T7SS of Sgg and show that this T7SS is an important player in colonization of the colon, interactions with CRC cells, and promotion of colon tumors. To our knowledge, this is the first characterization of T7SS in Sgg, the first direct evidence that T7SS is a critical determinant for Sgg pathogenicity in the colon, and the first identification of a molecular pathway important for the pro-tumor activity of Sgg.

We found that SggT7SS\textsuperscript{T05} displays a strong similarity to S. aureus T7SS in both genetic organization and amino acid sequence of the core components. Further analysis of the proteins encoded by genes within this locus identified six putative secretion substrates of SggT7SS\textsuperscript{T05}: SggEsxA, and Sgg507 to Sgg511. The first five of these proteins display features indicative of WXG100 proteins: ~100 amino acid residues in length, a centrally positioned W/LXG motif, a predicted four-helix bundle structure typical of WXG100 proteins, and a C-terminal conserved sequence motif HxxxD/ExxxH [46,50]. The last of the six proteins (Sgg511) shows an N-terminal similarity to the WXG100 proteins and a low-level similarity to bacteriocin, suggesting that it may belong to the LXG polymorphic toxin family [47–49].

The ability of Sgg to interact with the colonic epithelium and influence epithelial homeostasis and function is key to its pathogenicity in the colon. Sgg was previously shown to adhere to colonic epithelial cell lines but not invade these cells [12,53,68]. The Pil3 pilus of Sgg is able to bind to mucin and fibrinogen [69] and mediates Sgg adherence to HT29-MTX, a high mucin producer, but not to HT29 [68]. Our results indicate that SggT7SS\textsuperscript{T05} is important in Sgg adherence to HT29 cells, suggesting that SggT7SS\textsuperscript{T05}-mediated adherence likely involves a novel mechanism distinct from that of Pil3. The fact that CS from TX20005 enhances the adherence of TX20005 but does not increase the adherence of TX20005Δesx further suggests that SggT7SS\textsuperscript{T05}-mediated adherence involves both secreted and bacterial surface associated factors. It is possible that SggT7SS\textsuperscript{T05}-secreted factors act as adaptors linking bacterial surface associated proteins to host cell receptors, as proposed previously from structural analysis of WXG100 proteins [70]. The inability of TX20005 CS to restore the adherence capacity of the mutant suggests that the relevant bacterial surface associated factors also depend on a functional T7SS. This can happen in a number of ways. Recent work in S. aureus suggests that
T7SS secretes or stabilizes peripheral membrane proteins [71]. Work from Lou et al. suggests that there may be a T7SS secretion needle [72,73]. In addition, disruption of Ess-5 in mycobacteria affected bacterial surface hydrophobicity, which can influence bacterial interaction with host cells or the association of bacterial proteins with the surface [74].

Sgg was previously shown to stimulate the proliferation of colon cancer cells in vitro and in vivo and this effect requires β-catenin [12]. The Sgg molecules responsible for this pro-proliferative effect were unknown. Here we demonstrate that disruption of T7SS abolishes the ability of Sgg to stimulate cell proliferation, or to upregulate β-catenin or PCNA. Thus, SggT7SS<sup>TOS</sup> is required for Sgg to stimulate host cell proliferation. The fact that TX20005 CS alone stimulated host cell proliferation whereas CS from the mutant did not suggest that Sgg stimulation of host cell proliferation is mediated by SggT7SS<sup>TOS</sup>-secreted proteins. This is consistent with a recent study showing that CS from several Sgg strains were able to directly promote HT29 cell proliferation in the absence of bacteria [75]. At the moment, it is unclear whether the SggT7SS<sup>TOS</sup>-secreted factor mediating cell proliferation is the same as the one that enhances Sgg adherence to host cells. We previously showed that Sgg strains that adhered poorly were also unable to stimulate cell proliferation [26]. It is possible that the secreted factor responsible for stimulating cell proliferation is the same as the one that bridges the bacteria to the host cells. However, the possibility that distinct factors are involved in adherence and proliferation cannot be excluded. Further investigations to identify the specific secreted factors involved in these processes are needed.

Colonization of the colon by Sgg affords Sgg the opportunity to exert its influence in the colon. Our data indicates that SggT7SS<sup>TOS</sup> is important for Sgg to colonize the colon. Deletion of SggT7SS<sup>TOS</sup> significantly reduced Sgg load in the fecal materials and the colonic tissues. Previous work indicated that gallicin, a bacteriocin produced by Sgg, mediates Sgg colonization of the proximal portion of tumor-bearing colons [76]. Here we showed that SggT7SS<sup>TOS</sup> is involved in Sgg colonization of both the proximal and distal portion of normal colons. Thus, SggT7SS<sup>TOS</sup>-mediated Sgg colonization of the colon represents a novel colonization mechanism for Sgg. There are a number of ways SggT7SS<sup>TOS</sup> mediates gut colonization (Fig 9). The reduced Sgg load in the colonic tissues could be due to the impaired ability of Sgg to adhere to the colonic epithelium, as we showed that disruption of SggT7SS<sup>TOS</sup> impaired Sgg adherence to host cells. In addition, LXG toxins secreted by T7SS mediate interbacterial antagonism [49,77]. Sequence analysis of the SggT7SS<sup>TOS</sup> locus suggests that one of the genes (Sgg511) may encode a potential LXG toxin, thereby providing a competitive advantage and contributing to its survival in the gut. Lastly, T7SS is known to modulate host immune responses. It is important for the escape of mycobacteria from macrophage phagosomes [78–81] and the interaction with the cytosolic DNA sensor nucleotidyltransferase cyclic GMP–AMP synthase (cGAS) [82–84]. Effectors secreted by the T7SS of <i>S. aureus</i> stimulate IL-12 signaling [85] and control dendritic cell function [86]. Sgg is reported to induce subdued immune responses. The cytokine profile in the mouse colon following exposure to Sgg is similar to that following exposure to a common gut commensal <i>Lactococcus lactis</i> [12]. Sgg also selectively recruits bone-marrow derived suppressor cells and inhibits CD4<sup>+</sup> T cells, resulting in an immune tolerant microenvironment [27]. Transcriptomic analysis revealed that Sgg induces lower cytokine expression in macrophages compared to <i>S. aureus</i> [87]. Whether SggT7SS<sup>TOS</sup> plays a role in this subdued immune response is currently unknown. Further investigation is needed to gain insight into the involvement of SggT7SS<sup>TOS</sup> in regulating host immune responses.

Results from experimental models indicate that Sgg actively promotes the development of colon tumors [12,27], however, the Sgg molecules that mediate the tumor-promotion activity were unknown. Our results from the AOM-induced CRC model indicate that disruption of SggT7SS<sup>TOS</sup> impaired the ability of Sgg to promote colon tumors. This combined with the lack
of difference in the Sgg load between the WT and mutant bacteria-treated mice and the observed effect of TX20005 CS on cell proliferation suggest that SggT7SS<sup>T05</sup> contributes to the development of colon tumors through, at least in part, a direct effect of SggT7SS<sup>T05</sup>-secreted factors on tumor cell proliferation. Our results also suggest that Sgg factors other than SggT7SS<sup>T05</sup> are likely to be involved in tumor promotion as well. This is not surprising as previous studies suggest that Sgg promotes the development of colon tumors via multiple mechanisms. Sgg was shown to upregulate β-catenin signaling and the upregulation is essential for Sgg to stimulate cell proliferation and tumor growth [12]. Sgg also induces an immune tolerant microenvironment that favors tumor progression [27]. Further investigation into the biological activities mediated by specific SggT7SS<sup>T05</sup> effectors will be important to dissect the molecular mechanism underlying the pro-tumor capability of Sgg.

In summary, we report the first characterization of a T7SS in Sgg and demonstrate that it plays an important role in Sgg interactions with colonic epithelial cells, colonization of the colon and promotion of colon tumors. Based on the aforementioned results and discussion, a working model for how SggT7SS<sup>T05</sup> contributes to Sgg pathogenicity in the colon is proposed (Fig 9). Antibacterial toxins secreted by SggT7SS<sup>T05</sup> may inhibit the growth of other commensal bacteria in the gut, thereby affording a competitive growth advantage to Sgg. Adherence to the colonic epithelium further facilitates Sgg colonization of the colonic tissues. Close proximity to the colonic epithelium allows effectors secreted by SggT7SS<sup>T05</sup> to target host cell receptors and induce signaling activities involved in cell proliferation. Lastly, effectors secreted by SggT7SS<sup>T05</sup> may also modulate immune responses in the gut, creating an immune suppressive environment favorable for Sgg survival and tumor development. Further investigation into the activities of specific SggT7SS<sup>T05</sup> effectors will be important to elucidate the molecular pathways organized by SggT7SS<sup>T05</sup> that regulate keys aspects of Sgg pathogenicity in the gut. These specific effectors...
are likely candidates for biomarkers with strain-level resolution, and targets for clinical intervention.

Materials and methods

Bacterial strains, cell lines and growth conditions

Sgg strains [12] were grown in brain heart infusion (BHI) agar or broth (Teknova). For co-culture experiments and animal studies, stationary phase bacterial cultures were pelleted, washed with PBS, resuspended in phosphate-buffered saline (PBS) containing 15% glycerol, aliquoted and stored at -80˚C. Stocks were washed with PBS, and properly diluted to obtain the required concentration. Human colon cancer cell line HT29 (ATCC) was grown in Dulbecco’s Modified Eagle’s Medium F-12 50/50 (DMEM/F-12, GIBCO) supplemented with 10% FBS at 37˚C with 5% CO₂ in a humidified chamber. Cells between passages 20 and 30 were used in the experiments.

Genome Sequencing of TX20005

Genome sequencing of the Sgg strain TX20005 was performed using the PacBio Sequel platform (Pacific Biosciences). Read correction and de novo assembly was completed using Canu version 1.7.1 [88]. Assembly produced one circular contig with a length of 2,258,025 bp and nearly 174X depth of coverage. Genome polishing was conducted using Arrow (https://github.com/PacificBiosciences/GenomicConsensus; Pacific Biosciences), a tool that builds a final consensus sequence by mapping raw data against the assembled contig. The resulting sequence was subsequently annotated via the Rapid Annotations using Subsystems Technology (RAST) [44] server sponsored by the National Microbial Pathogen Database Resource (https://rast.nmpdr.org). Raw and corrected read metrics are shown in S1 Table.

Adherence assay

1) Plate assay. This was performed as described previously [12]. Briefly, HT29 cells were seeded at a density of 1x10⁶ cells/well in a 24 well plate and allowed to attach overnight. Cells were incubated with bacteria at a multiplicity of infection (MOI) of 10 for 1 hour at 37˚C with 5% CO₂. Cells were then washed with PBS, lysed with PBS containing 0.025% Triton-X 100 and dilution plated. Adherence was calculated as the percentage of adherent bacteria vs. total bacteria added.

2) Immunofluorescence microscopy. HT29 cells were seeded onto Nunc Lab-Tek II Chamber slides at a density of 5x10⁵ cells per well. Bacteria were added to the cells (MOI = 10) and incubated for 1 hour. Cells were washed with PBS, fixed in 100% ice-cold methanol for 10 minutes at -20˚C, and washed again with PBS. Cells were blocked in blocking buffer (PBS containing 1 mg/ml saponin and 2% (w/v) bovine serum albumin) for 30 minutes at room temperature, and then incubated with rabbit anti-Sgg (1:250) [12] and Alexa Fluor™ 594 conjugated wheat germ agglutinin (1:250) (Thermo Fisher) overnight at 4˚C in the dark, followed by incubation with goat anti-rabbit IgG Alexa Fluor 488 Conjugate and counterstained with DAPI (1:250). Slides were viewed in a DeltaVision confocal microscope. Fluorescence intensity at 488nm was quantified on 20 randomly selected fields for each group using ImageJ.

Cell proliferation assay

This was performed as described previously [12] with modifications. Briefly, HT29 cells were seeded in 96 well plates at a concentration of ~1x10⁴ or ~5x10⁴ cells/well and incubated overnight to allow cells to attach. Cells were then incubated in fresh DMEM/F-12 with 10% FBS containing Sgg bacteria (MOI = 1) for a total of 24 hours. Trimethoprim (1 µg/mL final concentration) was added after 6 hours of incubation to inhibit bacterial growth, as previously
described [12]. The number of viable cells was determined using the CCK-8 kit following the instructions of the supplier (Apex Bio).

**Preparation of Sgg culture supernatants**

Overnight cultures of Sgg grown in BHI broth were centrifuged. The supernatants were filtered through a 0.2 μm filter and then concentrated ~ 10-fold using Pierce™ Protein Concentrator PES, 3K Molecular Weight Cutoff, aliquoted and stored at -80˚C. For adherence and cell proliferation assays, concentrated supernatants were reconstituted in the appropriate tissue culture media to 1X.

**RNA extraction, cDNA synthesis and PCR**

1) *From colonic tissues*. Colonic tissues were collected one day after mice were orally gavaged with Sgg strain TX20005 (~1x10⁸ CFU/mouse) or saline. RNA was extracted using the DNA/RNA/Protein Allprep kit (Qiagen).

2) *From co-cultures with HT29 cells*. RNA was extracted from HT29 cells following 24-hour incubation with or without bacteria (MOI = 1) using the DNA/RNA/Protein Allprep kit (Qiagen).

3) *From Sgg cultures*. RNA was extracted from overnight cultures of Sgg grown in BHI using the E.Z.N.A. Bacterial RNA Kit (Omega). All RNA preparations were treated with DNase. cDNA was synthesized using a ProtoScript II First Strand cDNA Synthesis Kit (NEB). PCR primers are listed in Table 1. PCR reactions were performed using Taq polymerase and appropriate annealing temperatures.

**Deletion of SggT7S T05**

The mutagenesis system developed by Danne et al. [89] was used to delete SggT7S T05. Briefly, the ~ 1kb region upstream of Sgg_essA and the ~1kb region in the C-terminal portion of Sgg_essC were synthesized by Genscript and cloned into pUC57 (Genscript). The insert was then subcloned into a temperature sensitive conjugative plasmid pG1- oriT GBS1. The construct sequence was verified and introduced into S. galactiae NEM316 by electroporation and then into Sgg strain TX20005 by conjugation under the permissive temperature. Erythromycin-resistant transconjugants were then cultured under a non-permissive temperature to select

| Table 1. Oligonucleotides used in this study. |
|---------------------------------------------|
| **Forward (5’-3’)** | **Reverse (5’-3’)** |
| exxA-esaA | CTGAGGGAGCAACTCCGTACGTTG | ACCATATCTGCATGACGAG |
| esaA-esaB | TGCATCCTACTTGATGGGAAATCAGC | CCCCATTAAAGATCCTAAAAAGC |
| esaB-essB |GTGGGGCTACTTTTGCTACCTACCTTGAGC | GTTCCATACACTAATCTCATCAG |
| essB-essC | CGGGCGCCTCATTTTGAGC | CAGGCGCCTCATTTTGAGC |
| essC-507 | GATACTGCCTTAAATAGGGTTAAGAAGT | CATCTCCATCTTTAACG |
| 507–508 | GAGGGGCTTAAAGATGAAAGT | GCCATCTGTGTGTCATCTG |
| 508–509 | GCACAATTTCGAGCAGATATTCAAGT | GCCTCTCCTTGTGTATTTGGAG |
| 509–510 | GCACAACTTACTACCTCACCTAAAAATACATTACAGGAGAGC | CTAGTTGTGACCCCTCTTATTGAGT |
| 510–511 | GTGACTCTCAAACGCCTAATAGGCTAG | CTGTTCCATTCTGTGTGTCG |
| 511–512 | GAGGCTTATGCGGCTGTTGGGATTGGATAGAAG | CTGAAAGCTCTACAATTTAATTGGAATAG |
| 512–513 | CCGTAAAAATACCTTTTATATATACAAATAATAGAAGC | CCCCCACCCCTTTGATGCAAAG |
| Sgg_essA | TGAGGGAGCAACTCCGTACGTTG | GATCTCTGTGACGAG |
| Sgg_essC | TACAGCTTCTGCTGCTCA | GCTCTGTGACGAG |
| Sgg500 | GAAAATGTTGTATGGGTATCTTTGTATGGGTATGAGAAG | GTTGTTGCGGATATATGTAAGGTTAAG |
| Sgg507 | ATGTCAACAGAATATTACCCGTAGGATCTGTGT | CCGATTTTTTTTATATGAAGGTTTATTTTGTCTT |

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for single cross-over recombinants, followed by serial passage in antibiotic-free BHI and screening for double cross-over deletion mutants by PCR. Deletion was confirmed by PCR amplification of the regions spanning the deleted fragment and DNA sequencing of the PCR product, and by whole genome sequencing.

**Growth curve**

Overnight cultures of Sgg were inoculated into fresh BHI broth at 1:100 dilution and grown at 37°C with shaking. Samples were taken at 0, 3, 6, 9, 12, and 24 hours, dilution plated, incubated for 24 hours and colonies enumerated.

**Western blot**

For the detection of SggEsxA, TX20005 and TX20005Δesx were grown in BHI at 37°C with shaking for ~18 hours. Cultures were centrifuged, supernatants collected, filtered through a 0.2 μm filter, and concentrated using the Pierce™ Protein Concentrator PES, 3K Molecular Weight Cutoff. Bacterial pellets were washed and resuspended in PBS. Supernatants and bacterial suspensions were boiled in SDS loading dye and subjected to SDS-PAGE. Membranes were probed with anti-EsxA rabbit serum (1:100) (Pacific Immunological) and anti-FtsZ (1:250) antibodies (Abbexa, cat no. abx319936), followed by incubation with secondary antibodies conjugated to horse radish peroxidase (HRP) (1:3000). Blots were washed and immersed in Clarity Max ECL (Bio-rad). Images were acquired using a Fluorchem M chemiluminescent imager (Protein Simple).

Detection of β-catenin and PCNA was carried out as described previously [12]. Briefly, total cell lysates from HT29 cells cultured in the presence or absence of bacteria or CS were subjected to SDS-PAGE, transferred, and probed with antibodies against β-catenin (1:1000), PCNA (1:1000), and β-Actin (1:1000) (Cell Signaling Technology), followed by incubation with HRP-conjugated secondary antibodies (1:3000). Band intensities were measured using ImageJ and normalized to that of β-Actin.

**Animal experiments**

Animal studies were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the Texas A&M Health Science Center, Institute of Biosciences and Technology. Mice were fed with standard ProLab IsoPro RMH3000 (LabDiet). (1) **Colonization experiments.** This was performed as previously described [26] with slight modifications. Briefly, 6-week old A/J mice, sex matched (Jackson Laboratory), were treated with ampicillin at a concentration of 1 g/L in drinking water for 3 days and switched to antibiotic-free water 24 hours prior to administration of bacteria. Sgg was orally gavaged at a dose of 1 x 10⁹ CFU/mouse. Colons and fecal materials were collected at day 1, 3, and 7 post-gavage. Samples were homogenized in PBS in a TissueLyser (Qiagen), dilution plated onto Enterococcus Selective Agar (ESA) plates and incubated at 37°C for 24–48 hours to enumerate Sgg colonies, as previously described [76]. (2) **AOM-induced model of CRC.** This was performed as previously described [12] with slight modifications. Briefly, A/J mice were treated with 4 weekly i.p. injections of AOM (10 mg/kg body weight), followed by ampicillin in drinking water (1 g/L) for 7 days and switched to antibiotic-free water 24 hours prior to the first oral gavage with Sgg. Mice were orally gavaged with bacteria at ~ 1x10⁹ CFU/mouse or saline once per week for 12 weeks. Colons of mice were collected, cut opened longitudinally, and tumor number and size were recorded. Tumor burden was calculated as the sum of all tumor volumes per mouse. Visual evaluation of colons was carried out by a blinded observer.
Statistical analysis

GraphPad Prism 8 was used for statistical analyses. Two-tailed unpaired t-test was used for pairwise comparisons to assess the significance of differences between two groups in cell proliferation assays, western blot analysis, and adherence assays. The non-parametric Mann-Whitney test was used to assess the significance of differences in the bacterial load and tumor burden in animal studies. Two-way ANOVA was used to analyze the bacterial growth curves. Ns, not significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.

Supporting information

S1 Table. Raw and corrected read matrices for genome sequencing of TX20005. (DOCX)

S1 Fig. Expression and purification of recombinant SggEsxA (rEsxA). The DNA sequence encoding full-length SggEsxA was codon optimized, synthesized by IDT as a gBlocks gene fragment, and then cloned into the pWL613a vector (a pET28b-based vector with an N-terminal 6His tag and a TEV protease cleavage site) which was linearized with BamHI and XhoI, thus producing a construct which expresses 6His-SggEsxA driven by a T7/lac promoter. The integrity of the resulting plasmid was confirmed by DNA sequencing. The construct was transformed into E. coli strain Rosetta2 (DE3) (Novagen) and protein expression was induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside. Recombinant 6His-SggEsxA was purified using a HisTrap column (GE Healthcare), followed by a HiPrep 26/10 desalting column (GE Healthcare). The purified protein was digested with TEV to remove the His tag, and loaded onto a HisTrap 5 column to collect the flow-through, which was then further purified by size exclusion chromatography using a HiLoad 16/600 Superdex 200 pg column (GE Healthcare) (A). The purified tag-free rEsxA was examined via 4–20% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining (B). (TIF)

S2 Fig. Deletion of Sgg_esxA to Sgg_essC does not affect the transcription of upstream or downstream genes. RNA was extracted from stationary phase TX20005 grown in BHI, treated with DNase and reverse transcribed. PCR was performed using primer pairs internal to the gene upstream of Sgg_esxA (Sgg500) and the gene downstream of Sgg_essC (Sgg507) (Table 1). Genomic DNA (gDNA) from TX20005 was used as a positive control for PCR, and RNA without reverse transcriptase treatment (no RT) was used as a control for possible DNA contamination. (TIF)

S3 Fig. Western blot analysis of culture supernatants (CS) from TX20005 and TX20005Δesx. CS was prepared from Sgg grown in BHI broth with shaking for ~ 18 hours and analyzed as described in the Materials and Methods section. An equivalent of ~ 0.5 ml of overnight cultures was loaded onto an SDS gel. The membrane was probed with anti-EsxA antiserum followed by HRP-conjugated secondary antibodies. Purified rEsxA was used as a control for the protein. (TIF)

S4 Fig. Growth of TX20005 and TX20005Δesx in the cell culture conditions with trimethoprim. Stationary phase TX20005 and TX20005Δesx was inoculated into the appropriate cell culture media (time 0) and incubated following the procedure described for cell proliferation assays. Samples were taken at 0 hour, immediately after the addition of trimethoprim (6 hour),
and at the end of the incubation (24 hour). Bacterial titer was determined by dilution plating of the culture media onto tryptic soy agar and incubation for 24–48 hours.

(S5) Fig. Sgg burden in the proximal and distal portion of the colon. Colons collected at day 3 (A) and 7 (B) post bacterial gavage from the colonization experiment were separated into proximal and distal portions, weighed, homogenized and plated onto Enterococcus Selective Agar plates to enumerate Sgg bacteria. Data shown are the mean ± SD (n = 5/group).

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