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Comparative Analysis of *Salmonella enterica* Serovar Typhimurium Biofilm Formation on Gallstones and on Glass

A. M. Prouty[1] and J. S. Gunn[1,2]*

Department of Microbiology and Immunology, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78229,[1] and Department of Molecular Virology, Immunology and Medical Genetics, Division of Infectious Diseases, Department of Medicine, and Center for Microbial Interface Biology, The Ohio State University, Columbus, Ohio 43210[2]

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In this study, the roles of global regulators, motility, lipopolysaccharide, and exopolysaccharides were further characterized with respect to biofilm formation on both gallstones and glass surfaces. These studies show the complex nature of biofilms and demonstrate that characteristics observed for each biofilm are unique to the particular culture condition.

Biofilms are defined as communities of organisms attached to a surface and producing an extracellular matrix, in which the bacteria are imbedded (for a review see reference 4). The discovery of bacterial biofilms in medical and industrial ecosystems has created an urgency to identify and characterize factors that are necessary for biofilm development, which may serve as targets for biofilm prevention and treatment.

Salmonella enterica serovar Typhi is the etiologic agent of typhoid fever, and 3 to 5% of the population that are infected with *S. enterica* serovar Typhi will become chronic carriers of the organism. During the chronic-carrier state, bacteria reside in the gallbladder, the storage site for bile. Strong correlations between chronic carriers and individuals with gallbladder abnormalities, such as gallstones, have been drawn (7, 18). We previously reported that *S. enterica* serovars Typhimurium and Typhi can form mature biofilms on gallstones in vitro that require the presence of bile (23). It was also demonstrated that bacteria with an incomplete lipopolysaccharide (LPS), those that were nonmotile, and those deficient in quorum sensing were unable to form biofilms on gallstones. In addition, strains with mutations in important fimbrial genes and a strain with a mutation in ViAg, an exopolysaccharide capsule produced only by *S. enterica* serovar Typhi, exhibited biofilm formation similar to that of the wild type.

Most studies of biofilm formation are performed on glass or plastic, which provide an economical means to perform large-scale screening of mutants. Our studies suggest that salmonella biofilms formed on different surfaces have unique phenotypes. The present investigation further characterizes factors involved in salmonella gallstone biofilm formation and also provides evidence supporting the influence of the environment on biofilm development.

PhoP-PhoQ plays a significant role in biofilm formation. PhoP-PhoQ (PhoPQ) is a two-component regulatory system that is required for virulence in mice and humans and that has been implicated in bile resistance (9, 12, 19, 30). As such, the role of PhoPQ in gallstone biofilm formation was examined. A group of uniform cholesterol gallstones removed from a single patient were used for this study. Briefly, bacteria and a gallstone were incubated in Luria-Bertani broth plus 3% bile with aeration at 37°C for 4 or 14 days. Every 24 h, the medium was removed, the gallstones were washed two times in sterile phosphate-buffered saline, and fresh medium was added with antibiotics (as necessary). Gallstones were analyzed by scanning electron microscopy (SEM) as previously described (23), and bacterial strains used in this study are listed in Table 1. A strain with an insertion disrupting *phoP* was incubated with a human gallstone in 3% bile (a crude ox bile extract [Sigma Chemical Co., St. Louis, Mo.]) at 37°C. While previous data demonstrated that a wild-type strain takes approximately 14 days to form a mature biofilm (23), a *phoP*-null mutant was able to form a mature biofilm within 4 days (Table 2). These results suggest that PhoPQ plays an important role in gallstone biofilm formation. To further this finding, *prgH*, a PhoPQ-repressed gene carried on pathogenicity island I and involved in the formation of the type III secretion system (TTSS) apparatus (2), was tested for its role in the enhanced biofilm formation on gallstones observed in the *phoP*-null mutant. Analysis by SEM demonstrated that a *prgH* mutant is unable to form a mature biofilm in a wild-type or *phoP*-null background, as none of the previously described web-like strands were present, suggesting a lack of an exopolymeric substance (EPS) (Table 2).

A system was recently developed to analyze salmonella biofilm formation on glass coverslips (23). Briefly, 15-mm-diameter no. 1 microscope cover glass (Fisher Scientific, Pittsburgh, Pa.) was UV sterilized and incubated in the bottom of petri dishes with tryptic soy broth with gentle rocking at 37°C. Every 24 h, the medium was removed, the petri dish was washed two times with sterile phosphate-buffered saline, and fresh medium with antibiotics was added. The coverslips were removed at 6, 8, and 10 days, stained, and examined. Strains were incubated in broth for 10 days at 37°C and stained with ruthenium red as previously described (23); this preferentially stains acidic polysaccharides, an indicator of biofilm development. On glass, the *phoP*-null mutant exhibited enhanced biofilm formation, forming large expansive biofilms that could not be observed for a wild-type strain (Fig. 1). A PhoP constitutive (PhoP^c) strain

* Corresponding author. Mailing address: The Ohio State University, Tzagournis Medical Research Facility, 420 W. 12th Ave., Columbus, OH 43210. Phone: (614) 292-6036. Fax: (614) 292-5495. E-mail: gunn.43@osu.edu.

TABLE 1. Strains used in this study

Strain	Genotype	Source of reference
JSG210	14028s (parent for all strains)	ATCC[a]
JSG206	*phoP*::Tn*10*d-*cam*	19
JSG208	PhoP^c (*pho*24)	19
JSG648	*prgH*::Tn*phoA*	2
JSG790	*prgH*::Tn*phoA phoP*::Tn*10*d-*cam*	30
JSG1221	*zbi812*::Tn*10 galE*	Gift from Karl Klose
JSG1225	*fliA*::Tn*10*d-Tet	16
JSG1546	*wcaA*::*luc*	This study
JSG1547	*motA595*::Tn*10*	17
JSG1563	*wcaA*::*luc phoP*::Tn*10*d-*cam*	This study
JSG1564	*zbi812*::Tn*10 phoP*::Tn*10*d-*cam*	This study
JSG1577	*rpoS*::pRR10(Δ*trfA*)	8
JSG1675	*rfaD*::*luc phoP*::Tn*10*d-*cam*	This study
JSG1676	*rfaD*::*luc*	This study
JSG1746	*rpoS*::pRR10(Δ*trfA*) *phoP*::Tn*10*d-*cam*	This study
JSG1748	*bcs*::Kan	This study
JSG1758	*bcs*::Kan *phoP*::Tn*10*d-*cam*	This study
JSG1990	*fliA*::Tn*10*d-Tet *phoP*::Tn*10*d-*cam*	This study
JSG1991	*motA595*::Tn*10 phoP*::Tn*10*d-*cam*	This study

[a] ATCC, American Type Culture Collection.

(mimicking continual activation of the PhoP regulon) was unable to develop a biofilm, unlike the *phoP*-null mutant (Fig. 1). This result further demonstrated that the enhanced biofilm formation by a *phoP*-null mutant is PhoP dependent (Table 2). Furthermore, a *prgH* mutation eliminated the *phoP*-null phenotype on glass coverslips, as indicated by the absence of ruthenium red staining (Table 2). These results suggest that, similar to what is found for *S. enterica* serovar Typhimurium biofilms on gallstones, the presence of the TTSS apparatus is important for mature biofilm formation on glass.

While there is no current evidence for a TTSS apparatus being utilized for EPS delivery to the outer surface, secretion of EPS could be a novel function for the SPI1 TTSS in salmonellae. The apparatus associated with type III secretion possesses a needle-like structure that protrudes from the outer membrane and that is used to inject proteins into the cytoplasm of host cells (14, 15). Alternatively, the needle apparatus may be necessary for bacterium-surface or bacterium-bacterium interactions. It is also possible that the nature of this particular mutation in *prgH*, by disrupting one secretion system, causes other secretion systems to become congested, and therefore unable to translocate EPS to the outer surface. Our previous studies demonstrated that transcription of the *prgH* operon is repressed in the presence of bile (22). Therefore, a wild-type strain grown in the presence of bile, which is a part of the culture conditions for in vitro gallstone biofilm development, should display a phenotype similar to the that of the *prgH* mutant (immature biofilm), which is not the case. This suggests that the *prgH*::Tn*phoA* mutation has an indirect effect on mature biofilm formation. Additional studies will be needed to establish the mechanism for EPS translocation to the outer surface and how the SPI-1 TTSS needle complex could affect biofilm-dependent EPS secretion.

TABLE 2. Summary of the role of LPS, EPS, motility, and RpoS in glass versus gallstone biofilms

Mutant gene	Phenotype of gene mutant	Ability[a] to form biofilm on:	
		Gallstone	Glass
phoP	Loss of PhoPQ two-component system	++	++
prgH	Loss of type III secretion apparatus	−	−
motA	Inability to rotate flagellum	+	−
fliA	Loss of flagellum	−	−
galE	Pleiotropic; EPS synthesis defect and loss of LPS O antigen	−	−
rfaD	Inability to add O antigen to LPS	+	−
bcs	Inability to synthesize cellulose EPS	+	−
wca	Inability to synthesize colanic acid EPS	+	+
rpoS	Loss of stationary-phase sigma factor	−	−

[a] Ability of strain to form a biofilm on gallstones as observed by SEM and on glass slides as observed by ruthenium red staining. ++, enhanced biofilm formation as compared to that by the wild type; −, severe defect in or complete loss of biofilm formation capability; +, normal wild-type biofilm formation.

The flagellar filament, not motility, is necessary for biofilm formation on gallstones, but motility is required for biofilm formation on glass. Previous studies performed with a *fliA* mutant suggested that motility was necessary for gallstone biofilm development (23). To determine if the physical presence of the flagellar filament or the motility of the organism was required for biofilm formation, a mutant that maintained the flagella but was nonmotile (*motA595*::Tn*10*) was examined. *motA* encodes a motor protein required for flagellar rotation and does not interfere with flagellar biosynthesis (17). The *motA595*::Tn*10*-containing strain formed a mature biofilm on gallstones at 14 days as seen in a wild-type strain (Table 2). The *fliA* and *motA* mutants were also examined for biofilm formation on glass coverslips. On glass, the *fliA* mutant showed a deficiency in biofilm formation, which had also been observed on gallstones (Table 2). Interestingly, the *motA* mutant, which was able to form a full biofilm on gallstones, was able to form only a very weak biofilm on glass coverslips (Table 2). These results indicate that, on gallstones, the flagellum is necessary for attachment to the surface, but motility is not required. However, motility is required for biofilm development on glass.

Biofilms produced by *Escherichia coli* and *Burkholderia cepacia motA* mutants exhibit significantly reduced surface-associated biomass compared to wild-type biofilms in the first 8 h, but within 48 h the biofilms of the wild-type strain and the *motA* mutant strain are virtually indistinguishable (13). It is possible that the *S. enterica* serovar Typhimurium *motA* mutant may also show deficiencies in early biofilm formation on gallstones but is able to overcome the initial delay to form a mature biofilm.

An incomplete LPS does not affect gallstone biofilm formation but is important for interaction with glass. The role of LPS in biofilm formation has only recently begun to be characterized (10, 21, 29). Genevaux et al. hypothesized that an incomplete or rough LPS may interfere with attachment or development of external appendages, such as fimbriae or flagella (10). Previous studies demonstrated that a *galE* mutant which lacks sugars beyond the primary heptose of the core region was unable to form a biofilm on gallstones after 14 days (23). Because *galE* also interferes with synthesis of galactose, those data did not clarify whether the lack of O antigen or the defect in galactose biosynthesis was responsible for the strain's inability to form a biofilm. To answer this question, a mutation in *rfaD* that did not affect sugar synthesis but that prevented the attachment of O antigen to the core was constructed. DNA internal to *rfaD* was amplified by PCR with primers JG409 (5′

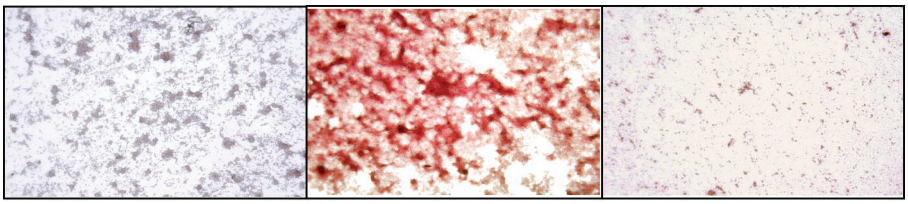

FIG. 1. The loss of PhoP (PhoP$^-$) significantly enhances biofilm formation on glass compared to biofilm formation by wild-type (WT) and PhoP^c strains. Ruthenium red staining of PhoP$^-$ strain biofilms was heavier and thicker, indicating mature biofilms. The WT biofilm possessed small microcolonies with moderate staining. PhoP^c strain bacteria were present on the glass slides, but biofilms did not develop, as indicated by the nearly complete lack of ruthenium red staining. Magnification, ×400.

CGGAATTCGGTCGCACGTCTGATTTCATCG 3′) and JG410 (5′ GGGGTACCCGCTAACTTGGAGCTGTGG 3′). The primers were designed with *Eco*RI and *Kpn*I sites at the 5′ ends. The fragments were cloned into the firefly luciferase-reporter suicide vector pGPL01 (11). Recombination on the chromosome accomplished a disruption in the operon. Biofilm development by the *rfaD* mutant was similar to that by the wild-type strain (Table 2). The *galE* and *rfaD* mutants were also examined for biofilm formation on glass coverslips, and neither mutant was able to form a biofilm on glass (Table 2). These results suggest that, on glass, a full-length LPS is necessary for biofilm formation. However, LPS O antigen does not play a role in gallstone biofilm formation, but a function of *galE* unrelated to LPS biosynthesis is necessary for gallstone biofilm development. This may suggest that synthesis of the extracellular matrix requires UDP-galactose, as has been observed for *Vibrio cholerae* biofilms (21). Other groups suggest that the hydrophobicity of the surface may be a more important factor. Mireles et al. (20) observed that, for *S. enterica* serovar Typhimurium LT2, all of the LPS mutants examined were able to form a biofilm on polyvinyl chloride but that none were able to attach to a hydrophilic surface such as glass (20). Gallstone surfaces and bile are hydrophobic, which may also partially explain differences in the binding of bacterial surface component mutants (e.g., LPS) to glass versus gallstones.

RpoS is necessary for biofilm formation on gallstones and glass. RpoS is a global regulator for stationary-phase-specific genes and has been implicated in biofilm formation in *E. coli* and *Pseudomonas aeruginosa* (1, 3, 32). Various laboratories have demonstrated that the role of *rpoS* in *E. coli* biofilms can be either inhibitory or enhancing depending on the surface and media to which the bacterium is exposed (1, 3). A *P. aeruginosa rpoS* mutant produced a thicker biofilm than its wild-type parent, while an *E. coli rpoS* mutant exhibited the opposite behavior, i.e., it was greatly inhibited in biofilm formation (32). However, other laboratories have demonstrated that an *E. coli* strain with a mutation in *rpoS* showed enhanced exponential biofilm growth on polyvinyl chloride in Luria-Bertani broth (3), while a strain with a similar mutation exhibited greatly reduced ability to form biofilms on glass in glucose-limited defined media (1). In *S. enterica* serovar Typhimurium, it has been observed that the components of the EPS of an *rpoS* mutant are different from those of a wild-type strain, suggesting that RpoS regulates a extracellular component(s) (25). Studies with the *rpoS* mutant and gallstones found that a small number of bacteria adhered to the surface, but no web-like strands (desiccated EPS) could be visualized (Table 2). Additionally, while individual bacterial cells on the glass coverslips were observed, they did not stain with ruthenium red, indicating a lack of EPS (Table 2). These results indicate that the presence of RpoS is required for proper biofilm formation to occur, perhaps by interfering with EPS production.

The components of the *S. enterica* serovar Typhimurium EPS differ depending on environmental conditions. The definition of a biofilm is a bacterial community encased in an extracellular matrix (4). An extracellular matrix is composed predominantly of polysaccharides, and these matrices have been well characterized for *E. coli*, *V. cholerae*, and *P. aeruginosa* (5, 24, 27, 31). As described previously, *Salmonella* spp. appear to produce an EPS in gallstone biofilms that can be clearly visualized by SEM upon preservation of the matrix by air drying (23). Composition of the EPS within biofilms is heterogeneous and can change depending on the environmental conditions (26). To determine the EPS involved in salmonella gallstone biofilms, three known EPS-producing operons were analyzed. Previously, we determined that the ViAg, which constitutes the *S. enterica* serovar Typhi capsule, was not necessary for biofilm formation (23). Colanic acid is the predominant EPS in *E. coli* biofilms (5, 27), and *S. enterica* serovar Typhimurium possesses the homologous gene cluster encoding colanic acid (28). A polar mutation in *wcaA*, the first gene of the colanic acid operon, was constructed, and the strain was tested in the gallstone biofilm assay. DNA internal to *wcaA* was amplified by PCR with primers JG368 (5′ GGAATTCATAC CCATAACGCCAGG 3′) and JG369 (5′ GGGGTACCTAA TCTGCATCTCGC 3′). The primers were designed with *Eco*RI and *Kpn*I sites at the 5′ ends. The fragments were cloned into the firefly luciferase-reporter suicide vector pGPL01 (11). Recombination on the chromosome accomplished a disruption in the operon. A colanic acid mutant had no effect on gallstone biofilm formation, with temporal development and appearance similar to those of a wild-type biofilm, suggesting that colanic acid is not a major EPS component in gallstone biofilms (Table 2).

Recently Solano et al. (26) implicated cellulose as an important component in *S. enterica* serovar Enteritidis biofilms. Cellulose biosynthetic enzymes are encoded by divergently transcribed operons *bcsABZC* and *bcsEFG* (26, 33). To analyze the role of cellulose in gallstone biofilms, a polar deletion that eliminated the promoters and part of the structural genes for both operons was

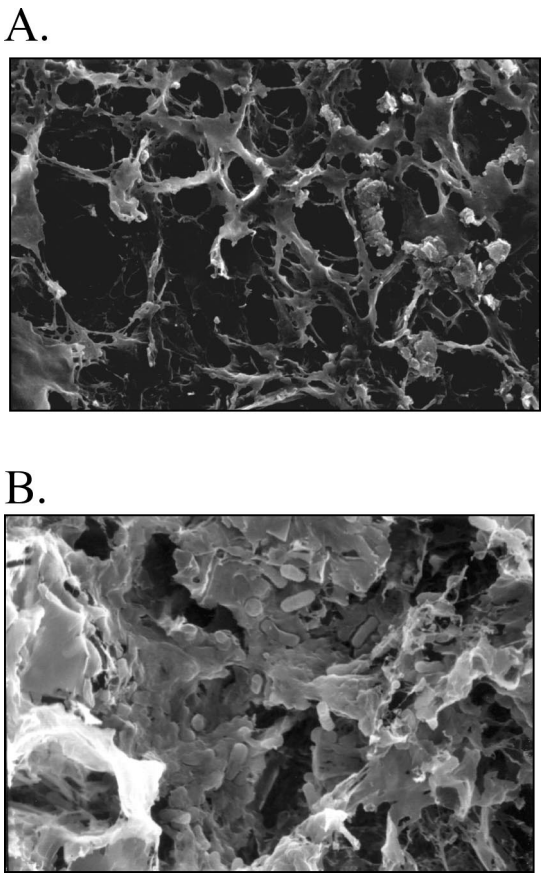

FIG. 2. The effect of cellulase on gallstone biofilms. (A) SEM micrograph of an air-dried gallstone incubated in the absence of *S. enterica* serovar Typhimurium. (B) *S. enterica* serovar Typhimurium wild-type gallstone biofilm incubated in the presence of 0.1% cellulase. The presence of bacteria embedded within a matrix indicated that cellulase did not affect biofilm formation on gallstones or EPS production. Magnification, ×5,000.

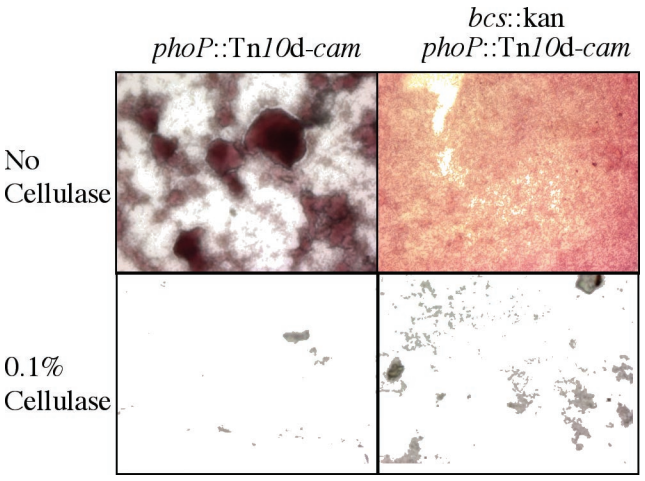

FIG. 3. Ruthenium red staining of salmonella biofilms on glass indicates that the primary component of the EPS is cellulose. A *phoP*::Tn*10*d-*cam* mutant and *bcs*::Kan *phoP*::Tn*10*d-*cam* double mutant were incubated with and without cellulase (0.1%). For the *bcs* double mutant, the presence of microcolonies and light ruthenium red staining demonstrates the presence of minor EPS components other than cellulose. The observance of limited bacteria in the presence of cellulase indicates that a primary component required for a mature biofilm structure on glass is cellulose. Magnification, ×400.

constructed (*bcs*::Kan) by means of λ red-mediated site-specific recombination, as described by Datsenko and Wanner, using primers JG417 (5′ GTTGCTAAGATGTCCCAGCTCTTTT TCCTTTTCCAGGTGCGTTGTGCGTGTAGGCTGGAGCT GCTTCG 3′) and JG418 (5′ GTTCATCCCATAATGATGAG ATGCCGAGAGAAAATACGGGGTCCACAGGTCCATTC CGGGGATCCGTCGACC 3′) (6). Mutations were confirmed by PCR. Strains were incubated with cellulase as previously described (26). Briefly, 0.1% cellulase (from *Trichoderma viride*) in 0.05 M citrate buffer solution (Sigma Chemical Co.) was added daily with the fresh medium in both the gallstone biofilm assays and the glass coverslip biofilm assay. For a control, samples were also incubated in 0.05 M citrate buffer alone.

The *bcs* mutant was incubated with gallstones and examined by SEM. This mutant displayed a phenotype similar to that of the wild type, suggesting that cellulose is not a necessary component of gallstone biofilms (Table 2). To further extend these results, wild-type *S. enterica* serovar Typhimurium, a *phoP*::Tn*10*d-*cam* mutant, and a *bcs*::Kan mutant were incubated in 0.1% cellulase for 14 days during biofilm formation. Incubation in cellulase did not have an observable affect on gallstone biofilm formation by the wild-type, *phoP*::Tn*10*d-*cam*, and *bcs*::Kan strains (Fig. 2). These results further indicate that cellulose is not a major component of gallstone biofilm EPS.

The *wcaA* and *bcs* mutants were also tested on glass coverslips in biofilm assays. The colanic acid mutation in a *phoP*::Tn*10*d-*cam* or wild-type background strain did not have an effect on biofilm formation, suggesting that, similar to the results found for gallstones, colanic acid does not play a role in biofilm formation on glass (Table 2). However, the cellulose mutant exhibited a significant decrease in biofilm formation on glass coverslips in the wild-type or *phoP*::Tn*10*d-*cam* background. This result was further confirmed by incubation of the *phoP*::Tn*10*d-*cam* mutant and a *bcs*::Kan *phoP*::Tn*10*d-*cam* double mutant in 0.1% cellulase during biofilm development. The presence of cellulase significantly reduced biofilm formation of the *phoP*::Tn*10*d-*cam* mutant (Fig. 3). These results suggest that cellulose is the primary EPS in biofilms formed on glass coverslips, but as shown above, cellulose is not important in EPS production by gallstone biofilms. Interestingly, *bcs* mutant microcolonies that stain with ruthenium red (although less intensely) could still be observed before incubation with cellulase, but not after (Fig. 3). Most likely salmonella biofilms are composed of multiple sugars, as has been demonstrated for other bacterial biofilms (5, 24, 27, 31). The observed ruthenium red staining indicates that these minor components allow for early microcolony development in the *bcs* mutant without cellulase but are lost due to lack of further biofilm development. Alternatively, the EPS associated with this early microcolony may be nonspecifically eliminated upon cellulose digestion.

Biofilms are very diverse and unique, not just to the organism, but to the particular environment in which the biofilm is being formed. This makes in vitro characterization of biofilms difficult and requires the establishment of laboratory conditions that mimic the natural setting being studied. Imitation of

the natural milieu is also very complicated because the microenvironment in which the bacteria infect or replicate may be impossible to recreate. Interestingly, the media and support matrix seem to provide many of the signals that determine the components of the biofilm. This report, while providing important information about *Salmonella* sp. biofilms on gallstones, also reiterates the complex nature of biofilms.

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