Oral Polyphosphate Suppresses Bacterial Collagenase Production and Prevents Anastomotic Leak Due to *Serratia marcescens* and *Pseudomonas aeruginosa*

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Objective: The objective of this study was to determine the effect of polyphosphate on intestinal bacterial collagenase production and anastomotic leak in mice undergoing colon surgery.

Background: We have previously shown that anastomotic leak can be caused by intestinal pathogens that produce collagenase. Because bacteria harbor sensory systems to detect the extracellular concentration of phosphate which controls their virulence, we tested whether local phosphate administration in the form of polyphosphate could attenuate pathogen virulence and prevent leak without affecting bacterial growth.

Methods: Groups of mice underwent a colorectal anastomosis which was then exposed to collagenolytic strains of either *Serratia marcescens* or *Pseudomonas aeruginosa* via enema. Mice were then randomly assigned to drink water or water supplemented with a 6-mer of polyphosphate (PPi-6). All mice were sacrificed on postoperative day 10 and anastomoses assessed for leakage, the presence of collagenolytic bacteria, and anastomotic PPi-6 concentration.

Results: PPi-6 markedly attenuated collagenase and biofilm production, and also swimming and swarming motility in both *S. marcescens* and *P. aeruginosa* while supporting their normal growth. Mice drinking PPi-6 demonstrated increased levels of PPi-6 and decreased colonization of *S. marcescens* and *P. aeruginosa*, and collagenase activity at anastomotic tissues. PPi-6 prevented anastomotic abscess formation and leak in mice after anastomotic exposure to *S. marcescens* and *P. aeruginosa.*

Conclusions: Polyphosphate administration may be an alternative approach to prevent anastomotic leak induced by collagenolytic bacteria with the advantage of preserving the intestinal microbiome and its colonization resistance.

Keywords: anastomotic healing score, mouse model of colonic anastomosis, polyphosphate, *Pseudomonas aeruginosa*, *Serratia marcescens*

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Despite decades of descriptive research, the etiology and pathogenesis of anastomotic leak remains unknown. Although there is compelling evidence that microbes are the primary drivers of the pathogenesis of anastomotic leak, few efforts have been aimed at understanding and controlling the microbes that may complicate anastomotic healing.[1,2]

A microbial cause for anastomotic leak was first proposed over 60 years ago,[3] and has been confirmed in multiple studies.[4] The main clinical evidence for a microbial cause of anastomotic leak is indirect and is based on clinical trials in which the use of oral nonabsorbable antibiotics significantly reduce the incidence of anastomotic leak.[5,6]

Clinical studies have demonstrated that despite the use of purgative cleansing agents and intravenous and oral nonabsorbable antibiotics, patients still remain colonized by strains of *Enterococcus faecalis* and *Pseudomonas aeruginosa* for as long as 7 days after surgery.[7] We have provided compelling evidence that these pathogens can produce collagenases that impair anastomotic healing.[8,9] Thus strategies other than antibiotics are needed to contain the virulence of these pathogens.

Here we present the concept of phosphate-based therapy as a method to attenuate bacterial virulence without affecting bacterial growth. This latter property may be highly desirable given the emerging data demonstrating the health-promoting effects of preserving the intestinal microbiome.[10] Previous work from our laboratory has demonstrated that bacteria harbor exquisitely sensitive sensory systems to detect the extracellular concentration of phosphate, which plays a major role in virulence expression.[11,12] Because we have shown that surgical injury depletes local intestinal phosphate concentrations,[13] maintaining local phosphate levels at anastomotic tissues may suppress the release of undesired bacterial virulence products.

In the present report, we identify a pathogen capable of producing a high degree of collagenase, *Serratia marcescens*, whose role in anastomotic leak has not been previously described. We show that 6-mer of polyphosphate (PPi-6) suppresses collagenase production in *S. marcescens* and *P. aeruginos*a in vitro. We then created a novel model of bacteria-mediated anastomotic leak by exposing mice to both preoperative and postoperative antibiotics followed by introduction of collagenolytic bacteria via enema. Mice drinking PPi-6 were protected against anastomotic leak due to the inoculated pathogens.

METHODS

Specific Aims

Aim 1: To demonstrate that PPi-6 can suppress the virulence of *S. marcescens* (S2) and *P. aeruginosa* (P2) in vitro as judged by their collagenolytic activity, biofilm formation, and motility.

Aim 2: To demonstrate that a collagenolytic strain of *S. marcescens* is capable of causing anastomotic leakage in mice when introduced via enema after a low colon anastomosis.

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Aim 3: To demonstrate that mice orally ingesting PPi-6 in their drinking water are protected against anastomotic leakage due to a collagenolytic strain of *S. marcescens* introduced via enema after a low colon anastomosis.
Aim 4: To demonstrate that other collagenolytic strains of gram-negative bacteria, that is, *P. aeruginosa*, can also cause anastomotic leak in this model and is prevented by PPi-6 when administered in the drinking water.
Aim 5: To demonstrate that the mechanism by which oral PPi-6 prevents anastomotic leak due to *S. marcescens* and *P. aeruginosa* in mice after low colon anastomotic surgery is by its ability to concentrate within anastomotic tissues and prevent bacterial collagenase production in vivo.

Bacterial Strains

An antibiotic-resistant *S. marcescens* strain was originally isolated from the stool of a critically ill patient.[14] This strain was characterized as collagenolytic and was termed S2-*S. marcescens* to denote it from other isolated strains of *S. marcescens* (i.e., S1-*S. marcescens*—vide infra) that do not produce collagenase. The S1-*S. marcescens* strain, also isolated from the stool of a second critically ill patient,[14] did not display collagenolytic activity and was used for comparison. *P. aeruginosa* strain previously isolated from leaking rat anastomoses (termed MPAO1-P2) was used, which displayed high collagenolytic activity, swarming motility, and biofilm formation.[9,15]

The Effect of PPi-6 on the In vitro Collagen/Gelatin-degrading Activity of *S. marcescens* and *P. aeruginosa*

Tryptic soy broth (TSB) was supplemented with sodium hexametaphosphate PPi-6 (Sigma) at a range of concentrations 0 to 0.2 mM to obtain suitable TSB/PPi-6 media. The TSB/PPi-6 medium was spiked with the bacterial inoculum and diluted 100-fold in freshly prepared TSB/PPi-6 with fluorescein-labeled gelatin or collagen [EnzChek Gelatinase/Collagenase assay kit (Fisher Scientific)] as previously described.[8]

The Effect of PPi-6 on Biofilm Production by *S. marcescens* and *P. aeruginosa*

Bacterial cultures were prepared in TSB/PPi-6 media as above. The PPi-6 concentration was adjusted to 8 and 16 mM. Three hundred microliter of the bacterial culture was plated on Costar 96 well plates (Sigma Aldrich) in triplicate. Plates were incubated overnight at 37°C. Wells were then washed 3 times with tap water, and 300 μL of 0.1% crystal violet was added into each well and incubated at room temperature for 30 minutes. The stained biofilm was then dissolved in 300 μL of 70% ethanol, and absorption was measured at 570 nm and normalized to bacterial cell density that was measured at OD600 nm before washing the plates for biofilm analysis.

The Effect of PPi-6 on *S. marcescens* and *P. aeruginosa* Motility

Swimming media contained 5 g peptone, 3 g yeast extract, and 3 g Bacto agar per liter with and without PPi-6 at concentrations of 8 to 48 mM. Swarming media contained 5 g tryptone, 2.5 g yeast extract, and 5 g Difco Bacto Agar per liter, with and without PPi-6 at concentrations of 8 to 48 mM. Bacteria were grown in TSB overnight, and 1.5 μL of overnight bacterial culture was inoculated at the center of a plate. The plates were incubated at 35°C for 8 hours.

Mouse Model

The C57BL/6 male mice aged 10 to 12 weeks (Charles River Laboratories) were maintained in accordance with the University of Chicago IACUC Protocol 72417. All animals were allowed to acclimate for 48 hours before experiments. Mice were allowed food and water ad libitum.

Mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). A midline abdominal incision was created and the colon transected at the peritoneal reflection. An anastomosis was performed using 7 interrupted sutures of 8-0 proline. Its integrity was tested by distending the distal colon with normal saline via enema using 22G blunt tip gavage needle. Finally, the abdomen was closed in 2 layers with 5-0 Vicryl and 5-0 Nylon. All animals were volume-resuscitated with 1 mL 0.9% normal saline administered as a subcutaneous injection (See supplemental video, http://links.lww.com/SLA/B181).

At postoperative days POD1, POD2, and POD3, 100 μL of a freshly prepared bacterial suspension (OD600 nm = 1.0 in 10% glycerol) was injected twice daily to the anastomosis site via rectal enema using a 22G blunt tip gavage needle (Fig. 1D).

Antibiotic Treatment

As it is well-known that mice are highly resistant to intestinal colonization by human pathogens due to the presence of diverse and abundant indigenous microbiota, a broad and prolonged regimen of antibiotics was needed to facilitate anastomotic tissue colonization by *S. marcescens*. To colonize the mouse gut with pathogenic bacteria, the prolonged administration of antibiotics in the drinking water is a common phenomenon.[16,17] Therefore, mice received oral clindamycin (100 mg/kg, ~50 μL oral gavage of 50 mg/mL) and a subcutaneous injection of cefoxitin (40 mg/kg, ~100 μL of 10 mg/mL) twice daily for 5 days, starting at preoperative day 1 to POD3 (Fig. 1D). Mice were randomly allocated to drink water or water supplemented with PPi-6 water beginning the day before surgery until the day of sacrifice on POD10.

Postoperatively, mice were clinically assessed for their health status based on an observational scoring system required by the Animal Care and Use Committee of the University of Chicago (Protocol 72417). Mice were scored as follows: score 0—alert, responsive, and normal activity; score 1—poor grooming or decreased activity; score 2—decreased activity but movement when stimulated, increased respiratory rate, or ruffled fur or hunched posture; score 3—fails to move when touched, difficulty breathing, or irregular respiratory pattern; score 4—moribund. Mice developed sepsis scored 3 or 4 were sacrificed earlier (POD5–POD7).

At sacrifice, an anastomosis healing score (AHS) was determined as previously described.[18] Briefly, 0 indicated normal healing, 1 - flimsy adhesions, 2 - dense adhesions without abscess or intraperitoneal contamination, 3 - dense adhesions with gross abscess at the anastomotic site, and 4 - gross leak with peritoneal contamination and a visible anastomotic dehiscence (Fig. 1A).

Anastomotic Tissue Sample Collection

At sacrifice, anastomotic tissues were inspected and their AHS was determined. Sterile instruments were used for tissue dissection to prevent contamination. Colonic contents and anastomotic tissues were collected. Each sample was homogenized in either 1 mL of sterile water (for tissue collagenase activity and PPi-6 content measurements) or 10% glycerol (for culturing bacteria). Samples were stored at −80°C.

Tissue Bacterial Culture

Given that the S2-*S. marcescens* strain is resistant to imipenem, we prepared selective plates to isolate S2-*S. marcescens* and distinguish it from indigenous strains of *S. marcescens* by supplementing MacConkey agar with imipenem, 1 mcg/mL. The selectivity of the plates was confirmed by plating homogenized anastomotic

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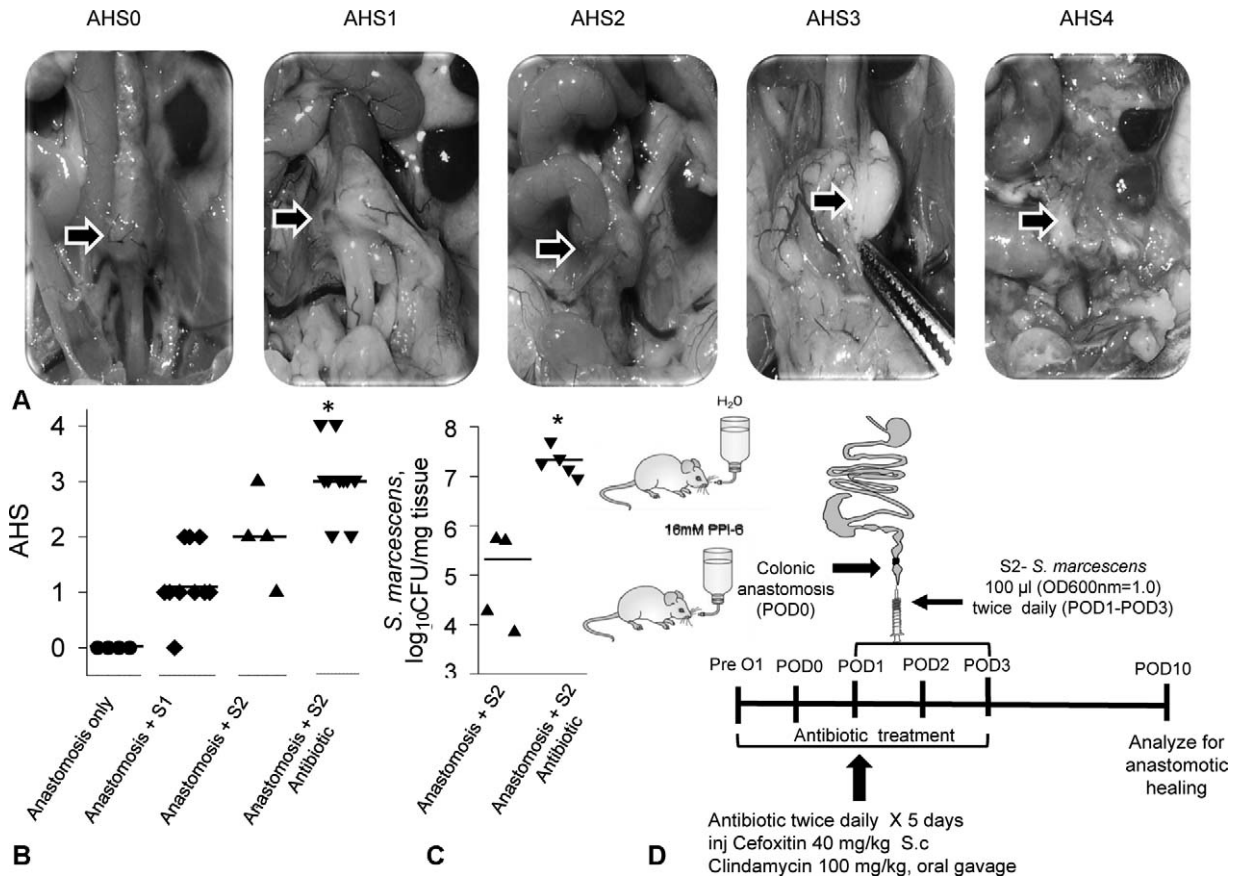

FIGURE 1. Mouse model of S2-*S. marcescens*-induced anastomotic leak. (A) Anastomosis healing score (AHS): 0 = normal healing, 1 = loose adhesions, 2 = dense adhesions and inflammation, 3 = abscess with dense adhesions/inflammation, and 4 = gross leak. Arrows indicate the line of the anastomosis. (B) Introduction of S2-*S. marcescens* to anastomotic tissues and antibiotic exposure induce anastomotic leak (n = 5 for anastomosis only, n = 10 for anastomosis + S1, n = 4 for anastomosis + S2, n = 10 for anastomosis + S2 + antibiotic; $^*P < 0.01$ for anastomosis + S2 +antibiotic (chi-square test). (C) Antibiotic treatment promotes higher colonization of anastomotic tissues by *S. marcescens* (n = 4 for anastomosis + S2, n = 5 for anastomosis + S2 + antibiotic; $^*P <$ 0.01 (Student *t* test). (D) Schematic presentation of the model and experimental protocol. On POD0, a distal colonic transection and anastomosis is performed. Antibiotics are administered twice daily, starting 1 day before surgery and continued until POD3. S2-*S. marcescens* is delivered by enema at POD1, POD2, and POD3, twice a day, 100 µL of *S. marcescens* solution in 10% glycerol, OD600 nm = 1.0.

tissues from mice not exposed to S2-*S. marcescens*. Tissues were homogenized, serially diluted in normal saline (0.9% NaCl), and 50 µL of each dilution was plated. The colony-forming unit (CFU) count was then normalized to the sample weight.

Tissue Collagenase Assay

Tissue collagenase was assayed as previously described.[8] Briefly, 10 mg of anastomosis tissue was homogenized in 650 µL assay buffer [EnzChek Gelatinase/Collagenase assay kit (Fisher Scientific)] supplemented with 1 mkM $ZnCl_2$; 30 µL of fluorescent pig gelatin was added to each sample and incubated for 24 hours in the dark at room temperature. Fluorescence was measured by M5 SpectraMax set at excitation 415 ± 15 nm and emission 550 ± 15 nm.

PPi-6 Assay

To determine whether the orally ingested PPi-6 was present at anastomotic tissues, an entire ring of anastomotic tissue was collected and centrifuged at 6000 rpm. The supernatant was then assayed for PPi-6 concentration using a Micromolar polyphosphate assay kit (Profoldin). Briefly, 30 µL of the sample and 30 µL of the kit fluorescence dye (PPD) was added to a 96-well plate and incubated for 30 minutes in the dark. PPi-6 was measured as a fluorescence at excitation 415 ± 15 nm and emission 550 ± 15 nm using M5 SpectraMax plate reader.

Statistical Analysis

Results are represented as mean ± SEM. Statistical analysis was performed by Student *t* test or chi-square test using GraphPad 4.0. $P < 0.05$ was considered statistically significant.

RESULTS

S2-*S. marcescens* Expresses a High Degree of Collagenolytic Activity

Results indicate that S2-*S. marcescens* expresses a high degree of collagenase activity after 10 hours of growth in TSB (Fig. 2A).

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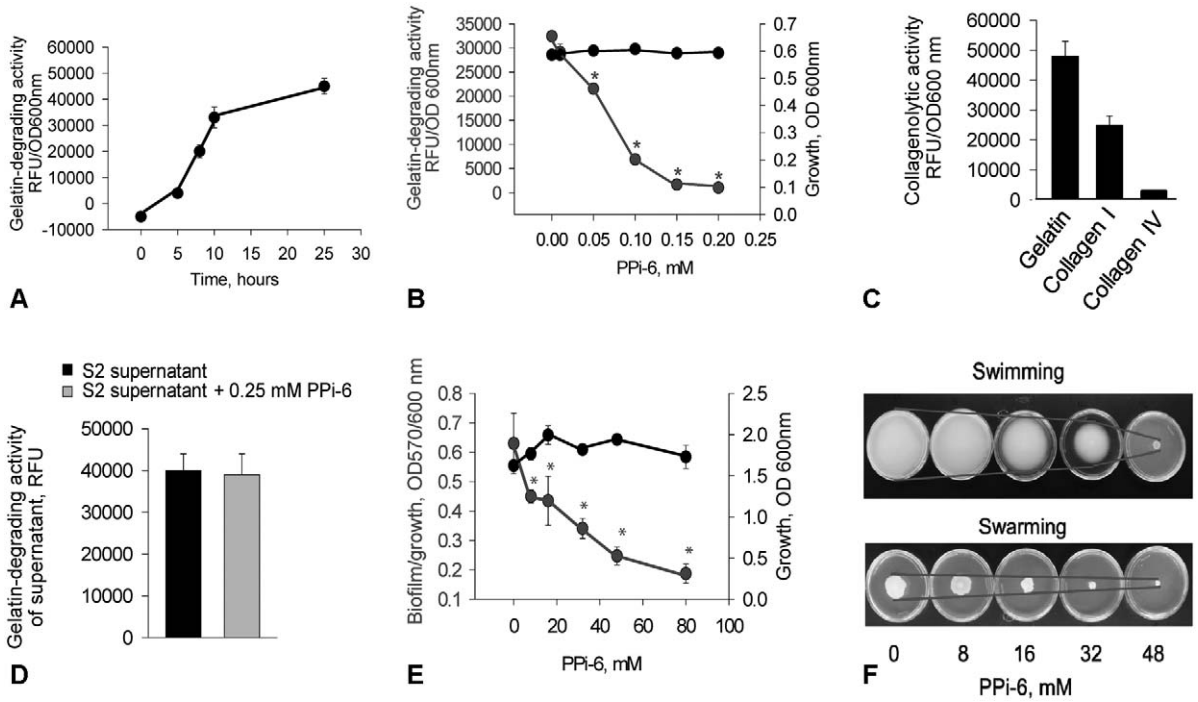

FIGURE 2. Polyphosphate suppresses the collagenolytic activity, biofilm production, and motility of S2-*S. marcescens*. (A) Dynamics of gelatin-degrading activity in *S. marcescens* grown in TSB (n = 6. (B) PPi-6 suppresses the collagenolytic activity of *S. marcescens* (blue color curve) in a concentration-dependent manner (n = 6/group; $^*P < 0.01$ between groups of PPi-6 = 0 and PPi-6 $\geq$0.05 mM) without affecting its growth (black color curve). (C) Gelatin and collagen I, but not collagen IV, are degraded by *S. marcescens* (n = 6/group). (C, D), PPi-6 does not affect the gelatin-degrading activity of the *S. marcescens* supernatant. (E) PPi-6 suppresses biofilm production by *S. marcescens* (blue color curve) in a concentration-dependent manner (n = 6/group; $^*P < 0.01$ between groups of PPi-6 = 0 and PPi-6 $\geq$10 mM) without affecting its growth (n = 4/group, $^*P < 0.01$). (F) PPi-6 suppresses swimming and swarming motilities in a concentration-dependent manner.

This isolate can degrade both gelatin and collagen I, a key protein in anastomotic healing (Fig. 2C). The S1-*S. marcescens* displayed negligible collagenase activity.

PPi-6 Suppresses the Collagenolytic Activity of S2-*S. marcescens*

Phosphate is a well-established extracellular cue that is known to suppress bacterial virulence across a broad range of pathogens via several conserved phosphoregulatory and phosphosensory systems present on bacterial membranes.[19] We tested several phosphate-containing compounds (data not shown) including potassium phosphate buffer (25 mM, pH 6.0), phosphorylated polyethylene glycol (Pi-PEG15–20, 5%–10%),[17] and the 6-mer polyphosphate herein termed as PPi-6, and identified that PPi-6 was highly effective at suppressing collagenase production in S2-*S. marcescens* (Fig. 2B). Importantly, PPi-6 suppressed collagenase production at concentrations that did not affect bacterial growth (Fig. 2B), suggesting that regulatory mechanisms rather than bacteriostatic effects are involved in this response. Given that polyphosphate may chelate Zn from the active site of collagenase,[20] we tested whether PPi-6 had a direct effect on the collagenase already secreted by bacteria. S2-*S. marcescens* was grown to stationary phase (12–14 hours) and then bacterial cells were removed by centrifugation and the supernatant filtered through a 0.22 μm filter for sterilization. When PPi-6 was added to the supernatant containing the secreted collagenase(s), it did not suppress its collagenolytic activity (Fig. 2D). These data suggest that the mechanism by which polyphosphate suppresses collagenase activity is by affecting its production rather than by chelating Zn and denaturing the collagenase.

PPi-6 Suppresses Biofilm Production and Inhibits Swarming and Swimming Motility in S2-*S. marcescens*

We next tested if PPi-6 may provide more global antivirulence effect by affecting biofilm production and motility, two phenotypic features important for tissue colonization. As shown by others, both swarming and swimming motility can be induced by phosphate limitation.[21,22] PPi-6 was added to TSB, and S2-*S. marcescens* was grown overnight, and then plates were prepared with low agar concentrations to analyze the motility of *S. marcescens* at varying concentrations of PPi-6. Results demonstrated that PPi-6 suppressed biofilm production (Fig. 2E), swimming motility (Fig. 2F, top panel), and swarming motility (Fig. 2F, bottom panel) in a concentration-dependent manner.

S2-*S. marcescens* Complicates Anastomotic Healing in Mice Treated With Antibiotics

We next developed a novel mouse model of anastomotic leak induced by *S. marcescens*. The operative procedure was carried out as indicated in the "Methods" section. The majority of mice were sacrificed on POD10. There were no systemic signs of gross infection at the time of sacrifice, and all mice appeared healthy without evidence of peritonitis from the time of anesthesia to the time of sacrifice. The

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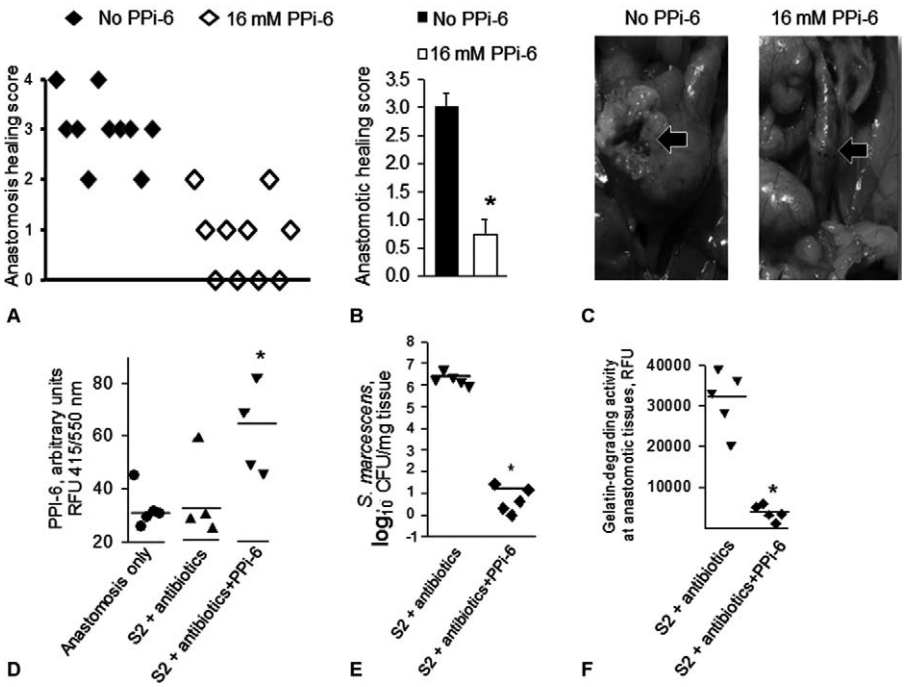

FIGURE 3. PPi-6 promotes healing in mouse model of S2-*S. marcescens*-induced anastomotic leak. (A–C) AHS is improved by oral PPi-6 as seen by the absence of gross leaks (AHS3 and AHS4) (A), significant decrease of the mean AHS ($n = 10$, $P < 0.05$) (B), no abscesses in healed tissues (C). (D) PPi-6 concentration is increased at the site of anastomosis in mice drinking PPi-6 [$n = 5$ for anastomosis only, $n = 4$ for S2 + antibiotic, $n = 4$ for S2 = antibiotic + PPi-6; $^*P < 0.05$ (Student *t* test)]. (E) colonization of anastomotic tissues by *S. marcescens* is attenuated by oral PPi-6 [$n = 5$/group; $^*P < 0.01$ (Student *t* test)]. (F) Total collagenase activity at anastomotic tissues is decreased by oral PPi-6 [$n = 5$; $^*P < 0.01$ (Student *t* test)].

procedure for anastomotic tissue inoculation was as follows: 100 μL of bacterial suspension in 10% glycerol, OD600 nm = 1.0 (2×10^9 CFUs/mL) of S1-*S. marcescens* (noncollagenolytic strain; n = 10) or S2-*S. marcescens* (collagenolytic strain; n = 4) were introduced at site of the anastomosis after its surgical completion via enema with a feeding needle and syringe. Enema inoculation of bacteria was carried out on POD1, POD2, and POD3, twice daily. Results demonstrated that S1-*S. marcescens* caused minimal healing complications (AHS = 1.2), never exceeding AHS2. Mice inoculated with S2-*S. marcescens* displayed a mean AHS of 2.0, with several mice reaching an AHS of 3 (abscess formation) (Fig. 1A, B). To create a more severe form of poor anastomotic healing due to collagenolytic *S. marcescens* that more closely mimicked the clinical condition, we treated mice with broad and prolonged antibiotics to increase *S. marcescens* tissue colonization and observed worse AHS in the 3 and 4 range (n = 10, chi-square test, $P = 0.004$; Fig. 1A, B). This antibiotic regimen and dosing schedule was required to achieve a 100-fold increase in *S. marcescens* colonization at anastomotic tissues ($P < 0.01$, n = 5) (Fig. 1C) compared with those mice not administered antibiotics. The model was then standardized to include antibiotic treatment for 5 days, starting at the preoperative day 1, and infection of anastomotic site with S2-*S. marcescens* starting on POD1 (Fig. 1D). It is important to note in this model that only 2 out of a total of 55 mice subjected to the protocol died. Both died on POD2 due to sepsis and were excluded from study. Furthermore, 2 mice from the S2 antibiotic group developed sepsis with score 4 due to gross leakage and were euthanized on POD5. Three mice developed sepsis with a score 3 due to abscess formation and were sacrificed on POD7. The remaining mice appeared healthy and were sacrificed on POD10.

PPi-6 Attenuates *S. marcescens* Colonization and Collagenase Activity at Anastomotic Tissues and Prevents Healing Complications

The PPi-6 was added to the drinking water at a final concentration of 16 mM, based on in vitro experiments indicating suppression of biofilm formation and motility of S2-*S. marcescens* (Fig. 2). Mice were randomly assigned to receive tap water supplemented with PPi-6 versus tap water only starting the day before surgery and continuing through the length of the experiment. PPi-6 dramatically improved anastomotic healing as compared with tap water-only controls (Fig. 3A–C). Luminal contents at the anastomotic sites of mice drinking PPi-6 contained higher concentration of PPi-6 compared with the water-only drinking group ($P < 0.001$, n = 4/group), suggesting that PPi-6 was delivered to the site of the anastomosis (Fig. 3D). Colonization of anastomotic tissues by *S. marcescens* (Fig. 3E) and total collagenase activity in the anastomotic site were significantly decreased in mice drinking PPi-6 (Fig. 3F).

Generalizability of PPi-6 to Other Gram-negative Pathogens Known to Cause Anastomotic Leak (*P. aeruginosa*)

Based on previous work with a P2 phenotype strain (high collagenase) of *P. aeruginosa* that were isolated from rats with anastomotic leak and fully characterized, reiterative experiments were performed with this strain, which is termed MPAO1-P2.[9,15] Results indicated that PPi-6 at 8 mM had a potent inhibitory effect on collagenase production in MPAO1-P2 (Fig. 4A). In addition, PPi-5 inhibited MPAO1-P2 biofilm formation (Fig. 4B) and swarming motility (Fig. 4C). Swimming motility was suppressed at 48 mM of PPi-6 (Fig. 4D). Similar to *S. marcescens*, PPi-6 did not affect the growth of MPAO1-P2 when exposed to collagenase inhibitory concentrations. We next introduced the *P. aeruginosa* MPAO1-P2 strain into the mouse model of low colon surgery. All parameters of the model, including antibiotic treatment, were the same as for *S. marcescens*. Results indicated that PPi-6 improved healing ($P < 0.05$, n = 5; MPAO1-P2 + antibiotic $\bar{x}$ = AHS 2.25 ± 0.25 vs MPAO1-P2 + antibiotic + PPi-6 $\bar{x}$ = AHS 1.2 ± 0.37) (Fig. 4E). The improved healing was associated with a 100-fold decrease in *P. aeruginosa* colonization at anastomotic tissues (n = 5, $P < 0.001$)

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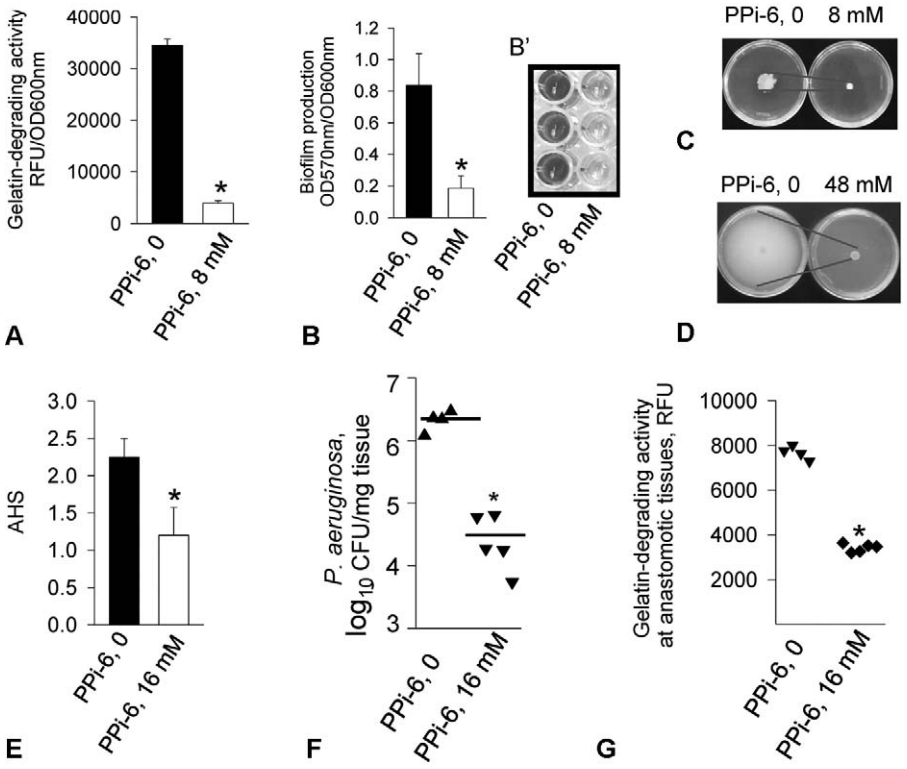

FIGURE 4. PPi-6 promotes healing in *P. aeruginosa*-induced anastomotic leak. (A) PPi-6 suppresses the gelatin-degrading activity of *P. aeruginosa* MPAO1-P2 grown in TSB (n = 6). (B) PPi-6 suppresses biofilm production by *P. aeruginosa* MPAO1-P2. (C, D) PPi-6 suppresses swarming (C) and swimming (D) motility in MPAO1-P2. (E–G) Oral PPi-6 causes a significant decrease in the mean AHS (n = 10; $P < 0.05$ (E); attenuation of *P. aeruginosa* colonization at anastomotic tissues (n = 5; $^{*}P < 0.01$) (F); and decrease of total collagenase activity at anastomotic tissues (n = 5; $^{*}P < 0.01$ (G).

(Fig. 4F) and attenuated total collagenase activity in anastomotic tissues (n = 5, $P < 0.001$) (Fig. 4G).

DISCUSSION

Animal models of anastomotic leak are often criticized for lacking clinical relevance as the leaks that these models produce are not considered similar to those observed clinically (i.e., abscess formation and gross dehiscence of the anastomosis). Furthermore, the conditions needed to produce leaks in animals (production of gross intestinal ischemia, inadequate suturing with major tissue gaps, etc) are not those that well-trained experienced surgeons would ever allow to occur. Whereas our model could also be criticized for its lack of clinical relevance, 3 features make it highly useful to the field of anastomotic leak research: anastomoses are technically well-constructed and fully intact at the time of closure; the leaks produced are clinically relevant (i.e., abscesses, gross leaks); and clinically relevant leaks in this model occur at low frequency as is typical of the clinical situation.

As in our previous studies, here we demonstrate that collagenolytic bacteria play a major role in the pathogenesis of anastomotic leak.[8] Recent studies have confirmed that there is an emerging trend of antibiotic-resistant pathogens present at sites of anastomotic leaks, the most common of which express the collagenolytic phenotype (i.e., *P. aeruginosa*, *E. faecalis*).[23] Our model involved the use of a nonroutine scheduling dose of oral and parenteral antibiotics to achieve significant colonization by collagenolytic multidrug-resistant *S. marcescens*. Although this departs from routine clinical practice, it nonetheless suggests that loss of the microbiota with subsequent colonization by healthcare-acquired pathogens can complicate anastomotic healing if they achieve a certain density at anastomotic tissues.

In defense of our use of both preoperative and postoperative antibiotics in this model is the observation that the mouse gut is known to be highly resistant to colonization by exogenous microbes, with most mouse models of intestinal infection requiring antibiotic exposure for up to 1 month[24,25] and repeated oral inoculations to achieve mucosal colonization. Humans, however, may be more vulnerable in this regard given that patients undergoing colon surgery, usually harbor cancer, receive preoperative chemotherapy and radiation, are older, eat a western high-fat diet, are obese, and are repeatedly exposed to antibiotics, factors that are known to significantly and dramatically change the microbiome.[23,26,27]

Here we isolated antibiotic-resistant *S. marcescens* strain from the stool of a critically ill patient that we had previously characterized. This strain was exposed to multiple selective pressures as a result of the patient being confined in an intensive care unit for over 1 month. The relevance of using this strain is based on recent reports demonstrating that resistant pathogens are both prevalent and growing in incidence in anastomotic tissues,[23,28] especially those that experience a leak. The relevance of this pathogen and other resistant organisms is real given that surgeons today are operating on older and sicker patients with more chronic diseases.[14,29,30]

The biological importance of inorganic polyphosphate (polyP) has been extensively studied.[31] It has been demonstrated that polyP is produced by almost all living organisms, from microbes to animals, and serves multiple important biologic functions[32] including those involved in the stress response.[33,34] In animals, polyP is found mainly in brain tissue and within heart nuclei at the μM concentration,[33,35] which is dramatically lower compared with microorganisms.[36] Microorganisms can synthesize polyP at concentrations as high as 100 to 200 mM.[31] Subsequently, numerous reports have described that multiple microorganisms can accumulate polyP in their cytosol especially during conditions unfavorable for growth.[37]

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Lactobacillus brevis can produce polyphosphate which has been shown to enhance epithelial barrier function[38] and down-regulate the expression of inflammation and fibrosis-associated genes in the intestinal epithelium.[39] Previous work from our laboratory has shown that after anastomotic surgery in rats, the relative abundance of *Lactobacillus* at anastomotic tissues is profoundly diminished.[40,41] Therefore, the provision of polyphosphate to patients in preparation for intestinal anastomotic surgery may represent a novel approach to prevent anastomotic complications.

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