Roles of bacterial physiology in inter- and intra-species ecology of microbes

by

Julia Frances Nepper

A dissertation submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

(Biophysics)

at the

UNIVERSITY OF WISCONSIN-MADISON

2017

Date of Final Oral Examination: 12/07/2017

This dissertation is approved by the following members of the Final Oral Committee:
    Qiang Cui, Professor, Chemistry
    M. Thomas Record, Professor, Biochemistry and Chemistry
    Alessandro Senes, Associate Professor, Biochemistry
    James Weisshaar, Professor, Chemistry
    Douglas B. Weibel, Professor, Biochemistry, Chemistry and Biomedical Engineering

To the family I found.

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Acknowledgements

I would like to thank my advisor, Dr. Doug Weibel. True that my time in his lab wasn't easy—though I've heard most PhDs are actually quite difficult, so perhaps it's no surprise. While I was here I learned to be independent, confident, and enthusiastic, not just in research, but everything I do. He gave me the freedom to choose my own path in graduate school, and for that I am incredibly grateful. I am also grateful to him for always pushing me to achieve more, even when I was sure it was impossible.

This has been the most difficult journey in my short life. I struggled with anxiety and depression, with constant failure, with being only average for the first time. And without the absolutely incredible friends I have made, I would never have made it this far. Truly, I love each and every one of you for being there for me when I needed advice, or help, or simply a shoulder to cry on (profusely). Even the friendships that didn't last, I am grateful for, and will always remember.

Matthew Stilwell, Rishi Trivedi, you guys were my closest buds, and I hope our friendships last forever—you know far too many of my secrets. You always believed in me, except when I was doing something stupid, in which case you told me in no uncertain terms to stop that. You were my cheerleaders, my partners in crime, my classmates, and my brothers.

Antje Pokorny Almeida, even though I haven't worked in your lab since 2011, you still took the time to reply to my very long emails about life, the universe, and other things. You were there at the beginning, and you stayed with me until the end (in spirit).

Christopher James Michael Bell, you went above and beyond the call of duty to keep me sane during the toughest years of grad school, even while bearing the weight of a ridiculously

long name. Though our relationship didn't last, I still count you among my friends, and I owe you so much for everything you did for me.

Mom, thank you for forgiving me for moving to Wisconsin, and then not calling you often enough. Those five-hour conversations about nothing in particular really helped me keep it together towards the end.

Dad, thank you for endowing me with a portion of your superhuman intelligence, and all of your incredible stubbornness. And thank you for teaching me math. I know I hated it at the time, but it's actually been quite useful. I use it at least once a month.

Roles of bacterial physiology in inter- and intra-species ecology of microbes

Julia Frances Nepper

Under the supervision of Professor Douglas B. Weibel

Department of Biochemistry

At the University of Wisconsin-Madison, Madison, WI

Biofilms are multicellular communities that are the natural state for most if not all bacteria. The processes by which they are formed and are regulated are of broad interest to the scientific community. The roles of phospholipids and cell membranes in general in biofilm formation are largely enigmatic. In the first part of the thesis, I detail how modulation of the phospholipid cardiolipin in *Escherichia coli* affects its ability to form biofilms.

In the second part of the thesis, I describe a method for detection of *Mycoplasma capricolum* subsp. *capripneumoniae*, a major pathogen affecting small ruminants in Africa, Asia, and the Middle East. This method is highly sensitive and specific, and with future work can be adapted into an assay applicable at the point-of-care.

Chapter 4 of the thesis describes a classroom-accessible activity utilizing microfluidics to study the behavior of entomopathogenic (insect-eating) nematodes. The activity provides an inexpensive method for K-12 teachers to incorporate concepts from multiple disciplines, including ecology, engineering, and chemistry, into their curricula.

In the appendix, I report a study investigating attitudes and engagement in public science

engagement in UW-Madison graduate students. Perceptions of PSC have not been investigated

in this population; this study will be helpful in designing interventions to increase PSC

engagement in UW graduate students, and in guiding design of future studies.

CHAPTER 1

An introduction to microbial lipids, communities, and ecology

Regulation and synthesis of bacterial lipids

The first committed step in the synthesis of straight-chain lipids is the carboxylation of acetyl-coenzyme A (acetyl-CoA) by ACC, the acetyl-CoA carboxylase (made up of AccABCD) to form malonyl-CoA (1). The malonate moiety is then transferred to acyl carrier protein (ACP) by FabD in order to make it recognizable to the enzymes in the elongation module of lipid synthesis (2). In bacteria that produce branched-chain fatty acids such as *Staphylococcus aureus*, the precursors for those lipids are branched acyl-CoA molecules such as isovaleryl-CoA, which are derived from amino acids (3). In both schemes, the precursors are elongated by FabH to form various β-ketoacyl-ACPs.

FabH specificity plays an important role in the final composition of acyl chains in bacterial membranes. *Escherichia coli* produces both saturated and unsaturated straight-chain fatty acids with even-numbered chains, while many Gram-positive bacteria produce branched-chain, odd-numbered fatty acids (3, 4). These differences in acyl chain distribution are due to the substrate specificity of FabH. *E. coli* FabH preferentially uses acetyl-CoA, but FabH of *Bacillus subtilis* and *S. aureus* prefer to utilize branched-chain acyl-CoA molecules. According to structural data, this is due to the shape of the active site binding pocket in the different FabH variants; in *E. coli*, the FabH pocket is too small to fit anything larger than propionyl-CoA, while Gram-positive counterparts can easily accommodate the bulkier branched-chain molecules (5, 6).

Following elongation of malonyl-ACP by FabH, fatty acid precursors are fed into the elongation module, which adds two carbons to the fatty acid with each cycle. Either malonyl-ACP or β-ketoacyl-ACP can be utilized for elongation. FabF adds two carbons to malonyl-ACP using another molecule of malonyl-ACP as a donor, forming β-ketoacyl-ACP, which is then

reduced by FabG and NADPH to form β-hydroxy-acyl-ACP, which is dehydrogenated by FabZ (this step can also be performed by FabA in *E. coli*) to create *trans*-2-enoyl-ACP (7).

The next step in fatty acid elongation can be performed by multiple different enzymes, depending on the bacterial species. In *E. coli*, this step is performed by FabI, the target of several antibacterial compounds, including the soap additive triclosan (8). However, there are at least three other enzymes discovered to date that can carry out this reaction: FabL, found in *B. subtilis* (9); FabV, found in multiple species, including *Vibrio cholera* and *Pseudomonas aeruginosa* (10, 11); and FabK, which is found in many species of *Streptococcus* (12).

At this stage, the acyl-ACP can either get elongated further or continue to the acyltransferase module of lipid synthesis. If the fatty acid re-enters the elongation cycle, it can be elongated again by FabF or—if it is 10 carbons or more—FabB (13). If the fatty acid is destined to become an unsaturated phospholipid, after reaching 10 carbons and being dehydrogenated by FabA or FabZ, FabA will isomerize the *trans*-2-decenoyl-ACP to *cis*-2-decenoyl-ACP (14) (this step is performed by FabM in *S. pneumoniae* (15)). This can then be elongated further by FabB, but not FabF. Most bacteria use only FabF for acyl-ACP elongation (16).

After elongation, acyl-ACPs are fed into the acyltransferase system to be converted into phospholipids. The chain length of fatty acids is determined by competition between the elongation and acyltransferase enzymes. The lower limit of chain lengths, generally 12-14 carbons, is a results of the substrate specificity of the acyltransferase enzymes, while the upper chain length limit—20-22 carbons—is derived from the substrate specificity of the elongation module enzymes (16).

In *E. coli* and other γ-proteobacteria, the first step in the acyltransferase module is carried out by PlsB. PlsB attaches a fatty acid to the first position of glycerol-3-phosphate (G3P), then

PlsC attaches another fatty acid to the two position of G3P, creating phosphatidic acid, the universal bacterial phospholipid precursor (16, 17). The PlsB/C system can use acyl-ACPs from the elongation module or exogenous fatty acids which have been ligated to CoA by FadD (18). The G3P is derived from glycolysis; GpsA dehydrogenates dihydroxyacetone phosphate to produce G3P (19).

Most bacteria use the PlsX/Y/C system to create phosphatidic acid (16). PlsX exchanges ACP with phosphate, creating an acyl phosphate which is then ligated to the first position of G3P by PlsY. Ligation of a fatty acid to the two position of G3P is again accomplished by PlsC, which utilizes acyl-ACP for this step (17). *E. coli* does possess PlsX and PlsY enzymes, though their function in this organism is not currently known. While single deletions of either gene do not negatively affect growth, double deletion seems to be lethal, indicating some crucial function for these enzymes (20, 21).

Phosphatidic acid is then further modified to create phospholipids with a diversity of different headgroups. Bacteria generally have some combination of anionic and zwitterionic phospholipid in their membranes, which is important for the correct folding and function of many membrane proteins (22). This membrane composition also plays a role in host-pathogen interactions; antimicrobial peptides—which are secreted by all domains of life—are usually cationic, hence their preference for prokaryotic membranes.

Following synthesis of phosphatidic acid, the phospholipid is usually converted to CDP-diacylglycerol (CDP-DAG) by CdsA (23). From here, CDP-DAG can either follow the synthesis pathway for anionic or zwitterionic phospholipids. In most bacteria, phosphatidylglycerol (PG) and cardiolipin (CL) are the major anionic phospholipids. PgsA adds a G3P to CDP-DAG, forming phosphatidylglycerolphosphate, which is then dephosphorylated by PgpA, B or C to

achieve PG. PG can be further modified by cardiolipin synthase (CL) enzymes to form CL (24). In most bacteria, CL is formed by the conjugation of two PG molecules; this reaction liberates glycerol. In eukaryotes, CL is typically formed by combining CDP-DAG and PG, liberating CMP (25). These pathways were thought to be conserved within their respective domains, but recently there have been reports of "bacterial" type CL synthesis in *Trypanosoma brucei* (26) and "eukaryotic" CL synthesis in Actinobacteria (27). In 2012, the Raetz lab published evidence for a third type of CLS found in *E. coli*; ClsC was discovered to use PG and phosphatidylethanolamine (PE) as substrates for CL synthesis (28). Recently, another group showed that *Xanthomonas campestris* produces an enzyme that makes CL and PE; both reactions use CDP-DAG as a substrate (29).

Zwitterionic phospholipids such as phosphatidylserine (PS) and PE are created via a different pathway. The addition of serine (and liberation of CMP) to CDP-DAG by PssA results in the formation of PS, which can then be decarboxylated by Psd to create PE. Virtually all PS created in *E. coli* proceeds along the path to become PE (24).

Phospholipids can also be incorporated into other cellular structures or further modified. MprF ligates a lysyl moiety to PG in Gram-positive species, resulting in lysyl-PG (30). PG can also be incorporated into membrane-derived oligosaccharides (MDOs) by MdoB in *E. coli*, liberating diacylglycerol (DAG) which is then converted back to phosphatidic acid by DgkA (31).

In *S. aureus*, the LtaS enzyme forms lipoteichoic acid (LTA) by polymerizing G1P residues from PG onto glycolipids (32). This process releases DAG, which can then be phosphorylated back to phosphatidic acid by DgkB or used for the synthesis of glycolipids by YfpP (33, 34).

Phosphatidylcholine (PC) and phosphatidylinositol (PI) are significantly less common in bacteria, but there is precedent for their existence in certain species. *P. aeruginosa* uses Pcs to synthesize PC by condensing choline with CDP-DAG (35). There are also PC synthesis pathways in *Agrobacterium tumefaciens* and *Sinorhizobium meliloti* (16). Some Actinobacteria, including various species of Mycobacteria, synthesize PI from condensation of inositol with CDP-DAG. Phosphatidylinositol synthase (PIS, encoded by the *pgsA* locus) is essential in *Mycobacterium tuberculosis* (36).

Other bacteria are able to produce phosphate-free lipids. In addition, there have been reports of bacterial sphingolipids, namely in *Sphingomonas* and *Sphingobacterium* species. *Bacteriodes fragilis*, a notable member of the natural human intestinal microbiome, has also been shown to produce sphingolipids (37). Species such as *S. meliloti, Brucela abortus*, and *P. aeruginosa* are able to synthesize lipids using amino acids rather than G3P as a backbone when grown under low phosphorous conditions (38, 39). *S. meliloti* also produces betaine lipids and sulfolipids under these conditions (40).

Because fatty acids have no fate besides lipid synthesis in the majority of bacteria, much of the biochemical regulation of lipid synthesis occurs at the initiation step. ACC is feedback-inhibited by acyl-ACP in *E. coli* (41), and there is evidence for a similar phenomenon in *S. pneumoniae* (42). FabH is also inhibited by acyl-ACP; unlike ACC, the strength of the inhibition increases dramatically as the length of the acyl chain increases (43), ensuring that fatty acids of the correct length are synthesized.

Little is known about the mechanisms that coordinate lipid synthesis with the synthesis of other biomolecules. Inhibition of phospholipid synthesis will eventually stop RNA, DNA, and protein synthesis (44). There has been data that suggests an increase in ppGpp (which also

increases when amino acid availability is low) following lipid synthesis inhibition (45) is involved in the cellular coordination of macromolecule synthesis, but this link is not yet clearly defined.

Regulation of phospholipid headgroup composition is also important for cellular homeostasis. PgsA, which carries out the first committed step in PG synthesis, seems to be feedback regulated by PG itself. PssA is regulated by a somewhat more interesting mechanism. PssA is usually associated with ribosomes *in vivo*, but will associate with the membrane if enough anionic phospholipids are present (46). Because PssA is only active when membrane-associated (46), this is a useful detection mechanism to maintain the balance of anionic and zwitterionic phospholipids.

Headgroup modification can also be influenced by factors such as antibiotic stress. In *S. aureus*, the ApsRSX three component system senses the presence of antimicrobial peptides and turns on synthesis of lysyl-PG in response (47). Multiple organisms can produce lysinylated PG using MprF (30, 48), including *B. subtilis*.

In addition to biochemical regulation mechanisms, there are also genetic control systems to maintain appropriate phospholipid synthesis. In Gram-negative bacteria, FadR is a classical repressor of several genes involved in fatty acid degradation. FadR will dissociate from DNA in the presence of long-chain acyl-CoA molecules (49), resulting in the expression of genes in the β-oxidation pathways. These proteins will break down the acyl-CoA into acetyl-CoA molecules that can be used in the citric acid cycle. Only bacteria that have β-oxidation pathways have FadR.

P. aeruginosa has the DesT repressor that controls transcription of *fabAB* and *desBC* to maintain the appropriate ratio of saturated and unsaturated fatty acids (50). DesBC are desaturases that can insert a double bond into saturated acyl-CoAs (51). DesT will bind to DNA,

repressing these loci, when bound to an unsaturated acyl-CoA, but its affinity for DNA weakens when bounds to a saturated acyl-CoA, thus it acts as a sensor of the fraction of unsaturated fatty acids.

FabR is another repressor found in several γ-proteobacteria—including *E. coli*—that controls expression of *fabA* and *fabB* (16). Unsaturated acyl-CoAs and acyl-ACPs increases the affinity of FabR for DNA (52), ensuring that the unsaturated to saturated fatty acid ratio remains constant.

FapR is a transcriptional repressor commonly found in Gram-positive bacteria such as *B. subtilis* and *Staphylococci* (53). FapR dissociates from DNA in response to increased levels of malonyl-CoA, de-repressing a host of genes involved in phospholipid synthesis, including *fabI*, *fabD*, *fabG*, and *plsX*.

In Gram-positive bacteria that produce unsaturated phospholipids such as *S. pneumoniae*, there is another repressor, FabT, that maintains the correct level of unsaturation in membrane lipids (54). FabT is dislodged from DNA in response to signs of decreasing membrane fluidity, rather than vice versa. FabT binding to long-chain acyl-ACP increases its affinity for DNA (55). FabT represses two operons, comprised of *fabT-fabH-acpP* and *fabK-fabD-fabG-fabF-accD-fabZ-accC-accB-accA*, respectively (55).

There are also two interesting genetic regulatory systems in *B. subtilis*: the DesRK two component system and the alternative sigma factor σ^W. DesK appears to be a "thermometer" that detects a decrease in membrane fluidity—for example during cold shock—and responds by activating DesR through phosphorylation (56). The phosphorylated response regulator binds the *des* promoter region, activating its transcription. The Des protein then inserts a double bond into existing phospholipids at the $\Delta 5$ position (57). σ^W, in contrast, responds to increasing membrane

fluidity (58). σ^W downregulates *fabHa* while upregulating *fabF*, resulting in longer, straight-chain fatty acids. *B. subtilis* has two FabH variants, FabHa—which prefers branched-chain acyl-CoA substrates—and FabHb—which prefers the straight-chain substrate acetyl-CoA (3). By decreasing *fabHa* expression, σ^W tips the balance towards straight-chain fatty acids.

Phospholipid biosynthesis largely takes place on the cytosolic face of the cytoplasmic membrane, thus lipids must be flipped to the periplasmic or extracellular face of the membrane. Maintenance of lipid asymmetry is important for maintaining the appropriate membrane curvature as well as for the function of membrane and membrane-associated proteins (59) and balanced membrane growth (60). There have been reports of lipid flippase activity in *B. subtilis* membrane and *E. coli* inner membrane extracts, which seems to be at least partly protein dependent (61, 62). Recently, MurJ and FtsW were demonstrated to be flippases of lipid II in *E.coli* (63, 64), and it has been shown that LplT is capable of flipping some lysophospholipids (65, 66). In *S. aureus*, MprF has been established as a flippase of lyslated PG (67). Despite decades of investigation into this topic, no proteins have been identified in bacteria that can be definitively connected to general flipping of phospholipids such as PG and PE across the cytoplasmic membrane, although reports suggest the *E. coli* protein MsbA may be able to flip a variety of lipid molecules (68).

Lipids of *Escherichia coli*

The *E. coli* inner membrane contains three major phospholipids: phosphatidylethanolamine (PE) (~70%), phosphatidylglycerol (PG) (20-25%) and cardiolipin (CL) (5-10%) (Fig. 1) (69). PE is a zwitterionic lipid that is synthesized by the decarboxylation of phosphatidylserine (PS) by Psd (70). PS occurs at a very low concentration in the cell

membrane (<0.1%) and is synthesized by PssA from CDP-diacylglcerol (CDP-DAG) and L-serine (71). PE undergoes a transition from the lamellar liquid ordered phase (L_O) to the non-bilayer inverted hexagonal phase (H_{II}) as temperature increases (Fig. 2). The physiological relevance of this phenomenon has yet to be fully explained, however several possible functions may exist for including nonbilayer lipids into membranes—for example, nonbilayer lipids may promote membrane fusion (72). CL also has a propensity to form non-bilayer structures in the presence of divalent cations (e.g., Ca^{2+}, Sr^{2+}); interestingly, a *pssA* null mutant is only viable if grown with a divalent cation at a millimolar concentration (73). It is thought that cells maintain the membrane near the transition from the L_O to the H_{II} phase, possibly to take advantage of the elastic stress induced by the presence of PE (a "frustrated" bilayer) to modulate the activity of membrane proteins (74, 75). Many classes of proteins related to different stages in biofilm formation are integral or peripheral membrane proteins and may be affected.

PG is the major anionic phospholipid in *E. coli*. The initial step in its formation is the replacement of the cytidine monophosphate moiety of CDP-DAG with glycerol-3-phosphate—catalyzed by PgsA—to form phosphatidylglycerophosphate (16). This product is then de-phosphorylated by PgpA, PgpB, or PgpC (16, 76). PgsA is essential under most conditions, but can be deleted if it is complemented by a disruption in *lpp*, the gene encoding murein lipoprotein, and the most abundant protein in *E. coli* at ~300,000 molecules per cell (77). Lpp anchors the outer membrane to the peptidoglycan layer of the cell wall and is synthesized as a pre-lipoprotein and inserted into the inner membrane. Maturation of the protein requires transfer of diacylglycerol from PG (16). Without this modification, Lpp remains in the inner membrane and crosslinked to the peptidoglycan, causing cell lysis (16). PG is also essential for the synthesis of CL (Fig. 1), and involved in the formation of osmoregulated periplasmic glucans by OpgB (16).

It was previously shown that inhibition of PG synthesis represses the synthesis of flagellin—the major structural protein in the bacterial flagellum—by upregulating expression of the stress response sigma factor *rpoS* (78, 79). This finding implies a role for PG in biofilm formation (and suggests a possible role for CL, too), as flagella are important for initial attachment to a substrate at the start of biofilm formation (80).

CL is the third most abundant phospholipid in the membrane of *E. coli*, is anionic, and can be synthesized by three cardiolipin synthase (CLS) enzymes: ClsA, ClsB, and ClsC.(28) Most bacteria with characterized CLS proteins, including *E. coli,* synthesize CL by combining two PG molecules and liberating glycerol (24). ClsC is anomalous in that it catalyzes the conversion of a PG molecule and a PE molecule into CL (28). The concentration of CL in membranes is dependent on growth phase and approximately doubles as cells enter into a stationary phase (16, 28).

E. coli ClsA is the best characterized CLS protein, but work in recent years by Ziqiang Guan and colleagues has begun to shed light on the function and synthetic mechanisms of ClsB and ClsC (28, 81). Still, little research exists on any of these synthases, and their regulation and function is largely unknown beyond the product that they synthesize. Until recently, the only phenotype associated with *cls* deletion was an increased sensitivity to the aminocoumarin antibiotic novobiocin (82). This result is surprising because the redundancy of these genes suggests that they are involved in important cellular functions (16, 83, 84). Many other bacterial species, including *P. aeruginosa, Bacillus subtilis,* and *Staphylococcus aureus* also have multiple putative CLS's, indicating that this redundancy may be evolutionarily conserved (85, 86). This year, Rowlett and colleagues published a comprehensive study investigating a number of physiological parameters in cells lacking PE, PG, and/or CL (87). This study showed that a

number of systems are affected by depletion of CL, including LPS synthesis and activity of cellular dehydrogenase enzymes. However, the mechanisms behind many of these phenotypes have yet to be determined empirically.

Biofilm formation and development

Biofilms are highly structured, multicellular bacterial communities (88-90). Although many foundational studies of bacterial genetics and physiology observed cells in a free-living planktonic state, it has become clear that those growth conditions are rarely representative of natural environments (88, 90). Biofilms have been the subject of much study in recent years because of their relevance to human health; they are frequently found on medical devices such as catheters and synthetic implants, and can cause persistent infections and prolonged hospital stays (91). It is widely accepted that most bacteria live in biofilms and communities. For reasons that are still not well understood, bacteria in biofilms are up to 1000 times more resistant to a variety of environmental stresses, such as exposure to heavy metals, desiccation, phagocytosis, and antibiotics, and consequently they are particularly challenging to eradicate (88, 92-94). Mechanisms underlying biofilm formation and homeostasis—and how they achieve such remarkable resiliency—are of interest in both fundamental and applied science and technology.

A number of studies have investigated proteomic changes associated with biofilm development (95, 96). Others have investigated the expression and function of specific proteins involved in stress adaptation and extracellular polysaccharide synthesis (97-100). These studies have identified numerous cellular factors that are important for bacterial biofilm formation.

There are multiple broad stages of biofilm development, the first of which is attachment to a substrate (Fig. 3) (90). In *E. coli*, flagella and type I pili—also known as fimbriae—are

largely responsible for this step in development (97). Recent work suggests that after coming into contact with a hydrophobic surface, flagella will "zip" onto the substrate, progressively removing water from the surface and increasing van der Waals interactions between the substrate and the flagellum (97, 101). Fimbriae, which are much smaller than flagella, have been shown to be important for adhesion to hydrophilic abiotic surfaces and biological substrates such as epithelial cells (102).

Flagellar expression is controlled by transcription factors that are regulated by a number of different conditions, including nutrient availability. FlhDC is a heterotetrameric transcriptional regulator that is principally responsible for regulation of flagellum biosynthesis (103). The FlhDC operon relies on RNA polymerase sigma factor 70 (σ^{70}) to be transcribed (104). During exponential growth, σ^{70} is the major sigma factor used to transcribe genes. If cells are stressed—for example, upon entry into stationary phase—increased production of guanosine tetraphosphate (ppGpp) leads to upregulation of *rpoS*—the gene encoding sigma factor S (σ^{S})—causing σ^{S} to outnumber molecules of σ^{70} and a decreased expression of promoters under the control of σ^{70} (105).

Expression of fimbriae is regulated by reversible phase variation; inversion of the 314 bp *fimS* element by FimB or FimE switches cells from fully fimbriated to afimbriate, and vice versa. (106) Growth of cells under static conditions increases fimbrial production, while growth on agar results in very few fimbriated cells (107). In general, regulation of the *fim* switch is not well understood.

Biofilm formation is often induced by environmental stress, making *rpoS* a crucial player in this process (108). Many steps in biofilm development are regulated by σ^{S}, notably organelle synthesis (as noted above) and polysaccharide production. Following attachment of cells to a

surface and the formation of a monolayer, cells excrete extracellular polysaccharides in a process that is correlated with an increase in *rpoS* expression (90). Colanic acid, cellulose, and poly-β-1,6-N-acetyl-D-glucosamine (PNAG) are the three primary polysaccharides produced by *E. coli* biofilms (89, 109). Colanic acid anchors cells to surfaces, while cellulose and PNAG tether cells together within the biofilm. Each of these extracellular matrix (ECM) components acts as a protective barrier between the bacterial community and the outside, chemical and physical world (110). For example, electrostatic interactions sequester antibiotics and heavy metals away from cells growing in the outer layers of the biofilm, while the hydrophilicity of these molecules traps water and protects cells from desiccation (111). Secreted extracellular polysaccharides also protect the biofilm from predation and host immune responses (111). Additionally, cells increase expression of diguanylate cyclases to synthesize cyclic di-GMP (c-di-GMP), which repress flagella biosynthesis and activate the production of additional cell-cell adhesion proteins (89). In *E. coli*, c-di-GMP production activates transcription of CsgD, the regulator of the curli biosynthesis operon, which produces families of protein-based fibers that help maintain cell-cell interactions within the biofilm (112).

Despite its critical role in maintaining cellular integrity, the role of the cell membrane—and specifically the composition of phospholipids—in biofilm physiology has not been carefully studied. The cell membrane is a chemical insulator of the extracellular environment, and the ability to sense and respond to external conditions depends on careful regulation and fine-tuning of the phospholipid composition (113, 114). Studies in the opportunistic pathogen *Pseudomonas aeruginosa* have shown clear differences in membrane composition between planktonic and biofilm-associated cells (115, 116). These limited studies have found that a sessile lifestyle leads to a greater diversity of phospholipid species in *P. aeruginosa*, and it is also likely that the

fluidity of the membrane increases because the proportion of unsaturated and branched-chain fatty acid tails increases (116).

Lipopolysaccharide (LPS) composes the majority of lipid in the outer leaflet of the Gram-negative outer membrane and is the best-studied lipids in the context of biofilm formation. Many Gram-negative bacteria—including *E. coli, P. aeruginosa,* and *Klebsiella pneumoniae*—increase palmitoylation of the lipid A moiety of LPS in an animal model of biofilm infection (117). Further evidence for a role for lipids in biofilms can be found in genetic expression data; a transcriptomic study demonstrated that 15% of genes known or predicted to be involved in lipid transport and metabolism are differentially expressed in biofilms versus planktonic cells (118). Despite these tantalizing findings, the role of membranes in biofilm formation is largely unknown.

Envelope stress responses in *E. coli*

Five signaling pathways are known to be associated with envelope stress in *E. coli*: Psp (phage shock protein), Bae (bacterial adaptive response), σ^E, Rcs (regulation of colanic acid synthesis), and Cpx (conjugative pilus expression) (119). These systems initiate cellular responses to disruptions of the cell envelope, i.e. the inner and outer membranes, peptidoglycan, and periplasmic space. A number of stressors, including antibiotics, heat shock, oxidative damage, and altered lipid composition, can activate one or more of these pathways.

The Psp system was first discovered in *E. coli* infected with f1 (filamentous) phage (120). The *pspABCDE* operon is transcribed by σ^{54} with the help of the activator PspF (121, 122). Under normal conditions, the cytosolic negative regulator protein PspA binds to PspF and sequesters it from σ^{54}; the presence of an inducing signal causes PspA to be recruited to PspB

and PspC in the inner membrane, allowing PspF to interact with σ^{54} (123-125). One common feature among Psp-activating conditions—which include phage infection and ethanol stress—is a reduction in membrane potential, which drives the proton motive force (PMF) (126, 127). Thus, it is unsurprising that induction of the Psp response results in a switch to anaerobic respiration and downregulation of motility, reducing the cell's dependence on the PMF (128).

Induction of the Bae stress system increases the expression of the multiple drug resistance operon *mdtABCD*, as well as another gene encoding an efflux pump, *acrD* (129-131). This system is not well characterized, but some known activators are indole, copper, PapG pilus protein overexpression, and spheroplast formation (132, 133). The BaeSR pathway is a relatively simple two-component system, consisting of the sensory histidine kinase BaeS and response regulator/transcriptional activator BaeR (134). BaeSR mediates resistance to drugs such as novobiocin in *E. coli* and *Salmonella enterica* (131, 132).

The σ^E pathway is the best characterized envelope stress response (127). This subunit of RNA polymerase (RNAP) was first identified in association with the heat shock response in 1989 (135). Under normal conditions, the anti-sigma factor protein RseA inhibits the activity of σ^E (136, 137). This inhibition is enhanced by the protein RseB (138). Under conditions that cause unfolding of outer membrane proteins (OMPs)—such as heat stress, overproduction of OMPs, or metal ion exposure—RseP, DegS, and ClpXP act in concert to degrade RseAB, releasing σ^E into the cytoplasm (139-142). From there, σ^E activates transcription of genes such as *htrA* (*degP*), which encodes a periplasmic protease; *fkpA*, a periplasmic chaperone; and *rpoH*, the heat-shock sigma factor (σ^{32}) (135, 143-145).

Initiation of the Rcs signaling system causes the phosphorylation of RcsA and RcsB, enabling them to function as transcriptional regulators. RcsB (which can form a homodimer in its

phosphorylated state) and RcsAB regulate a diverse array of genes. The Rcs regulon includes a number of genes involved in acid resistance, as well as the colanic acid and curli synthesis operons, osmotically inducible peroxidase *osmC*, and the flagellar master regulator *flhDC* (146). Production of colanic acid and other secreted polysaccharides promotes later stages of biofilm maturation by providing structure and protection to the developing community. While improperly timed activation of Rcs impairs the functioning of other systems required for biofilm initiation, including expression of flagella (147-149), it is essential for normal biofilm development and is activated by growth on a solid surface (150).

The CpxAR system has been linked to pathogenesis and monitoring of surface organelle assembly. Misfolding of envelope proteins like NlpE, high osmolarity, and alkaline pH all serve as inducing signals for this pathway (151-154). More recently, it was shown that activation of Cpx response in phosphatidylethanolamine (PE)-deficient *E. coli* mutants is a direct results of altered membrane physical properties, i.e. surface charge and bilayer thickness (155). CpxP is a periplasmic protein that inhibits the autophosphorylation activity of CpxA; in the presence of membrane or extracytoplasmic stress, CpxP is degraded by DegP (156). Under these conditions, CpxA transfers its phosphoryl group to CpxR, which then modulates the transcription of a number of factors associated with protein folding and degradation, drug efflux, and biofilm formation (130, 157, 158).

Infectious disease diagnosis at the point of care

A key challenge in infectious disease control is quick and accurate identification of pathogens (159). Most diagnostic tools have significant limitations that reduce their overall usefulness. Growth-based assays are the slowest to achieve results, taking many hours to identify

the most rapid-growing pathogens, and as much as a week or more for slow growing microbes such as *Mycobacterium tuberculosis* (160, 161). Antigen-based assays, like enzyme-linked immunosorbent assays (ELISA), are much more rapid, usually taking less than an hour to complete. However, techniques of this nature are not available for all pathogens, and can suffer from a lack of specificity—especially when it is necessary to distinguish between several closely-related organisms or different strains of the same species.

Polymerase chain reaction (PCR) provides a reasonable compromise between speed and accuracy; PCR takes only a few hours to perform, and targeting highly polymorphic regions of the genome helps to ensure accurate identification even to the level of specific serovars of a single species. By targeting multiple genomic regions, it is possible to further increase the accuracy and precision of pathogen identification. PCR also overcomes problems associated with limited sample or low titers of pathogens, as amplification is possible theoretically with a single copy of the target nucleic acid molecule, and in practice can often be achieved with fewer than 10 nucleic acid molecules.

Despite these strengths, traditional PCR remains unfeasible for diagnostics in resource-limited areas. It requires trained personnel, dedicated laboratory space, and expensive equipment such as thermocyclers. The recent development of isothermal PCR methods addresses the latter issue. Recombinase polymerase amplification (RPA) and loop-mediated isothermal amplification (LAMP) can both be performed at constant temperature, eliminating the need for automated thermocyclers (162, 163).

RPA employs recombinase-driven oligonucleotide binding to a template sequence followed by DNA polymerase-facilitated strand extension (162). DNA amplification can be monitored in real time using a sequence specific fluorescent probe. This technique offers high

sensitivity comparable to that of traditional PCR with the advantage that double-stranded DNA is exponentially amplified at constant and low temperatures (optimal temperature range 37°C to 42°C). LAMP uses 4 to 6 primers, making it highly specific. This method takes advantage of the strand displacement that occurs during DNA synthesis to auto-cycle synthesis of the target DNA (163). An inner primer, which contains sequences from both the sense and antisense strands, begins the process. DNA synthesis initiates and the complementary strands are displaced, forming a stem-loop. This loop then serves as the template for other primers, initiating cycles of loop formation and DNA synthesis. LAMP can be used to amplify 130-300 bp targets at an optimum temperature of 60-65°C.

While these methods still require technicians and high-cost equipment to perform, they can be made 1) applicable for point-of-care measurements, and 2) accessible to resource-limited areas with adaptation for a microfluidic platform. Microfluidic systems offer many advantages over traditional diagnostic methods: ease of fabrication, portability, reduced use of reagents, and low cost per assay. A microfluidic diagnostic utilizing isothermal PCR for pathogen detection would thus be inexpensive, highly specific and sensitive, easy-to-use, and usable in the field.

Entomopathogenic nematodes

Nematodes have adapted to almost every ecological niche on the planet, and are so abundant that they represent four out of five animals on the planet (164). Nematodes form mutually beneficial relationships with bacteria to acquire nutrients. While numerous species are animal or plant parasites, many nematodes and their mutualistic bacterial symbionts are useful for pest control in agriculture, making them excellent model organisms for academic studies of symbiosis (165, 166). For example, the soil-dwelling, entomopathogenic (insect-eating)

nematode *Steinernema feltiae* forms a symbiotic relationship with the bacterium *Xenorhabdus bovienii*, which helps it kill and digest a wide range of insects (167, 168). Entomopathogenic nematodes carry their bacterial symbiotes in specialized pockets in their intestines (168, 169).

Nematodes sense chemical compounds (chemoeffectors) produced and released by the insect prey and use this information to track prey in a process known as *chemotaxis* (*chemo* as in chemical, *taxis* as in movement) (170). After catching its prey, the nematode burrows into natural openings in the insect such as its mouth, anus, or spiracles (breathing openings) (171). Once inside, nematodes release their symbiotic bacteria, which multiply and produce toxins and other compounds that kill and help digest the insect, which is then eaten by the nematode and the bacteria to provide energy for survival and reproduction. After the insect nutrients are depleted, the nematodes re-associate with the bacteria and enter a non-feeding, developmentally arrested state called the infective juvenile (IJ) (171). IJ nematodes move away from the insect carcass and traverse the soil in search of their next insect prey (Fig. 4).

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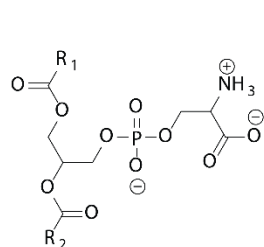
Figures

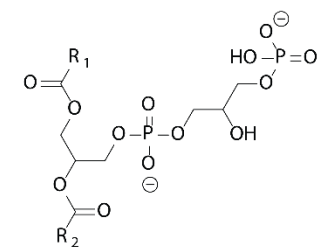**Figure 1: Biosynthetic pathways of phospholipids in *E. coli*.**
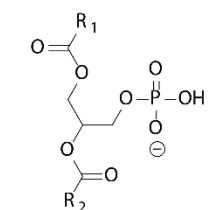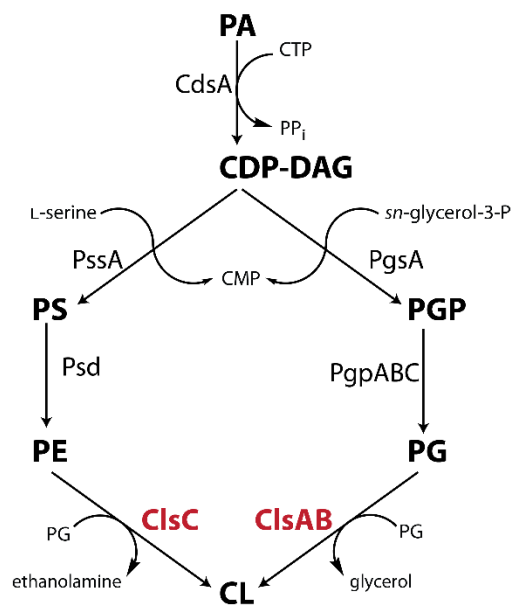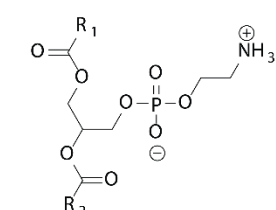
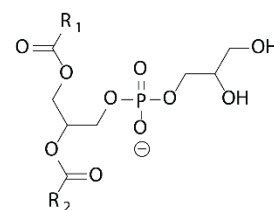Phosphatidic acid (PA)
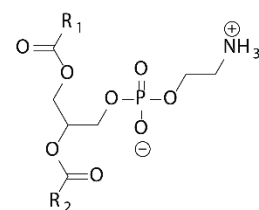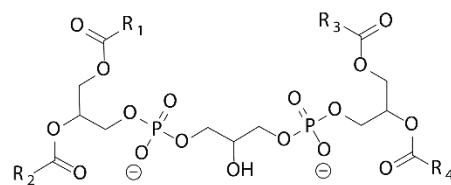
CDP-diacylcglycerol (CDP-DAG)

Phosphatidylserine (PS)

Phosphatidylglycerophosphate (PGP)

Phosphatidylethanolamine (PE)

Phosphatidylglycerol (PG)

Cardiolipin (CL)

Figure 1.

Figure 2: The relationship between lipid shape and preferred phase. Lipids with a small headgroup relative to the size of the acyl chains (e.g., PE, CL), have a tendency to adopt the inverse hexagonal phase as the lowest energy configuration. Cylindrical lipids such as PG readily form the familiar lamellar phase, while lipids with large headgroups and small acyl tails will form a tubular (micellar) structure. Taken from (74).
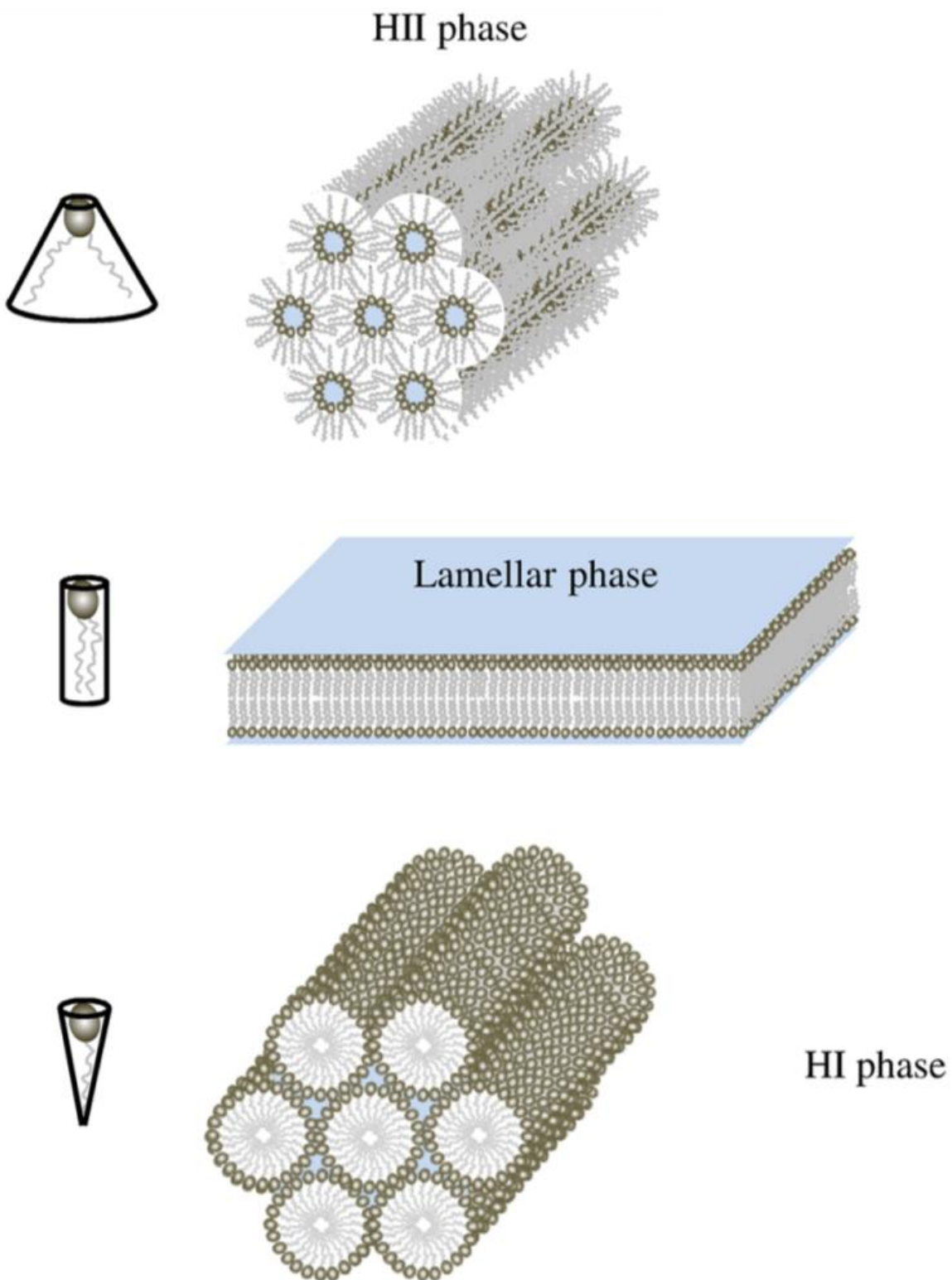
Figure 2.

Figure 3: Stages of biofilm development. (1) Initial substrate attachment. (2) Secretion of extracellular polysaccharides. (3) The biofilm is composed of multiple layers of cells. (4) Development of mature three-dimensional structures, such as mushroom clouds. (5) Biofilm dispersal. From **(172)**.
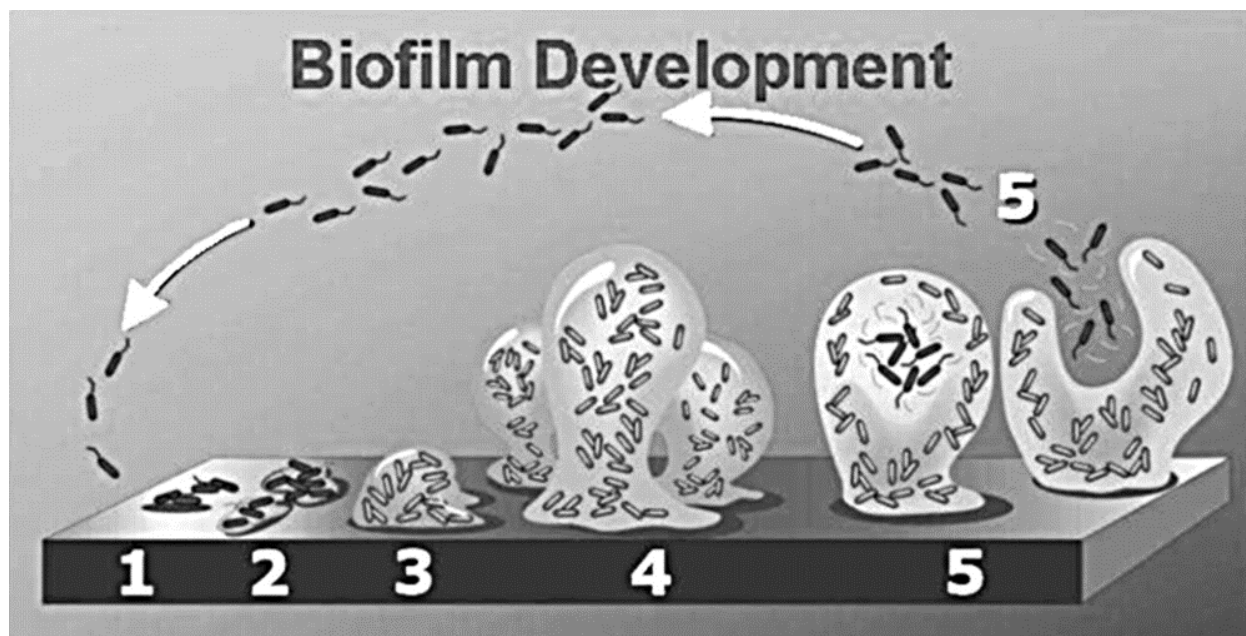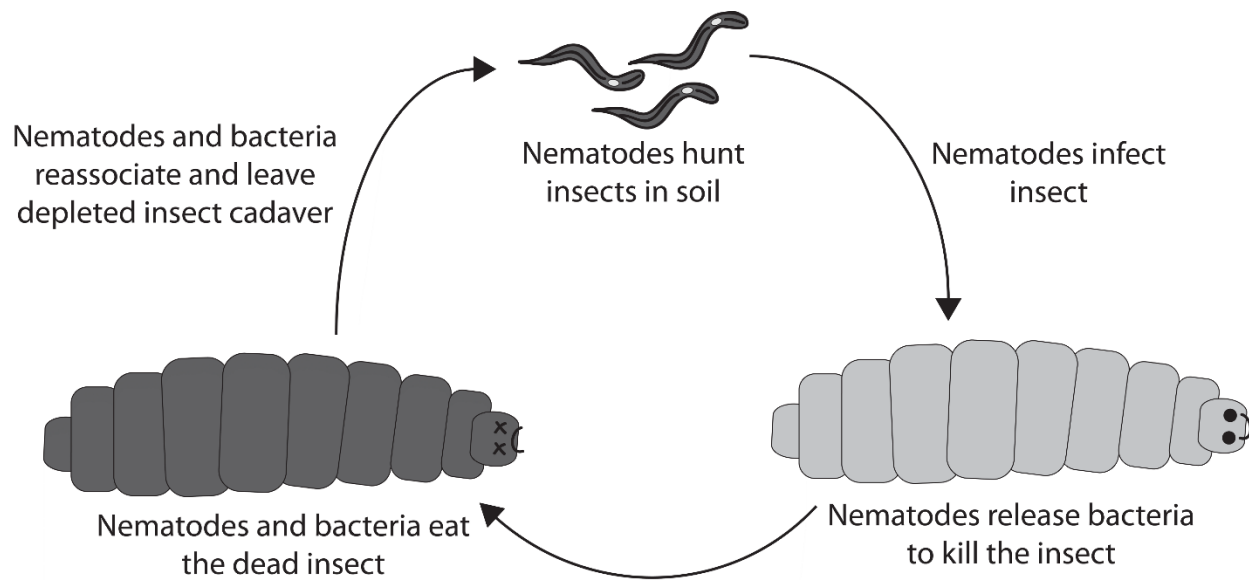
Figure 3.

Figure 4: The lifecycle of entomopathogenic nematodes. Nematodes traverse soil in search of insect prey, infect the insect, and subsequently release their symbiotic bacteria to kill the insect. The nematodes and bacteria feed on the dead insect until the nutrients are depleted, at which point they re-associate and exit the insect cadaver in search of new prey.

Figure 4.

CHAPTER 2

The role of cardiolipin in *Escherichia coli* biofilm development

Adapted from

Nepper JF, Lin YC, Weibel DB (2018). "Defective protein translocation in cardiolipin-deficient

Escherichia coli causes Rcs phosphorelay activation and aberrant biofilm formation." *J Bacteriol*

(*submitted*)

Abstract

Bacterial biofilm formation is a complex process that involves a number of transcriptional and proteomic changes. The role of the lipid membrane in biofilm development remains relatively unexplored. We describe the effect of cardiolipin depletion on biofilm formation in *Escherichia coli*. The absence of this lipid leads to the activation of the Rcs envelope stress response. The Rcs phosphorelay represses production of flagella and disrupts initial biofilm attachment. We show that in the absence of cardiolipin, translocation of proteins across the inner membrane is impaired. We hypothesize that defective protein translocation activates the Rcs pathway through RcsF. Our study provides a detailed exploration of the physiological significance of cardiolipin in *E. coli* as it relates to the crucial biological process of biofilm formation.

Introduction

Bacteria in nature are usually found in multicellular communities referred to as biofilms (1, 2). Cells within biofilms are physiologically heterogeneous and produce an extracellular polymer matrix that protects cells from hostile environments (e.g., dessication, antibiotics, oxidants, and shear forces) (3, 4). Biofilms are ubiquitous and persistent structures with a complexity and economic impact that has drawn broad attention to studying processes involved in their development.

Attachment to a substrate is the initial step in biofilm formation, and is facilitated by extracellular organelles, including pili and flagella (1, 5-7). As the biofilm grows and matures, cells within the community produce and secrete a polymer matrix (i.e., extracellular polymeric substance, EPS; or extracellular matrix, ECM) that encases, protects, and provides a three-dimensional structure to the microbial consortium. In organisms such as *Escherichia coli*, the ECM is dispensable for the first steps of biofilm formation, and required for later stages of development (8).

Many of the physiological changes that occur during biofilm formation involve processes associated with the cell membrane (e.g., an increase in secretion of polysaccharides or nucleic acids) or structures attached to it (e.g., pili and flagella). The cell membrane is a largely overlooked connection to biofilm-relevant factors and phenotypes. The membrane is a participant in the activation of stress responses, including those propagated by $\Box^S$ and Rcs, which are components of signaling pathways that play important roles at different stages of biofilm development (9, 10). Many of the signaling pathways that impact biofilm development— e.g. Rcs and Cpx—involve proteins directly associated with the membrane, are activated by disruption of the lipid membrane, or both (11, 12). In addition, the extracellular organelles that

participate in the attachment of cells to surfaces and different families of molecules that contribute to the ECM are transported across and/or inserted into the cell envelope to enable biofilm formation.

A number of observations highlight the importance of bacterial membranes and biofilm formation, and recent work has sought to deepen our understanding of the interplay of these two factors. Benamara *et al.* showed that the Gram-negative opportunistic pathogen *Pseudomonas aeruginosa* undergoes significant changes in inner and outer membrane lipid composition when adapting to a biofilm state of growth, and other groups have elucidated important roles for rhamnolipid production at every stage of biofilm development in *P. aeruginosa* and *Agrobacterium tumefaciens* (13-16). Specific lipids present in the ECM are important for late stages of biofilm formation in *Mycobacterium tuberculosis* (17). In *Listeria monocytogenes* biofilm attachment has been correlated with production of the straight-chain fatty acids hexadecanoic acid and octadecanoic acid (18). With the exception of a single recent paper (Ref 20 below), the connection between membrane composition and properties and biofilm formation is largely unstudied in the model gram-negative bacterium, *Escherichia coli* (19).

E. coli cells growing exponentially under typical laboratory conditions and at exponential phase produce membranes consisting of the following lipids: 70% phosphatidylethanolamine (PE), 15-25% phosphatidylglycerol (PG), and 5-10% cardiolipin (CL) (20) (Figure 1). CL is an unusual four-tailed anionic phospholipid synthesized by three nonessential phospholipase D type enzymes: **c**ardio**l**ipin **s**ynthase A (ClsA), ClsB, and ClsC (20). Recently, Rowlett *et al.* (2017) showed that deletion of all three cardiolipin synthases decreased biofilm formation in *E. coli*, providing the first direct link between phospholipid composition and biofilm formation in this model bacterium (19). Concomitant with this study, we discovered that depleting CL causes a

drastic decrease in both early and late stage biofilm formation. In our characterization of this phenotype, we discovered that disruption of CL biosynthesis activates the Rcs (regulation of colanic acid synthesis) envelope stress response and leads to downstream phenotypes that alter biofilm formation.

Initiation of the Rcs signaling system leads to the phosphorylation of RcsB, enabling it to dimerize and function as a transcriptional regulator (21). Phosphorlyated RcsB can also form a heterodimer with the auxiliary protein RcsA (22); the RcsB-RcsB and RcsA-RcsB complexes control the expression of a number of genes involved in acid resistance, as well as the colanic acid and curli synthesis operons, osmotically inducible peroxidase *osmC*, and the small regulatory RNA rprA (23-25). We found Rcs pathway activation has little or no effect on Δ*cls* cells growing planktonically, however disruption of the Rcs signaling pathway is sufficient to restore biofilm formation in *cls* mutants, suggesting that Rcs activation is responsible for the biofilm defects we observe. We hypothesize that CL depletion impairs the translocation of proteins across the cytoplasmic membrane, which initiates the Rcs stress response, leading to a downstream reduction in biofim formation.

Materials and Methods

Strains and growth conditions. All bacterial strains used in this study are listed in Table S1 (Supplemental Information). We created mutants using protocols for P1 phage transduction, chemical transformations, and lambda-red recombination (26-28). To construct strains of *E. coli*, we grew cells at 37°C in lysogeny broth (LB) consisting of 1% [w/v] tryptone, 0.5% [w/v] yeast extract, and 1% [w/v] NaCl, or on LB plates containing 1.5% agar and infused with LB. For all other purposes, cells grew at 30°C in M9 minimal media (3.4% [w/v] Na_2HPO_4, 1.5% [w/v]

KH_2PO_4, 0.25% [w/v] NaCl, 0.5% [w/v] NH_4Cl, 0.05% [w/v] thiamine HCl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.4% [w/v] glucose) supplemented with a defined amino acid mixture (500 mg/mL of each alanine, cysteine, glycine, histidine, aspartic acid, glutamic acid, phenylalanine, asparagine, glutamine, methionine, leucine, isoleucine, proline, serine, threonine, lysine, and valine, 50 mg/mL tryptophan, and 50 mg/mL tyrosine). Antibiotics (50 µg/mL ampicillin, 20 µg/mL tetracycline, 30 µg/mL kanamycin, and/or 25 µg/mL chloramphenicol) were added to growth media as needed. To induce expression of genes from various plasmid constructs, we added L-arabinose to a final concentration of 0.2% (w/v).

***Biofilm growth assays*.** We quantified *E. coli* biofilm formation using a crystal violet assay by diluting cells from an overnight M9 culture 1:100 into fresh M9 in wells of polystyrene 96-well microplates (100 µL/well), and incubating at 30°C for 24 h unless indicated otherwise (29). We measured optical density by quantifying absorbance of the wells at $\lambda=600$ nm (OD_{600}). Liquid growth media was removed and discarded, plates were rinsed with distilled water to remove non-adherent cells, 125 µL of an aqueous solution of 0.1% crystal violet was added to each well, and plates were incubated for 15 min at 25°C. The liquid in each well was removed and wells were rinsed with distilled water 3 times; addition of 125 µL of 30% acetic acid (in water) released crystal violet trapped in biofilms, and the dye was quantified by measuring the absorbance at $\lambda=550$ nm. We normalized the absorbance of crystal violet to absorbance of cells in the starting culture (OD_{600}) and determined the statistical significance of the data using an unpaired *t* test.

***Quantitative polymerase chain reaction*.** We used a Zymo Research Direct-zol RNA Miniprep kit (Zymo Research Corp., CA, USA) to extract total RNA from *E. coli* cells. Genomic DNA

was removed using the ArcticZymes HL-dsDNase (ArcticZymes, Norway), and RNA was reverse transcribed using an Applied Biosystems High Capacity RNA-to-cDNA kit (Life Technologies, TX, USA). We treated newly synthesized cDNA with RNase H (New England Biolabs, MA, USA) to digest RNA hybridized to cDNA. A PowerUp SYBR Green Master Mix (Life Technologies, TX, USA) enabled us to perform quantitative PCR on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, CA, USA) using the manufacturer's instructions for a standard cycling protocol. We used primers for *gapA* and/or *idnT* as endogenous controls.

Swarming assays. For *E. coli* swarming assays, we prepared swarm plates by pipetting 15 mL of hot LB containing 0.6% Eiken agar (Japan) and 0.5% [w/v] glucose into 100 x 15-mm Petri dishes (BD). We cooled plates uncovered for 30 min in a laminar flow hood, then inoculated the center of each plate with 3 μL of a saturated *E. coli* culture ($\sim 10^9$ cells/mL). We waited <5 min for the excess liquid in the droplet to absorb into the agar, covered the plates, and incubated the inverted plates at 30°C for 24 h.

Immunofluorescence microscopy of flagella. Immunostaining of flagella was performed using an anti-FliC primary antibody and an AlexaFluor 488-tagged goat anti-rabbit IgG secondary antibody using a published protocol (30). We grew *E. coli* cells to an absorbance (λ=600 nm) of 0.6-0.8, diluted cells 1:5 in 1X PBS, and imaged them in chambers consisting of a glass coverslip attached to a glass slide with double-sided tape and treated with a solution of poly-L-lysine. After adding cells to the chambers, we fixed them with 1% formaldehyde in PBS then rinsed and incubated cells in blocking buffer [3% bovine serum albumin (BSA) containing 0.2% Triton X-

100 in 1X PBS] overnight at 4°C. We incubated *E. coli* cells in a solution of primary antibody

diluted 1:1,000 in blocking buffer for 1 h at 25°C, washed with washing buffer (0.2% BSA and

0.05% Triton X-100 in 1X PBS), then incubated in a solution of secondary antibody for 1 h. We

incubated cells in secondary antibody diluted 1:1,000 in blocking buffer for 1 h, washed with

washing buffer followed by PBS, and imaged using a Nikon TI-E Eclipse inverted

epifluorescence microscope.

PhoA translocation assay. We measured PhoA translocation as described previously (31).

Overnight cultures of *E. coli* were diluted 1:50 in fresh M9 and grown to an absorbance of 0.6-

0.8 (λ=600). We induced the expression of PhoA in *E. coli* cells from pBad33 using 0.2%

arabinose, removed 1 mL of culture at the indicated timepoints for analysis, and determined the

absorbance (λ=600). We arrested protein secretion by addition of iodoacetamide to a final

concentration of 2 mM, collected cells by centrifugation for 1 min at 12,000 × g at 4°C,

discarded the supernatant, and resuspended cells in 1 mL MOPS buffer (67 mM 3-(*N*-

morpholino)propanesulfonic acid, 83 mM NaCl, 16 mM NH$_4$Cl, 10 mM MgCl$_2$, pH 7.2). We

repeated the wash step once more, then added 100 μL of the *E. coli* cell suspension to a

microcentrifuge tube containing 900 μL TZ buffer (1 M Tris-HCl, 1 mM ZnCl$_2$, pH 8.1), 25 μL

0.1% [w/v] SDS, and 25 μL of CHCl$_3$. Cells suspensions were vortexed briefly, then 100 μL of 4

mg/mL *p*-nitrophenyl phosphate (New England Biolabs, MA, USA) was added and the mixture

was vortexed again. Assay tubes were incubated at 28°C until the solution began to turn yellow,

then centrifuged for 5 min at 12,000 × g at 4°C. We removed 800 μL of liquid from the upper

portion of the tube, measured the absorbance of this aliquot (λ=420 nm), and calculated units of

active PhoA using the equation: $(1000 \times A_{420})/(t \times V \times OD_{600})$, where t is the time after induction of PhoA expression in min and V is the volume of cell suspension added in mL.

Lipid extraction and thin layer chromatography. We extracted and quantified total membrane lipids from *E. coli* cells using a modified version of the Bligh and Dyer method (32). Briefly, we pelleted *E. coli* cells at 5,000 x g for 10 min, resuspended in 100 μL of water, and lysed cells by adding 125 μL $CHCl_3$ and 250 μL methanol and inverting tubes several times. We added 100 μL of H_2O and 100 μL $CHCl_3$, vortexed samples, centrifuged for 5 min at 13,000 rpm, collected the lower organic phase, and dried it under a stream of N_2. We dissolved dried lipid extracts in 40 μL of 1:1 methanol:$CHCl_3$ and used 5-10 μL of sample for thin layer chromatography (TLC) on TLC silica gel 60 plates (Merck, Germany) with a mixture of 65:25:10 $CHCl_3$:CH_3OH:CH_3COOH for the mobile phase. After drying, we sprayed TLC plates with a cupric sulfate solution (100 mg/mL $CuSO_4$ in 8% [w/v] H_3PO_4) and incubated TLC plates on heat plates at 145°C for 5 min. We imaged plates using the Cy3 fluorescence setting on a GE ImageQuant LAS 4010 (GE Healthcare Bio-Sciences, PA, USA) and quantified the intensity of bands using Image J version 1.51h.

Quantification of biofilm polysaccharides. To extract polysaccharides from *E. coli* biofilms, we grew biofilms in 96-well microplates as described. After discarding the waste media and rinsing the wells of the plates, we added 50 μL of 1.5 M NaCl to each well and detached *E. coli* cells from the microplate surface by sonicating for 10 min in a Branson 2510 bath sonicator (Branson, CT, USA). We collected resuspended *E. coli* cells, centrifuged them for 10 min at 5,000 × g, removed the supernatant (SN), and quantified polysaccharides. Colanic acid was quantified by

assaying the concentration of L-fucose present using a published procedure with several

modification (33). Briefly, 450 µL of 6:1 H_2SO_4:H_2O was added to 10 µL of SN diluted in 90 µL

of H_2O. The mixture was incubated at 100°C for 20 min. After cooling to 25°C, we measured the

absorbance (A-co) at λ=396 and 427 nm, added 10 µL of 1 M solution of cysteine hydrochloride

to each sample, and measured the absorbance at λ=396 and 427 nm (A-cy). The concentration of

L-fucose was calculated using the formula, $(A_{396}\text{-cy} - A_{396}\text{-co}) - (A_{427}\text{-cy} - A_{427}\text{-co})$. We created

a standard curve of L-fucose (in the concentration range of 0 to 25 mg/mL) in a similar manner.

Extracellular polysaccharides (EPS) quantified were using the phenol-sulfuric acid method by

following a published protocol (34).

Quantitative Western blotting. We grew *E. coli* cells to an absorbance (λ=600 nm) of 1.0-1.3,

then pelleted and lysed the cells by incubating in resuspension buffer (50 mM glucose, 25 mM

Tris pH 8.0, 10 mM EDTA pH 8.0, 100 µg/mL RNase A, 1% SDS) for 30 min at 37°C followed

by 10 min at 95°C. We determined the total protein concentration of cell lysates using a Pierce

Coomassie Plus Bradford Assay kit (Thermo Fisher Scientific). We mixed cell lysates with

Laemmli sample buffer (Bio-Rad, Hercules, CA), and performed electrophoresis on a 12%

polyacrylamide gel. We transferred proteins to a nitrocellulose membrane at 4°C for 75 min at

100V, rinsed the membrane with distilled water, and blocked the membrane in SuperBlock T20

(PBS) Blocking Buffer (ThermoFisher, USA) for 1 h at 25°C. We washed the membrane with

PBST for 5 min (3 times). Primary HRP-conjugated antibody against 6X-His was diluted 1:2,000

in a solution of 5% bovine serum albumin (BSA) in PBST. We incubated the membrane in the

solution of primary antibody for 1 h, and washed the membrane 3 times in PBST. We treated the

membrane with ECL Plus reagent (GE Healthcare) for 2 min, imaged the membrane using the

chemiluminescence setting on a GE ImageQuant LAS 4010, and quantified band intensity using ImageJ version 1.51h.

Determination of* fim *invertible element orientation. We grew *E. coli* cells in 96 well microplates and used sonication to harvest biofilm cells as described above. We used an Epicentre Master Pure Complete DNA and RNA Purification kit (Madison, WI, USA) to extract genomic DNA from cells. Multiplex PCR was performed using New England Biolabs Q5 High-Fidelity DNA Polymerase (Ipswich, MA, USA), following the manufacturer's instructions. Primers were included to amplify off-oriented *fimAp* (INV and FIME), on-oriented *fimAp* (INV and FIMA), and *ftsZ* (EcFtsZ 1 and EcFtsZ 2; loading control) (35, 36).

Statistical analysis. We used Student's *t* test for pair-wise comparisons and a Fisher's exact test for comparisons of categorical variables. We performed experiments with at least 3 independent biological replicates to ensure reproducibility. We considered *p* values less than 0.05 statistically significant. For qPCR experiments, we considered changes of at least twofold to be significant.

Results

Cardiolipin affects biofilm formation

Previous studies have established that in the exponential phase of growth, CL represents a small percentage (~5%) of total lipid composition in *E. coli* cells. Under certain environmental conditions, such as high osmolarity, low pH, or upon entry into stationary phase, CL content increases by as much as twofold (37). To determine how lipid composition changes in biofilm-associated cells, we extracted total lipids from cells grown statically in 96-well microplates. As

expected, TLC analysis of cells grown in minimal medium revealed a significant increase in CL

in stationary phase cells (17.6% of total phospholipids) compared to exponentially growing cells

(6.6% of total phospholipids; Fig. 2). Similar to stationary phase cells, CL was enriched in

biofilm-associated cells (19.2% of total phospholipids). Interestingly, the proportion of PE

decreased to approximately half of the total phospholipid in both stationary phase and biofilm-

associated cells, and was accompanied by a concomitant rise in PG levels.

To further investigate the effect of CL on biofilm formation, we used crystal violet to

quantify adherence of cells lacking one or more *cls* genes to the surface of polystyrene

microplates. In minimal medium—but not in LB—disrupting any combination of *cls* genes

resulted in a significant ($p < 0.05$, unpaired *t* test) decrease in biofilm formation relative to wild-

type (WT) cells as soon as 1 h after inoculation (Fig. 3*A*, Fig. 4, Fig. 12). Even after incubation

for 5 days, Δ*cls* cells produced less biofilm than WT cells (Fig 5). All three Cls enzymes (ClsA,

B, and C) contain 2 phospholipase D-type HKD motifs, and mutation of one or both of these

motifs renders Cls catalytically inactive. Using an arabinose-inducible expression vector to

complement single *cls* mutant strains, we were able to partially restore biofilm formation by

ectopic expression of WT, but not mutated *cls* (Fig. 3*B*).

While ClsA is active at all stages of growth, ClsB and ClsC do not contribute

significantly to CL production until stationary phase or under certain conditions of stress (20,

38). We noted that Δ*clsA* mutants exhibited a more drastic reduction in biofilm formation than

other *cls* deletion strains. To investigate the growth phase dependence of biofilm development in

cls mutants, we assayed the ability of stationary phase cells to adhere to surfaces. We used

saturated overnight cultures to inoculate 96-well microplates and measured crystal violet staining

after 24 h of incubation (Fig. 3*C*). Under these conditions, strains lacking a single *cls* gene had a higher number of cells adhered to microplate well surfaces than a mutant completely lacking CL.

Deletion of *clsA* has been associated with increased sensitivity to novobiocin (39). We determined the minimum inhibitory concentrations (MIC) of multiple classes of antimicrobial compounds, including two targeting the cell membrane: cecropin A, an antimicrobial peptide; and polymyxin B, a mixture of lipopeptides derived from the bacterium *Bacillus polymyxa* (Table 1). In general, Δ*clsABC* cells were slightly more susceptible to these compounds as planktonic cultures than in biofilms. Treatment of CL-deficient cells in a biofilm state with polymyxin B or cecropin A remarkably increased biofilm formation relative to a non-treated control (Fig. 6). Most other antimicrobials we tested had similar effects on both WT and *cls* mutant strains.

Cardiolipin impacts activation of the Rcs envelope stress response

We reasoned that alteration of membrane lipids may likely activate a membrane stress response. Depletion of PE or PG causes severe physiological defects, decreased cell viability, and activation of multiple stress responses (9). *E. coli* possesses five signaling pathways known to be associated with envelope stress: Psp (phage shock protein), Bae (bacterial adaptive response), σ^E, Rcs (regulation of colanic acid synthesis), and Cpx (conjugative pilus expression) (40). We measured the activation of these pathways using quantitative PCR (qPCR) of genes transcriptionally regulated by each specific signal transduction pathway (Fig. 7*A*). Rcs-activated transcripts were on average 20-fold more abundant in Δ*clsABC* cells than in WT cells. Further, disruption of the Rcs pathway was sufficient to restore surface attachment of cells to WT levels (Fig. 7*B*).

Initiation of the Rcs signaling system causes the phosphorylation of RcsA and RcsB, enabling them to function as transcriptional regulators (Fig. 8). RcsB (which can form a homodimer in its phosphorylated state) and RcsAB regulate a diverse array of genes. The Rcs regulon includes a number of genes involved in acid resistance, as well as the colanic acid and curli synthesis operons, osmotically inducible peroxidase *osmC*, and the flagellar master regulator *flhDC* (21). Production of colanic acid and other secreted polysaccharides promotes later stages of biofilm maturation by providing structure and protection to the developing community. Improperly timed activation of Rcs can impair the functioning of other systems required for biofilm formation, including expression of flagella. Further, PG deficient *E. coli* cells exhibit defects in flagella synthesis (9, 10, 41). By immunostaining with an antibody raised against FliC, we observed that cells lacking CL had significantly fewer flagella than WT cells (Fig. 9*A* and *B*) and displayed a decrease in both swimming and swarming motility (Fig. 9*C* and *D*). Deleting the RcsA transcriptional regulator, which binds phosphorylated RcsB to form a complex and represses *flhDC* increased production of flagella (by the *cls* mutants) and rescued the motility defects.

Cardiolipin enhances protein translocation in vivo

RcsF is an outer membrane lipoprotein that relays stress signals to the sensor kinase RcsC (Fig. 8). Newly synthesized RcsF is transported to the outer membrane (OM) β-barrel assembly complex by the periplasmic chaperone LolA, where it is assembled into a complex with OmpA (42, 43). This step sequesters RcsF and prevents it from binding IgaA, an inner membrane (IM) protein that downregulates the Rcs pathway (44). Defects in the maturation of OM proteins

increases the size of the pool of unbound RcsF that is able to bind IgaA, thus relieving IgaA's inhibition of Rcs activation.

The majority of periplasmic and membrane-associated proteins are translocated across the IM by the Sec translocon—a large, multisubunit complex that is stimulated by CL when reconstituted in proteoliposomes (45, 46). We hypothesized that depleting CL would have a negative effect on Sec-mediated protein translocation *in vivo*. As a reporter system to assay protein translocation activity in *clsABC* mutants, we chose alkaline phosphatase, PhoA, which is inactive until it is exported to the periplasm (Fig. 10). WT cells exported on average 20.3 U of active PhoA per hour. We found that cells lacking CL had significantly decreased levels of protein translocation activity (2.9 U/hr), producing active PhoA at approximately the same rate as a Δ*secG* strain (3.1 U/hr) (Fig. 10). Further, Western blot analysis of His-tagged OmpA showed that WT cells produced mature OmpA at a higher rate than Δ*clsABC* cells.

Discussion

Despite the redundancy of CL synthases, relatively little is known about the physiological roles of CL in *E. coli*. Various CL-protein interactions have been identified, including interactions with respiratory complexes, aquaporins, and the replication initiator protein DnaA (47-51). CL content has also been correlated with entry into stationary phase and osmotic stress (20, 52, 53). Despite these observations, studies have shown that the synthases are non-essential.

Many previous studies of CL centered on *clsA* mutants and almost all studies of CL and CL synthases have been performed using cells grown in optimized laboratory conditions (i.e., rich media, agitation, 37°C). We studied the effects of CL depletion on processes involved in biofilm development, a lifestyle commonly adopted by cells under stress, including starvation

and deprivation of specific growth factors. Many studies of CL synthases in *E. coli* have been conducted using the rich medium, lysogeny broth (LB). Enteric bacteria are likely to encounter fluctuating environments that are not well mimicked by persistently high concentrations of nutrients; instead these cells experience short bursts of high concentrations of nutrients interspersed with long periods of low nutrient concentrations (54). In minimal nutrient medium, we identified a biofilm phenotype and studied the impact of a CL deficiency in a more physiologically relevant context. Cells grown in LB have no noticeable impairment in biofilm formation in the absence of *cls* (Fig. 5).

We demonstrate that biofilm formation is impaired in CL-deficient mutants under nutrient-limiting conditions, and demonstrate that this impairment arises due to activation of the Rcs phosphorelay system. Rcs activity is an important step in later stages of biofilm formation, but alterations in the timing of Rcs pathway activation can inhibit organelle production and reduce the ability of cells to attach to surfaces. Accordingly, we find that CL-deficient mutants have fewer flagella (compared to WT cells) in addition to reduced swimming and swarming motility. Disrupting the transcription of components of the Rcs signal transduction pathway in *E. coli* restored biofilm formation to wild-type levels and improved production of flagella. Deletion of *rcsF* resulted in the greatest improvement in flagella production and swimming migration, however Δ*rcsA* was the only mutant to display significant improvement in swarming, suggesting that Rcs activity affects relevant factors other than the number of flagella in *cls* mutants. *E. coli* mutants lacking *rcsC* performed better than Δ*clsABC* only in biofilm assays.

Rcs activity also influences the formation of fimbriae in *E. coli* by affecting the transcription of *fimB* and *fimE* (36). The relative ratio of the FimB and FimE recombinases dictates the orientation of the *fimA* promoter (*fimAp*), and thus controls transcription of the entire

type I fimbriae operon (55). We did not observe any change in the ratio of phase-on (*fimAp* facing the *fim* operon) vs. phase-off (*fimAp* facing opposite to *fim* operon, preventing transcription) oriented cells in the absence of *clsABC* (Fig. 11); however, it is possible that an investigation of fimbriation at level of the proteins would tell a different story.

Rowlett *et al.* (2017) described the activation of multiple envelope stress pathways in the absence of CL, however we saw no significant activation of pathways other than Rcs (19). We used qPCR to directly quantify transcript levels of target genes while Rowlett and coauthors used fluorescent protein promoter fusions as a reporter of promoter activity and immunoblotting to measure protein levels. As each technique assays different steps in protein expression, our results do not contradict those previously published. A detailed investigation of pre- and post-transcriptional changes in CL-deficient cells would help further illuminate potential factors affecting biofilm development in these mutants.

The activity of stress pathways such as Rcs has widespread effects on cell physiology: e.g., *rcsB* overexpression affected transcript levels of multiple genes involved in drug efflux and resistance (56). We observed that planktonic Δ*clsABC* cells had increased susceptibility to a number of different antimicrobial agents (Table 4), however MIC measurements of these mutants in biofilms were variable and inconclusive.

Anionic phospholipids and Rcs activation have been discussed together in multiple other studies, however the connections between the two components have been largely speculative. Previous studies of CL and the Sec translocon suggested a physical link between these two cellular components that may lead to Rcs activation. CL is tightly bound to purified, recombinant SecYEG and has been reported to enhance the stability of the SecYEG complex and stimulate the ATPase activity of SecA *in vitro* (45).

The outer membrane lipoprotein RcsF monitors lipoprotein translocation and assembly, and thus is sensitive to SecYEG activity (42). Our results demonstrate that mutants deficient in CL display a dramatic reduction in protein translocation activity, suggesting a possible molecular mechanism leading from CL depletion to Rcs activation.

The Rcs phosphorelay system consists of integral membrane proteins that may have activities that respond to changes in membrane fluidity, as occurs with other classes of membrane proteins (57-59). Increasing the concentration of CL increases membrane fluidity *in vitro*, however the impact of depleting CL on the fluidity of membranes in living cells has not yet been demonstrated conclusively (60). A recent study found no significant reduction in membrane fluidity in Δ*clsA E. coli* cells using fluorescence recovery after photobleaching (FRAP) experiments of membranes doped with BODIPY-labeled lipids (61). This work did not examine fluidity in a strain that was entirely CL-null, nor did it investigate the effect of multiple growth conditions. FRAP is generally unable to detect small changes in cell membrane fluidity, and the structure of the bacterial membrane in live cells—containing large amounts of lipopolysaccharide—may confound the interpretation of data.

Several studies have demonstrated that saturated fatty acids (SFAs) inhibit swarming in multiple organisms, such as *Salmonella enterica* serovar *Typhimurium*, a pathogen closely related to *E. coli*. As swarming is reported to be an early stage in biofilm formation, the presence of SFAs—and thus membrane fluidity—may also play a role in biofilm formation (16, 30). A recent study demonstrated that cells of *Staphylococcus aureus*, *L. monocytogenes*, *P. aeruginosa*, and *S. Typhimurium* in biofilms are enriched in SFAs compared to planktonic cells (62). Our studies focus on the effects of phospholipid composition of cells in relatively early stages of biofilm development. A more detailed investigation of biofilm membrane composition that

includes an examination of various types of biofilms (e.g. flow cell and pellicle) at different stages of development, and analyzes changes in lipids and the composition of their acyl tail groups may reveal details on the interplay of lipids and biofilm formation in *E. coli*.

Several outstanding questions concerning the role of CL in cellular processes remain unanswered. First, disruption of CL synthesis has broad effects on transcriptional regulation of a variety of genes, however the mechanisms that control production of CL are unknown. Second, a better understanding of CL regulation may aid in discovering the specific function(s) of each individual CL synthase and understanding the purpose of their redundancy in *E. coli*. The presence of multiple CLS genes is widely conserved among bacteria; most species encode at least 2 synthases. The closely related bacterium *S. enterica* encodes 3 genes with high sequence homology to *E. coli clsABC*. It is clear that CL serves a variety of physiological functions; by expanding the scope of our exploration into cell biology, we will gain insight into the roles of CL and other membrane lipids in bacteria in their natural environments.

Acknowledgements

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Tables

Table 1. Strains of *E. coli* used in this study

Strain name	Relevant genotype	Reference
MG1655	Wild-type *E. coli*	Laboratory strain
PO10	MG1655 Δ*clsABC::FRT*	(63)
Δ*clsA*	MG1655 Δ*clsA::FRT*	This work
Δ*clsB*	MG1655 Δ*clsB::FRT*	This work
Δ*clsC*	MG1655 Δ*clsC::FRT*	This work
Δ*clsBC*	MG1655 Δ*clsBC::FRT-kan-FRT*	This work
Δ*clsAC*	MG1655 Δ*clsAC::FRT-kan-FRT*	This work
Δ*clsAB*	MG1655 Δ*clsAB::FRT-kan-FRT*	This work
Δ*flhD*	BW25113 Δ*flhD::FRT-kan-FRT*	(64)
Δ*cls* Δ*rcsA*	MG1655 Δ*clsABC::FRT* Δ*rcsA::FRT-cam-FRT*	This work
Δ*cls* Δ*rcsC*	MG1655 Δ*clsABC::FRT* Δ*rcsC::FRT-cam-FRT*	This work
Δ*cls* Δ*rcsF*	MG1655 Δ*clsABC::FRT* Δ*rcsF::FRT-cam-FRT*	This work
DH5α	F⁻ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, *hsdR17*(rₖ⁻ mₖ⁺), λ⁻	Laboratory strain
5-alpha F' *Iq*	F' *proA⁺B⁺ lacIq* Δ(*lacZ*)M15 *zzf::Tn10* (Tet^R) / *fhuA2*Δ(*argF-lacZ*)U169 *phoA glnV44* Φ80Δ(*lacZ*)M15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs

Table 2. Plasmids used in this study

Plasmid	Relevant genotype	Reference
pMP4641	TetR	(65)
pMP4658	TetR	(65)
pBad33	Low copy arabinose inducible expression plasmid	(66)
pBad33-*clsA*	clsA inserted between XbaI and SphI sites; CamR	This work
pBad33-*clsB*	clsB inserted between XbaI and HindIII sites; CamR	This work
pBad33-*ymdBC*	ymdB-clsC inserted between XbaI and HindIII sites; CamR	This work
pBad33-*clsA*(H224F H404F)	clsA with H224F and H404F substitutions	This work
pBad33-*clsB*(H113A)	clsB with H113A substitution	This work
pBad33-*clsB*(H290A)	clsB with H290A substitution	This work
pBad33-*ymdB-ymdC*(H130A)	clsC with H130A substitution, ymdB wild-type	This work
pBad33-*ymdB-ymdC*(H369A)	clsC with H369A substitution, ymdB wild-type	This work
pBad33-*phoA*	phoA inserted into X site; CamR	This work
pCP20	FLP recombinase expression plasmid; AmpR CamR; temperature sensitive	(27)
pKD3	AmpR CamR	(27)
pKD46	AmpR; temperature sensitive	(27)

Table 3. Primers used in this study

Name	Sequence (5'→3')
INV[1]	GAGGTGATGTGAAATTAATTTAC
FIMA[1]	GATGCGGTACGAACCTGTCC
FIME[1]	GCAGGCGGTTTGTTACGGGG
EcFtsZ 1[2]	TAGCGGTATCACCAAAGGACT
EcFtsZ 2[2]	GTGATCAGAGAGTTCACATGCT

[1]Schwan *et al.* (1992), *J Bacteriol*

[2]Schwan *et al.* (2007), *J Bacteriol*

qPCR		
Gene	**Forward (5'→3')**	**Reverse (5'→3')**
clsA	ATATAATCGTCCTGAACGGCGGC	TGGTATGCAGTAACCCGCCTTCA
clsB	TAAAAAGTGGAACGCCAGCACG	AGTGACAAACTGAGCGGATCGAGA
clsC	CCGAAATGGCAGAGCATACGCTC	CGCTTGCTTGACAGTCATCCACG
cpsB	GTTGGCTCCTGGTCTTCATTA	CAGGCCAGATTCAGCATACA
cpsG	TGACGTGCTGGATATTGGTATG	GATTATGGCTGGCGGTAACT
degP	GCCTTCAGTGGTCAGCATTA	GGCAGAACGGAGAATCATCA
htrA	TATCGCGCTGATCCAAATCC	ACCAATCGCTACGGTGTAATC
mdtA	CAAGCAGGTTGATGTTGGTAAC	CCGGCAGGGTAAAGACTAAAT
acrD	TGACCTCGCTGGCATTTATC	CGAAATCATCCCGCCCATTA
pspA	CTGATGATCCAGGAGATGGAAG	TGTTCAATACGGCGAGTCAG
pspC	CTGGTGGTGCTGTCGATTT	AAGGTAGCTGCTCACCAAAG
idnT[3]	CTGTTTAGCGAAGAGGAGATGC	ACAAACGGCGGCGATAGC
gapA[4]	GTGATCCGGCTAACCTGAAA	GTCCTGGCCAGCATATTTGT

[3]Zhou *et al.* (2011), *BMC Mol Biol*

[4]Uchiyama *et al.* (2010), *FEMS Microbiol Lett*

Table 4. Minimum inhibitory concentration (MIC) of antimicrobial compounds in *E. coli* grown

at 30°C in M9 minimal medium supplemented with amino acids. Shown are the averages of 3

independent biological replicates and the standard error of the mean associated with each

average.

Compound	**MG1655**			*ΔclsABC*		
Cecropin A[a]	12.5	±	0	4.1	±	1.1
Ampicillin[b]	16.0	±	2.7	13.3	±	2.7
Kanamycin[b]	16.0	±	5.3	10.7	±	2.7
EDTA[b]	64.0	±	10.7	16.0	±	0
Polymyxin B[b]	8.7	±	4.1	4.0	±	0
Cefuroxime[b]	32.0	±	0	8.0	±	4.0

[a] μg/mL

[b] mg/mL

Figures

Figure 1. Phospholipid biosynthesis pathways in *E. coli*. Chemical transformations for CL biosynthesis in *E. coli* cells. Abbreviations for phospholipids are: PA, phosphatidic acid; CDP-DAG, CDP diacylglycerol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; and CL, cardiolipin. The lipid composition of wild-type *E. coli* growing exponentially in typical laboratory growth conditions (37°C in rich media with agitation) is 70% PE, 15-25% PG, and 5-10% CL.
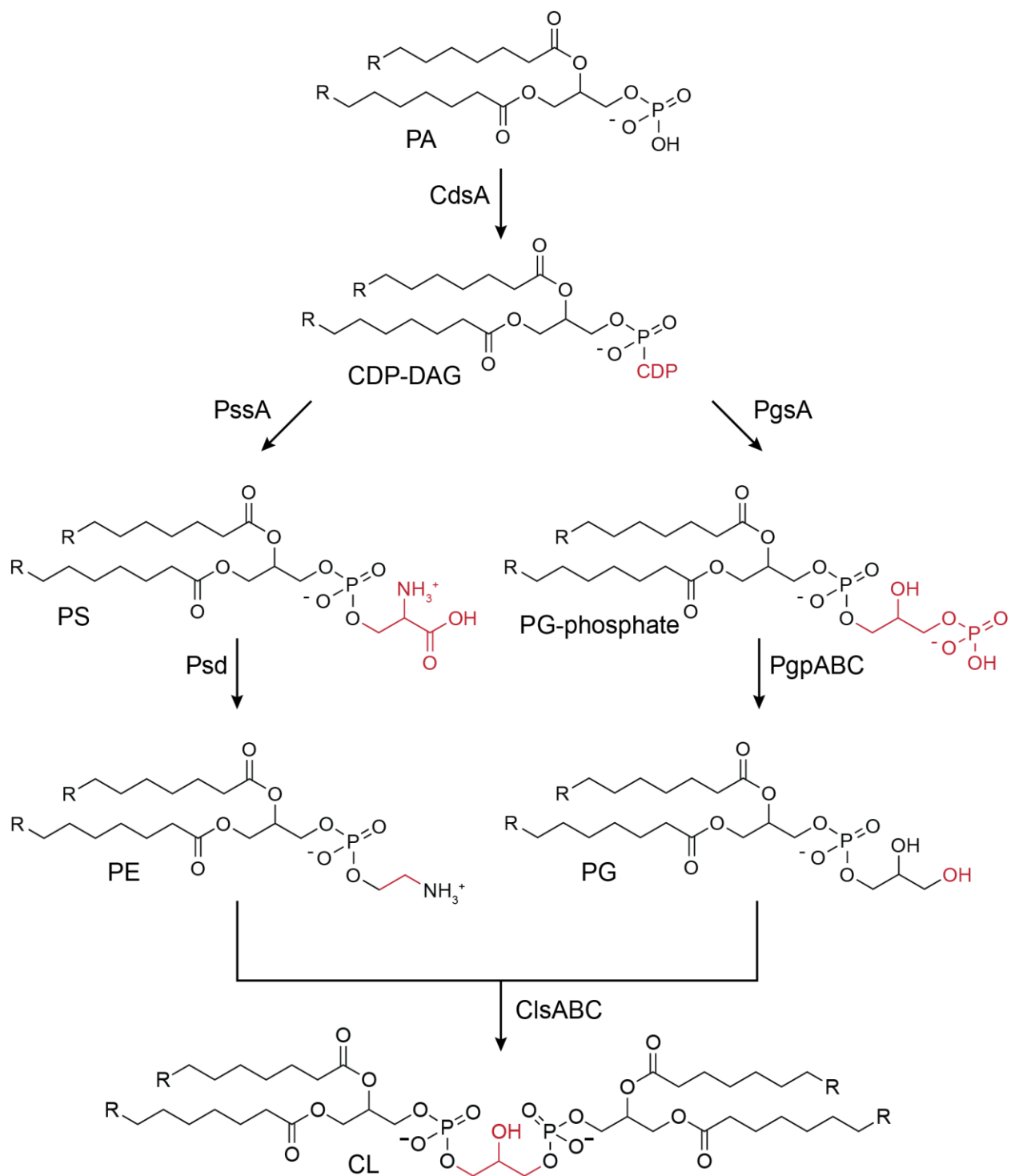
Figure 1.

Figure 2. CL is elevated in cells from early stage biofilms compared to cells in log phase.
Total membrane lipids were isolated from MG1655 cells grown in suspension to an absorbance ($\square$=600 nm) of 0.4-0.6 (log), for 24 h (stationary) or in 96-well plates without shaking for 24 h (biofilm). After separation by TLC, phospholipids were visualized by fluorescence imaging following treatment with cupric sulfate. ImageJ was used to quantitate lipid spots. Error bars indicate standard deviation of 3 biological replicates; ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, n.s. not significant; Student's t test.
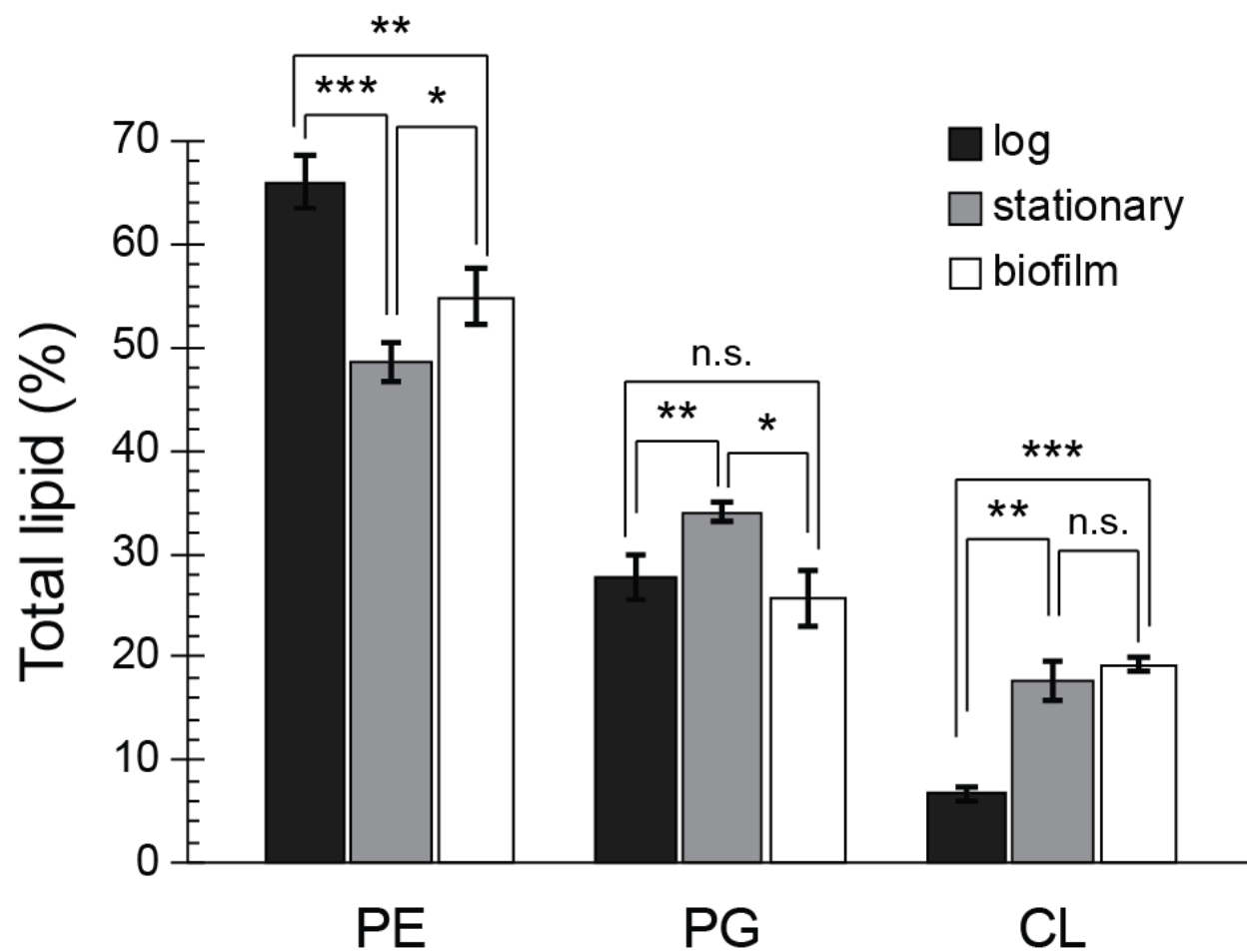
Figure 2.

Figure 3. Disruption of cardiolipin synthesis reduces biofilm formation. *E. coli* cells were grown in microtiter plates for 24 h at 30°C without shaking. Adherent cells were stained with crystal violet (CV), and CV absorbance was measured (λ=550 nm). Error bars indicate standard error; ***p < 0.001, **p < 0.01, *p < 0.05, n.s. not significant; Student's *t* test compared to WT. (A) CV staining of mutants with noted genotypes. (B) Δ*cls* strains were complemented with pBad33 (vector), full-length *cls*, or a catalytically inactive mutant (*cls**; *clsA* H224F H404F, *clsB* H113A, or *clsC* H130A). Cells were grown with 0.2% arabinose to induce protein expression; CV absorbance values of cells grown with arabinose were normalized to CV absorbance of uninduced cells. Error bars indicate standard error; ***p < 0.001, **p < 0.01, n.s. not significant; Student's *t* test compared to uninduced control. Inset: image of representative crystal violet staining of cells grown in M9 with 0.2% arabinose. From top: wild-type (MG1655); Δ*clsA*/pBad33; Δ*clsA*/pBad-clsA; Δ*clsA*/pBad-clsA*. (C) CV staining of biofilms started using saturated overnight cultures; CV staining of subcultured WT is shown for reference. Error bars indicate standard error; ***p < 0.001, **p < 0.01, n.s. not significant; Student's *t* test compared to WT.
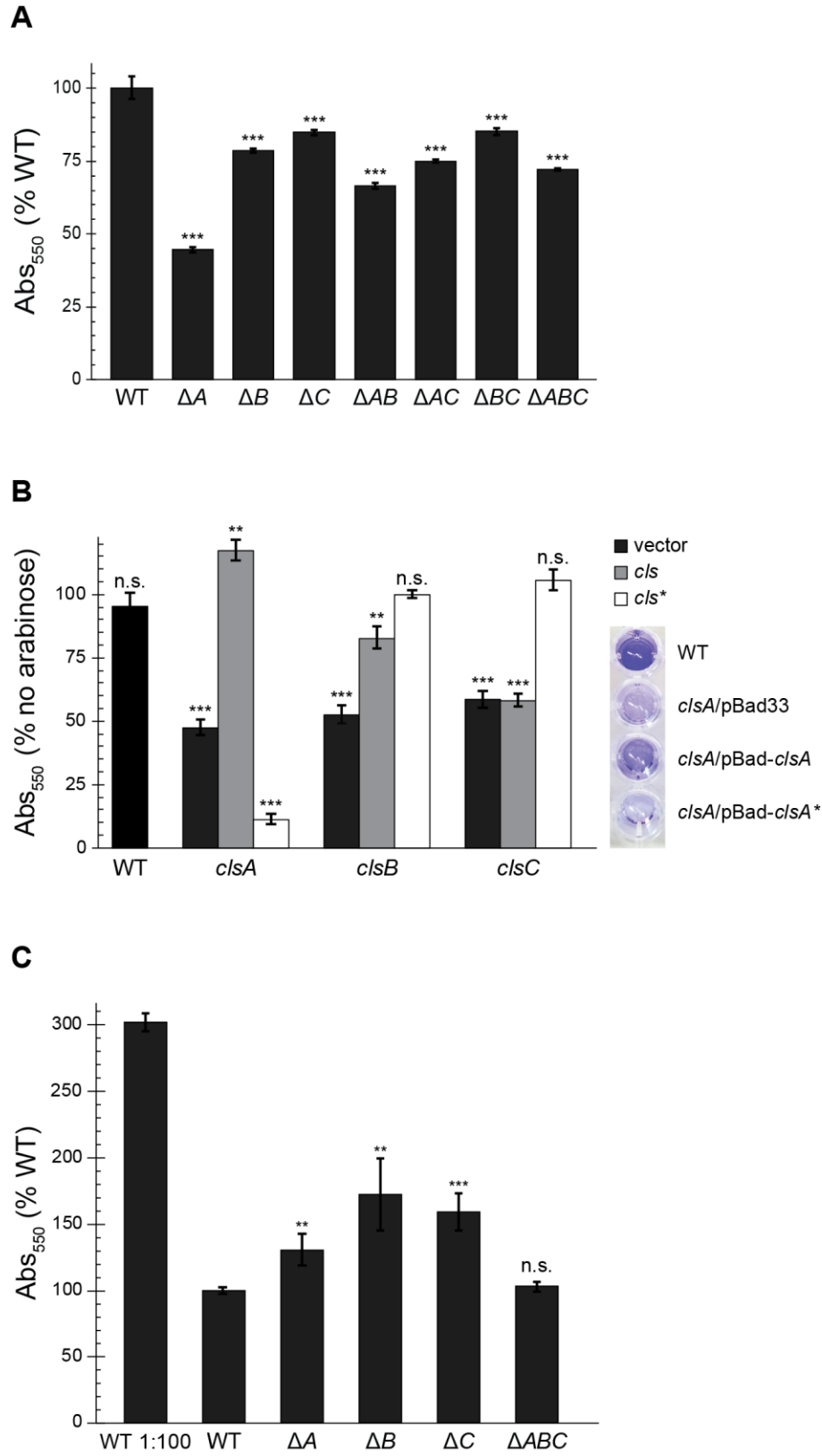
Figure 3.

Figure 4. Biofilm formation in rich medium. Cells were grown in lysogeny broth (LB) in microtiter plates for 24 h at 30°C without shaking. Adherent cells were stained with crystal violet (CV), and CV absorbance was measured at 550 nm. Error bars indicate standard error (N ≥6); differences between strains were not significant based on a Student's *t* test ($p > 0.05$), except WT vs. Δ*clsA* ($p < 0.0001$).
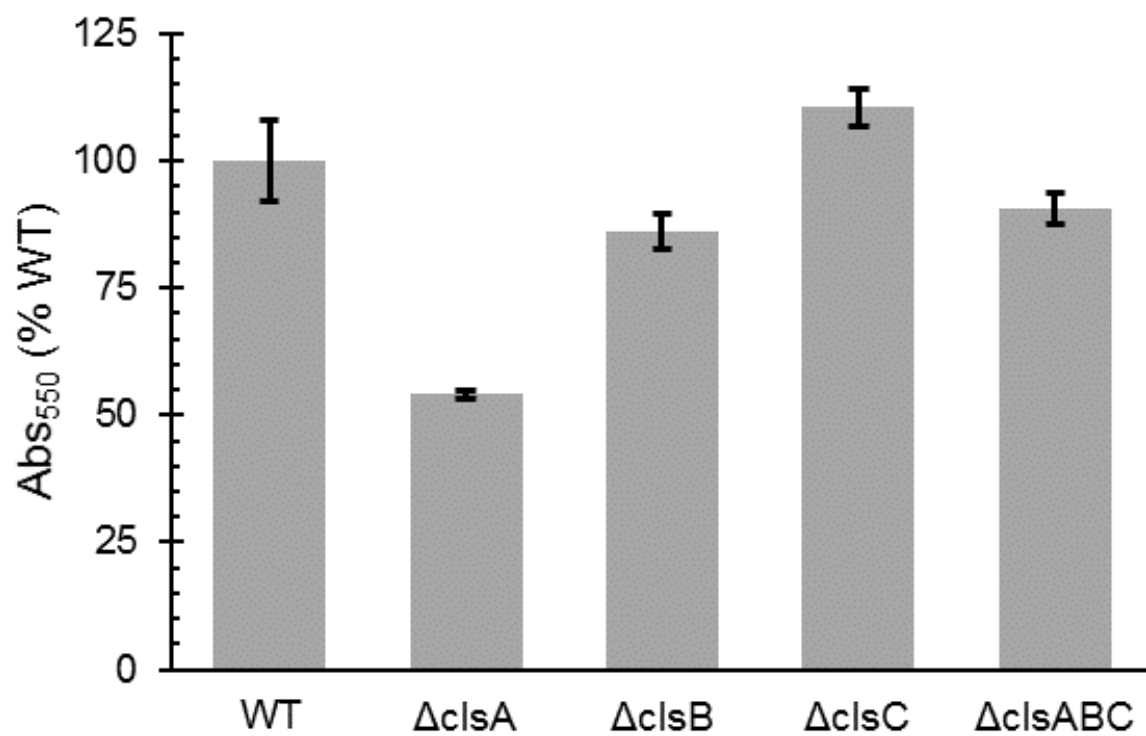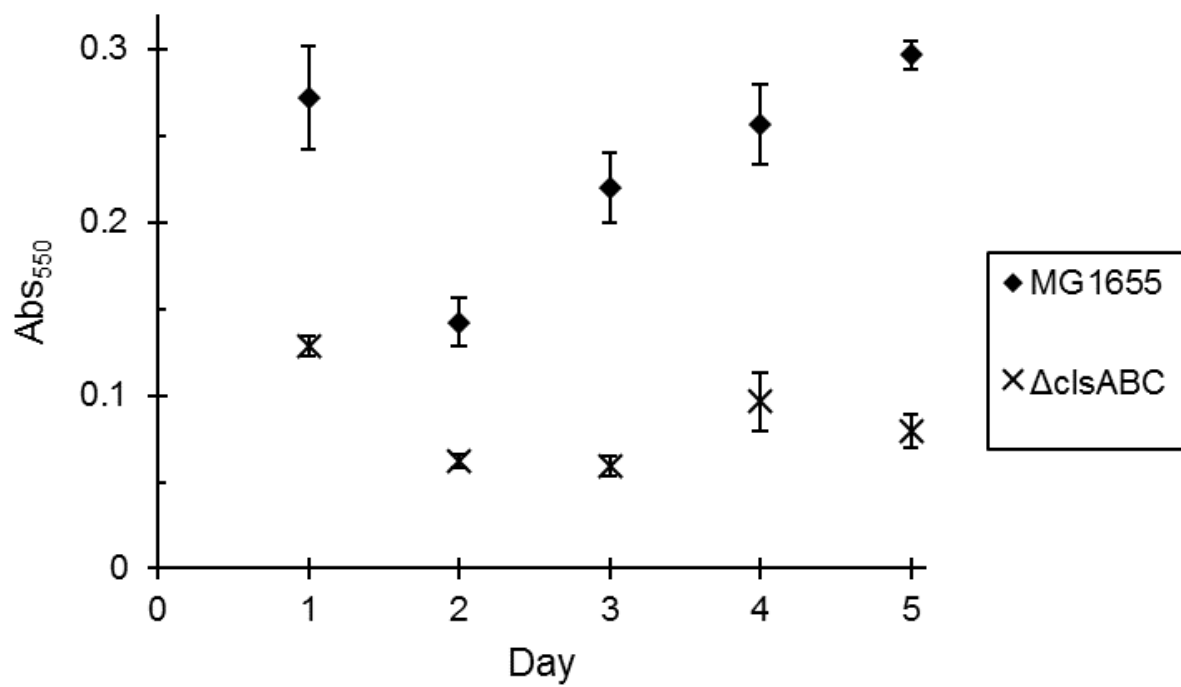
Figure 4.

Figure 5. Biofilm formation over multiple days. Cells were grown in microtiter plates for 24 h at 30°C without shaking. Adherent cells were stained with crystal violet (CV), and CV absorbance was measured at 550 nm. Error bars indicate standard error ($N \geq 6$).

Figure 5.

Figure 6. Effect of treatment with antimicrobials on biofilm formation in Δ*clsABC* cells.

Cells were grown in M9 minimal medium in microtiter plates for 24 h at 37°C without shaking, then treated with antimicrobial compounds for 24 h as described in the Materials and Methods. Adherent cells were stained with CV, and CV absorbance was measured at 550 nm. Error bars indicate standard error (N ≥ 16); **$p < 0.01$, *$p < 0.05$, Student's *t* test. (Poly B, polymyxin B; Cec A, cecropin A; Dapto, daptomycin; Amp, ampicillin; Nov, novobiocin; Cipro, ciprofloxacin; Kan, kanamycin; Erythro, erythromycin; Tet, tetracycline).
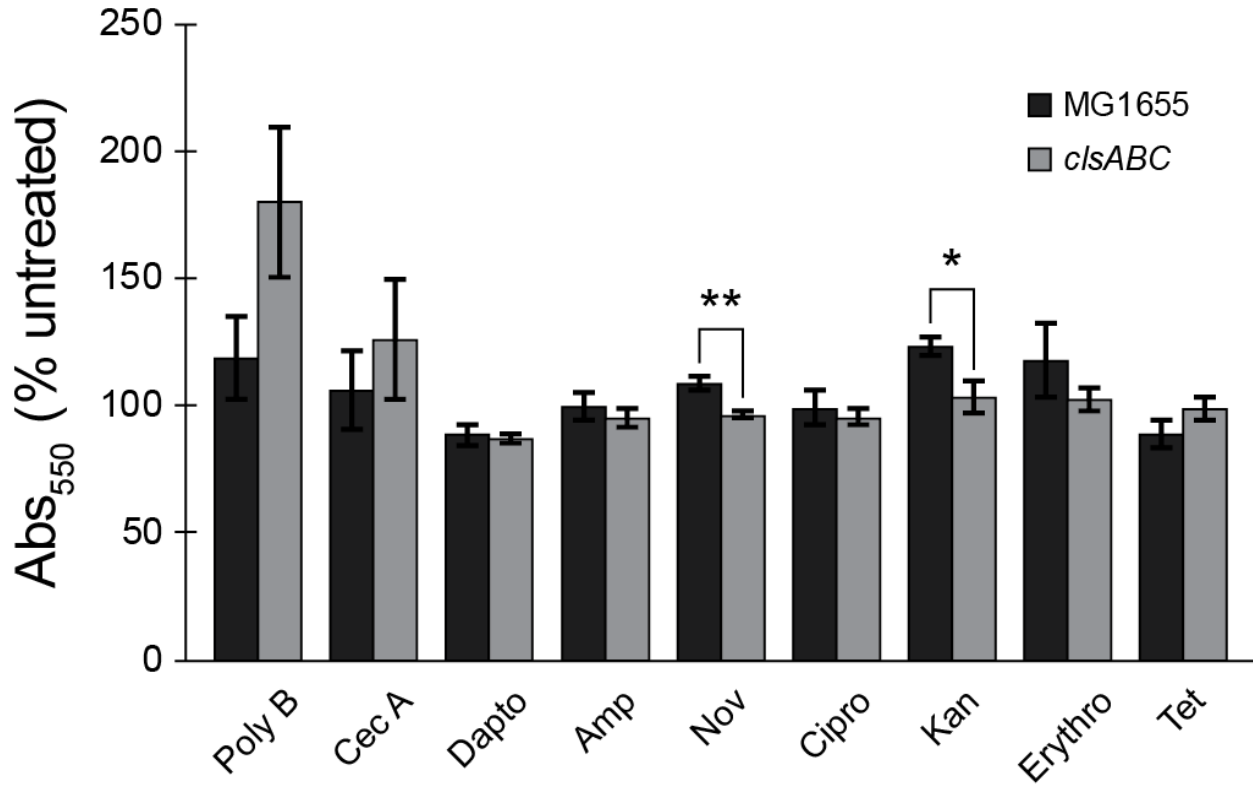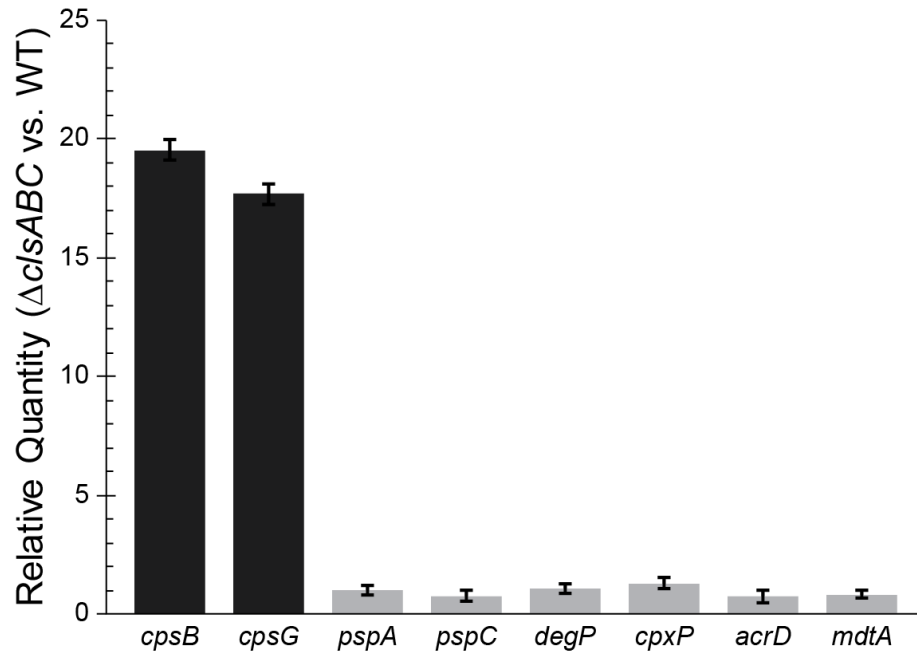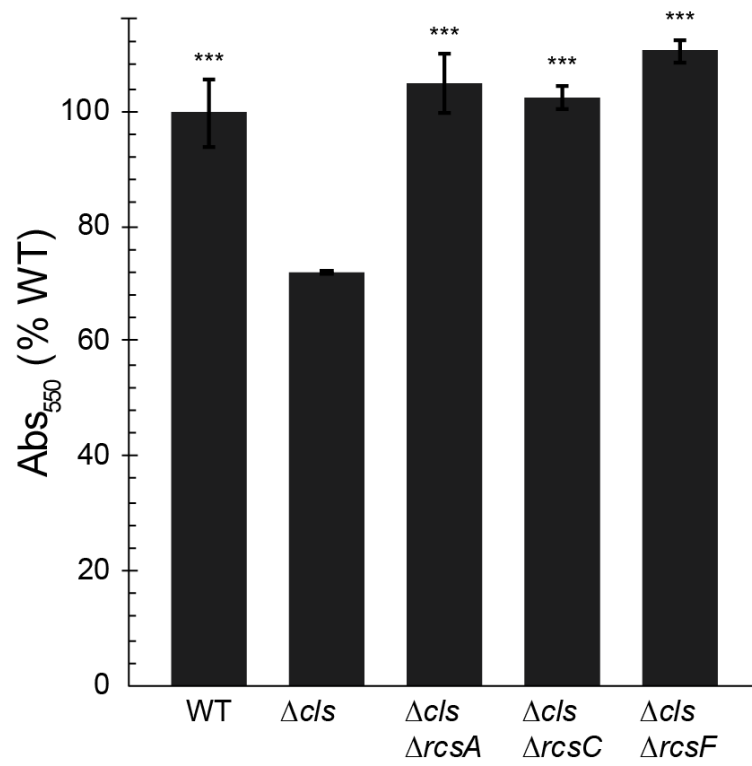
Figure 6.

Figure 7. The Rcs phosphorelay is activated in CL deficient cells. (A) We used qPCR to measure activation of 5 major envelope stress pathways in Δ*clsABC* (Δ*cls*) cells. The relative abundance of transcripts of the Rcs-controlled genes *cpsB* and *cpsG* was ~20-fold higher in Δ*cls* compared to wild-type MG1655 cells. Bae (*mdtA*, *acrD*), Psp (*pspA*, *pspC*), sigma E (degP), and Cpx (*cpxP*, *degP*) signaling pathways were not significantly activated ($0.5 \leq RQ \leq 2$). Error bars indicate standard deviation. (B) Crystal violet staining of Δ*cls* mutants, and Δ*rcs* mutants in a Δ*cls* background. Error bars indicate standard error; ***$p < 0.001$; Student's *t* test compared to Δ*cls*.

A

B

Figure 7.

Figure 8. Rcs monitors export of outer membrane proteins. (1) After its synthesis, RcsF is transported to the outer membrane by the chaperone LolA and associates with BamA, a component of the machinery that assembles β-barrel proteins; the immature, processed form of OmpA is referred to as 'imp-OmpA'. RcsF is then assembled into a complex with OmpA. (2) Under normal conditions, IgaA represses the Rcs phosphorelay. However, defects in protein translocation cause a buildup of free RcsF, which associates with IgaA and blocks its inhibition of the phosphorelay. (3) Once repression by IgaA is released, RcsC is autophosphorylated. The phosphate is transferred to RcsD, then to the transcription factor RcsB. (4) Phosphorylated RcsB interacts with itself and other transcription factors to regulate genes involved in a number of biofilm formation processes, as well as genes for acid resistance, cell division, and other transcriptional regulators.
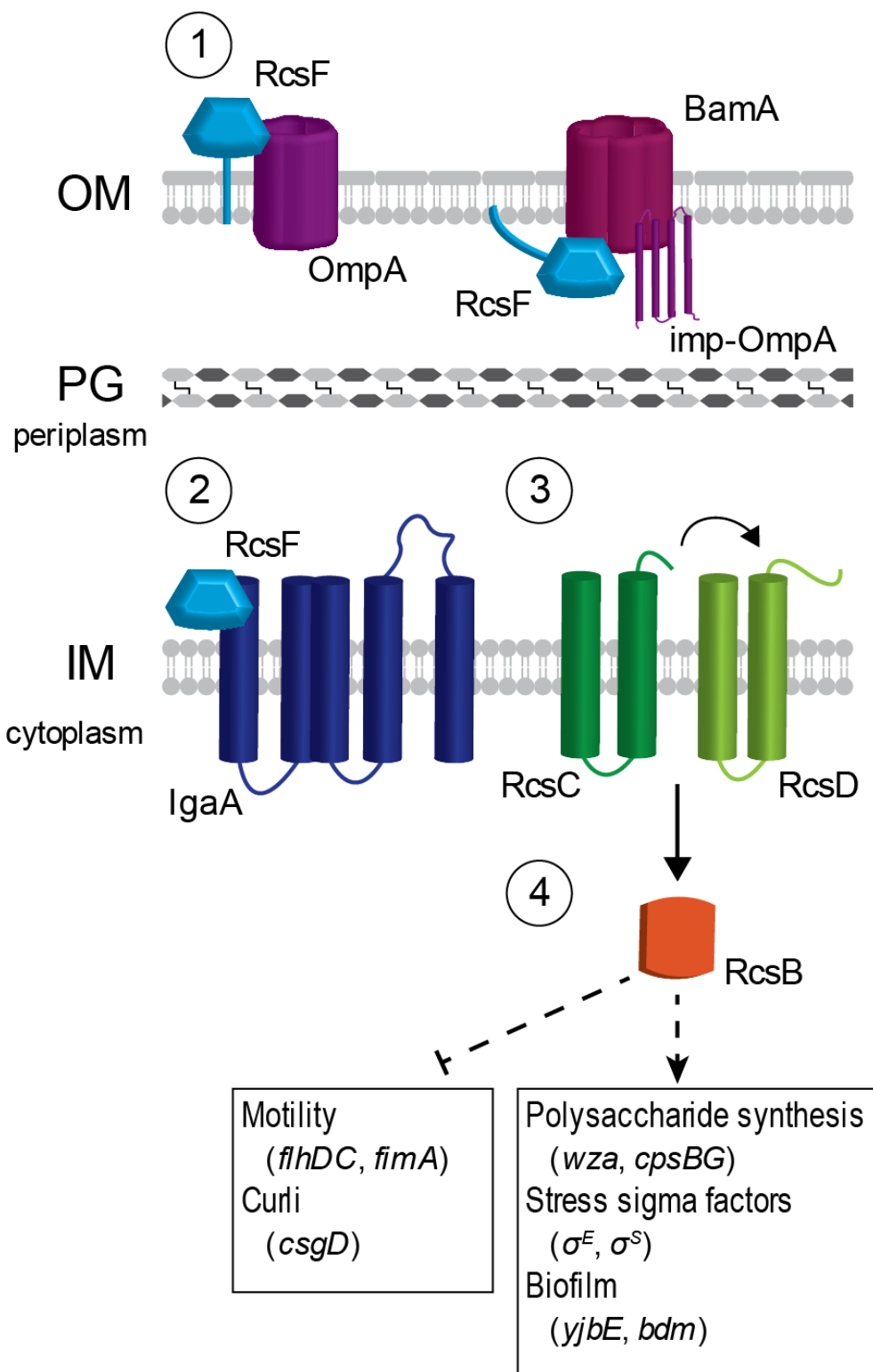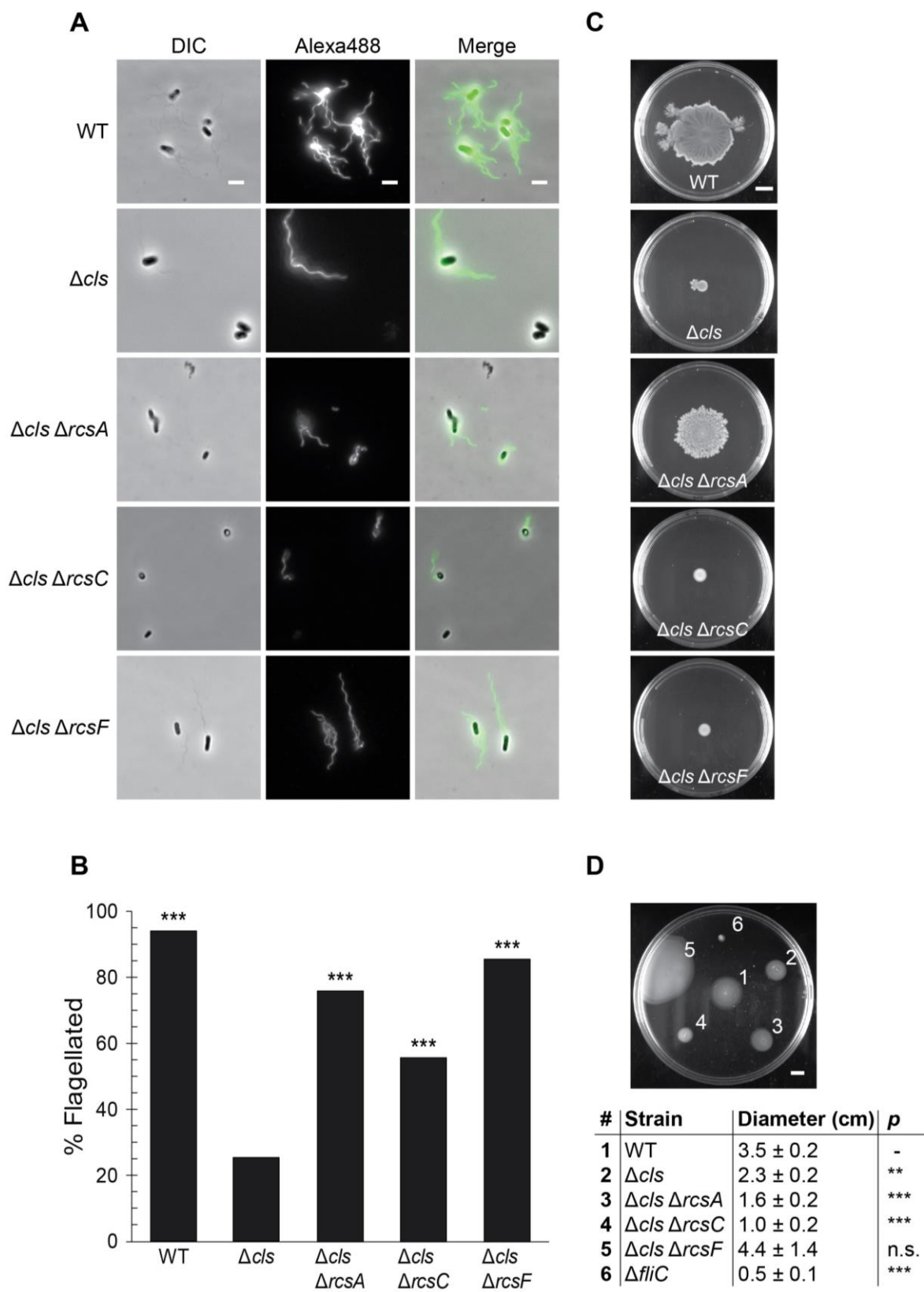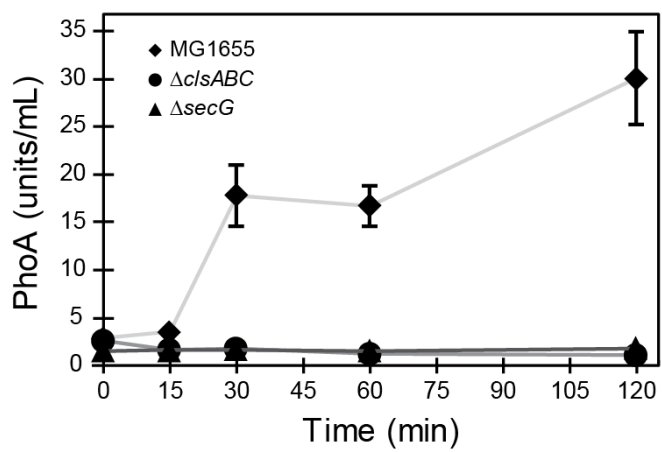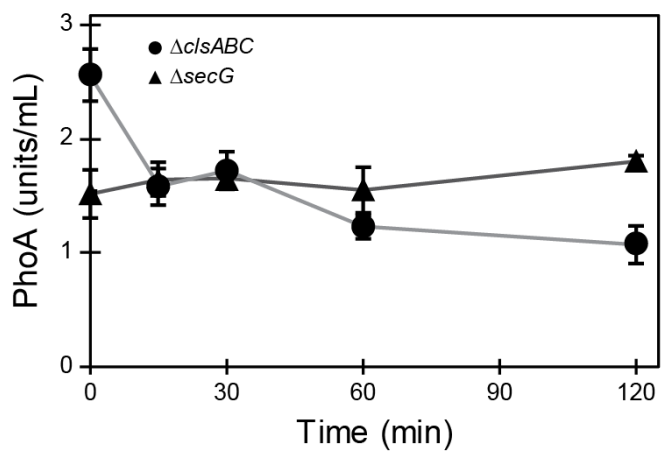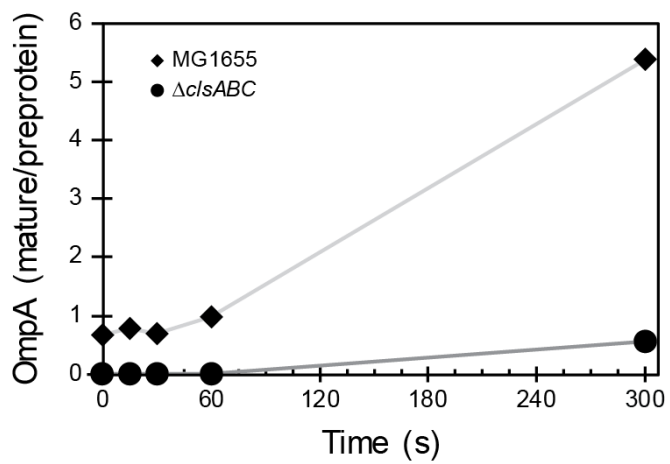
Figure 8.

Figure 9. Cardiolipin deficient cells produce fewer organelles that enable surface attachment and promote biofilm formation. (A) Using an antibody raised against FliC, we immunostained cells in the late exponential phase of growth and (B) determined the percentage of cells with at least one visible flagellum (N ≥ 100 cells). DIC = differential interference contrast. ***$p < 0.001$; Fisher's exact test, compared to $\Delta clsABC$. Scale bar is 3 μm. (C) Swarm and (D) swim plates were inoculated as described in the Materials and Methods, and imaged after 24 h at 30°C. Average swimming diameters after 24 h and standard deviations are indicated for each strain. **$p < 0.01$, ***$p < 0.001$, n.s. not significant; Student's t test compared to WT. Scale bar is 1 cm.

A

	DIC	Alexa488	Merge
WT			
Δcls			
Δcls ΔrcsA			
Δcls ΔrcsC			
Δcls ΔrcsF			

B

% Flagellated

C

WT
Δcls
Δcls ΔrcsA
Δcls ΔrcsC
Δcls ΔrcsF

D

| #	Strain	Diameter (cm)	p
1	WT	3.5 ± 0.2	-
2	Δcls	2.3 ± 0.2	**
3	Δcls ΔrcsA	1.6 ± 0.2	***
4	Δcls ΔrcsC	1.0 ± 0.2	***
5	Δcls ΔrcsF	4.4 ± 1.4	n.s.
6	ΔfliC	0.5 ± 0.1	***

Figure 9.

Figure 10. Protein translocation is reduced in the absence of CL. (A) and (B) Periplasmic

activity of PhoA in wild-type MG1655 (diamonds), Δ*clsABC* (circles), and Δ*secG* (triangles); 1

unit = 1 mol/min of hydrolyzed PNPP. Measurements represent the average of 3 biological

replicates and error bars indicate standard error of the mean. (C) Ratio of mature OmpA to pre-

OmpA.

A

B

C

Figure 10.

Figure 11: Fimbriation of CL deficient cells. Genomic DNA was extracted from biofilms as described in the Materials and Methods and PCR was used to determine the orientation of the FimA invertible element. (A) INV and FIMA primers amplify phase-on oriented DNA (450 bp product), INV and FIME primers amplify phase-off oriented DNA (750 bp product), and EcFtsZ1 and 2 primers amplify a 302 bp segment of the *ftsZ* gene. (B) ImageJ was used to determine the intensity of DNA bands, which were normalized to the *ftsZ* loading control. Error bars indicate standard error (N = 3); differences between strains were not significant based on a Student's *t* test ($p > 0.05$).

A
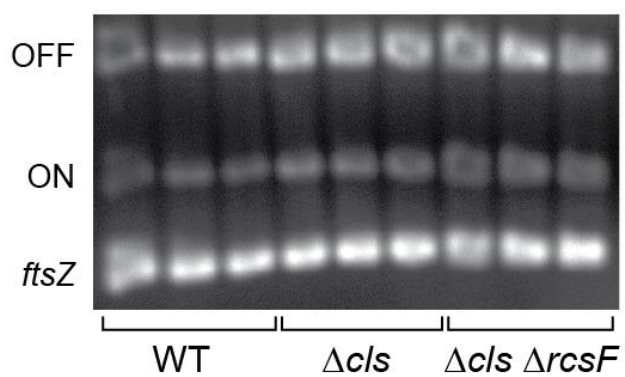
B
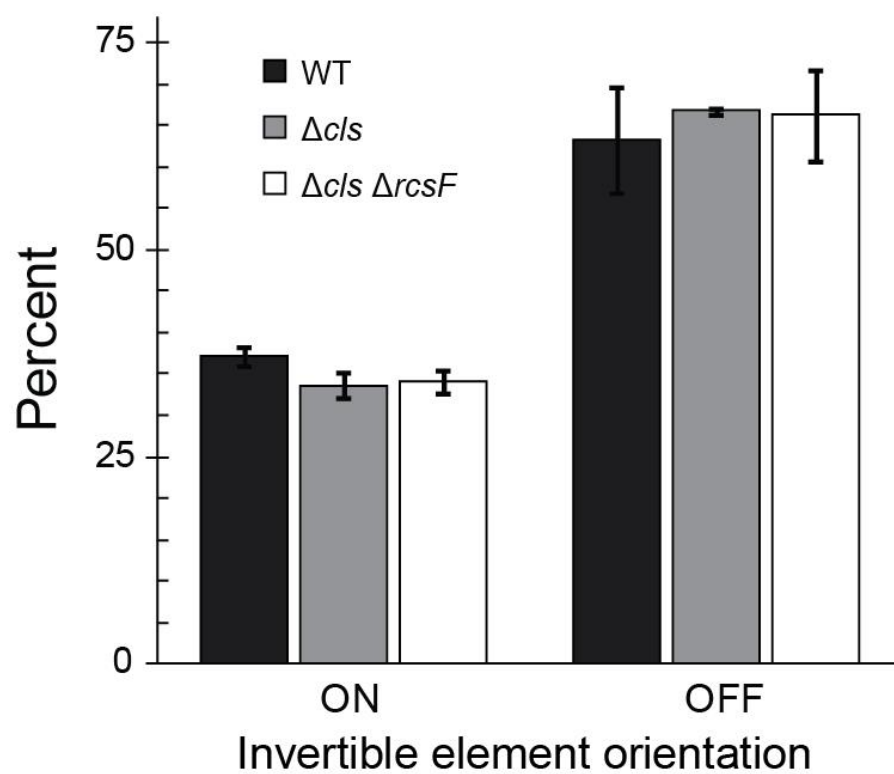
Figure 11.

Figure 12: CL depletion affects early stages of biofilm formation. *E. coli* cells were grown in microtiter plates for the indicated times at 30°C without shaking. Cell growth was measured by optical density (OD) (λ=600 nm). Adherent cells were stained with crystal violet (CV), and CV absorbance was measured (λ=550 nm) and normalized to OD. Error bars indicate standard deviation.
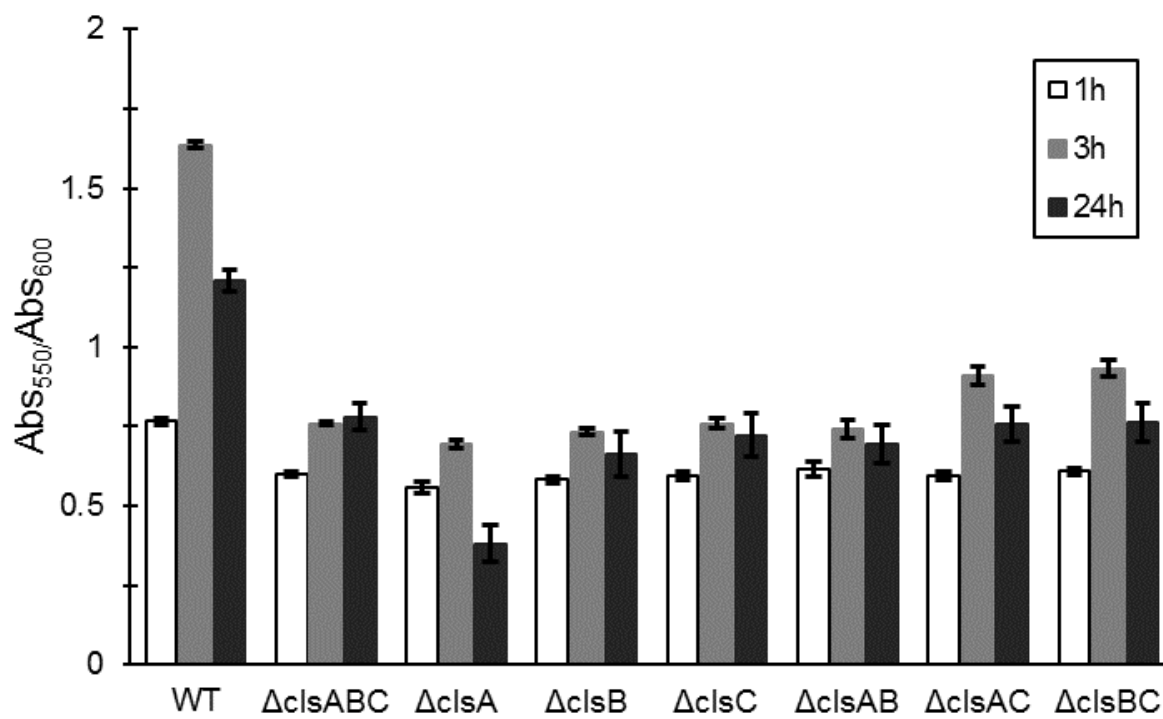
Figure 12.

CHAPTER 3

Developing an isothermal PCR-based diagnostic tool

Adapted from

Liljander A, Yu M, O'Brien E, Heller M, Nepper JF, Weibel DB, Gluecks I, Younan M, Frey J, Falquet L, Jores J (2015). "Field-applicable recombinase polymerase amplification assay for rapid detection of *Mycoplasma capricolum* subsp. *capripneumoniae*." *J Clin Microbiol* **53**(9):2810-2815.

JFN designed and performed experiments, analyzed data, and edited the manuscript.

Abstract

Mycoplasma capricolum subsp. *capripneumoniae* (*Mccp*) is the etiological agent of contagious caprine pleuropneumonia, a highly contagious disease that affects goats in Africa and Asia. Presently available methods for diagnosis of *Mycoplasma*, such as cultivation, serological assays and PCR, are time-consuming and require fully equipped laboratories. Here we report a rapid, specific and sensitive assay employing isothermal DNA amplification using recombinase polymerase amplification (RPA) for detection of *Mccp*. We evaluated the assay using genomic DNA and bacterial cultures. A detection limit between 5×10^3 and 5×10^4 cells/mL was achieved. No amplification was detected from 72 closely related *Mycoplasma* isolates, demonstrating a high degree of specificity. Fluorescence signals were obtained within 15-20 minutes and the method worked well using pleural fluid obtained directly from CCPP-positive animals without prior DNA purification. We show that definite diagnosis of CCPP can be achieved in less than 45 min at the point of care, with a short sample preparation time and a simple read-out device that can be powered by a car battery.

Introduction

A key challenge in infectious disease control is quick and accurate identification of pathogens (1). Most diagnostic tools have significant limitations that reduce their overall usefulness. Growth-based assays are the slowest to achieve results, taking many hours to identify the most rapid-growing pathogens, and as much as a week or more for slow growing microbes such as *Mycobacterium tuberculosis* (2, 3). Antigen-based assays, like enzyme-linked immunosorbent assays (ELISA), are much more rapid, usually taking less than an hour to complete. However, techniques of this nature are not available for all pathogens, and can suffer from a lack of specificity—especially when it is necessary to distinguish between several closely-related organisms or different strains of the same species.

Polymerase chain reaction (PCR) provides a reasonable compromise between speed and accuracy; PCR takes only a few hours to perform, and targeting highly polymorphic regions of the genome helps to ensure accurate identification even to the level of specific serovars of a single species. By targeting multiple genomic regions, it is possible to further increase the accuracy and precision of pathogen identification. PCR also overcomes problems associated with limited sample or low titers of pathogens, as amplification is possible theoretically with a single copy of the target nucleic acid molecule, and in practice can often be achieved with fewer than 10 nucleic acid molecules.

Despite these strengths, traditional PCR remains unfeasible for diagnostics in resource-limited areas. It requires trained personnel, dedicated laboratory space, and expensive equipment such as thermocyclers. The recent development of isothermal PCR methods such as recombinase polymerase amplification (RPA) addresses the latter issue, eliminating the need for automated thermocyclers.

RPA employs recombinase-driven oligonucleotide binding to a template sequence followed by DNA polymerase-facilitated strand extension (4). DNA amplification can be monitored in real time using a sequence specific fluorescent probe. This technique offers high sensitivity comparable to that of traditional PCR with the advantage that double-stranded DNA is exponentially amplified at constant and low temperatures (optimal temperature range 37°C to 42°C). RPA based diagnostic assays have been described for pathogen detection direct from biological material such as urine (5).

CCPP is one of the most severe diseases affecting goats throughout Africa, Asia, and the Middle East. CCPP is caused by *Mycoplasma capricolum* subsp. *capripneumoniae* (*Mccp*) (6) which belongs to the so-called "*Mycoplasma mycoides* cluster", comprising four additional closely-related mycoplasmas (*M. capricolum* subsp. *capricolum* (*Mcc*), *M. mycoides* subsp. *capri* (*Mmc), M. mycoides* subsp. *mycoides (Mmm)* and *M. leachii*) which all cause diseases in ruminants (7). CCPP is associated with major financial losses for goat producers and imposes trade restrictions on live animals. The disease is characterized by fever (>41°C), coughing and respiratory distress and is associated with morbidity and mortality reaching up to 90% and 60%, respectively (6). Macroscopic lesions of pleuropneumonia are often unilateral and a profound production of pleural fluid is often found post-mortem (8). The clinical symptoms of CCPP are often confused with infections caused by other mycoplasmas or *Pasteurella* spp. The current option for containing CCPP outbreaks is vaccination with a bacterin type of vaccine (9). Prompt diagnosis is paramount for effective disease control and monitoring. However, the distribution and burden of CCPP still remain largely unknown, mainly due to fragmented veterinary services, a lack of funds, an absence of infrastructure enabling swift sample transport, and the absence of point-of-care (POC) tests.

Recently, a competitive ELISA (cELISA) for CCPP was modified and resulted in a heat-stable laboratory diagnostic kit suitable for prevalence and vaccine efficacy screening, albeit not suitable for detecting acute disease (10). Thus, for diagnosis of CCPP, the gold standard remains the direct isolation and cultivation of *Mccp* from infected lung tissues or pleural fluid collected post-mortem. However, the process is cumbersome and time-consuming due to the fastidious nature of the organism. In addition, the isolation is often hampered by sample contamination and prior antibiotic treatment of the diseased animals. Moreover, DNA-based confirmation of the cultures using PCR methods is still needed (11-13). Thus culture, isolation, and molecular characterization of *Mccp* is not a feasible alternative for rapid containment of a CCPP outbreak.

A latex agglutination test (LAT)(14) has been developed for POC diagnosis of CCPP (15). This test is based on the detection of serum antibodies against polysaccharide antigens. Although rapid and applicable for field use, this test is not specific for CCPP (16). Alternatively, advanced molecular techniques such as real-time PCR are both sensitive and specific (12). However, methods such as these require a well-equipped laboratory with expensive equipment and trained personnel. A loop-mediated isothermal amplification (LAMP) assay for detection of *Mccp* has been reported recently (17). It does not depend on expensive thermocyclers but still requires DNA extraction protocols and separation and visualization of products via horizontal agarose gels, which limits the applicability of this method as a POC test. Here we describe the development of a specific and sensitive assay using RPA for rapid diagnosis of CCPP directly from crude clinical samples. This method is also suitable for field application as a POC test using lyophilized reagents and a car battery to power the read-out device.

Materials and Methods

Mycoplasma strains and growth conditions. For the optimization and evaluation of the RPA assay, *Mccp* strain ILRI181 was used. Eighty-six additional strains representing 25 species and sub-species were included to investigate the specificity of the RPA assay (Table 2). All strains were cultured in mycoplasma liquid medium (Mycoplasma Experience Ltd, UK) at 37°C for 24 h to 72 h until a colour change from red to orange was observed. Aliquots of *Mccp* ILRI181 cells were stored at -80°C until further use in spiking experiments. Colour change units/mL (CCU/mL) were determined in duplicates for strain *Mccp* ILRI181 by making 10-fold dilutions of the culture in mycoplasma liquid medium. The dilutions were grown at 37°C for one week prior to determining CCU/mL.

Isolation of genomic DNA. Bacterial strains were grown in mycoplasma liquid medium as described above. Cultures (10 mL) were centrifuged, the supernatants discarded and the cell pellets were re-suspended in 500 μL of TNE buffer (10 mM Tris-HCl, pH 8.5; 10 mM NaCl; 10mM EDTA). Afterwards 10 μL 10% SDS and 10 μL 20mg/mL Proteinase K was added and the cell suspensions were incubated at 37°C for 2 h. Thereafter 52 μL 100mM PMSF (in EtOH, stored at -20°C) was added followed by an additional incubation at room temperature for 15 min. Following the addition of 50 μL 10mg/mL RNase A the suspensions were incubated at 37°C for 1 h. Then 50 μL 1.5M sodium acetate, pH 5.2, and 550 μL buffered phenol was added; the solutions were vortexed and spun down at 14,000 x g for 10 min. The top phase was transferred to a new reaction tube and 550 μL phenol/chloroform/isoamyl alcohol (25:24:1) solution was added and the suspensions were vortexed for 20 sec. Following an additional centrifugation (14,000 x g for 10 min), the top phase was transferred to a new reaction tube and the DNA was precipitated using isopropanol, washed with 70% EtOH (18), air-dried and re-suspended in 100

µL 10 mM Tris-HCl, pH 8.5. DNA concentrations (ng/µL) were determined using Nanodrop (Thermo Scientific) and Qubit 2.0 (Life Technologies).

RPA primer and probe design. The full genome sequence of *Mycoplasma capricolum* subsp. *capripneumoniae* (*Mccp*) strain ILRI181, isolated during a recent outbreak in Kenya (19), (EMBL accession no. LN515339) was used for primer and probe design. Oligonucleotides were designed according to the TwistDX guidelines (TwistDx, UK) (Table 1). The *in silico* specificity of the primers and probe, was investigated using the pattern searching tool "fuzznuc" from the EMBOSS package (20) against selected bacterial genomes; *Mycoplasma capricolum* subsp. *capripneumoniae* (*Mccp*) *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*), *Mycoplasma mycoides* subsp. *capri* (*Mmc*), *Mycoplasma capricolum* subsp. *capricolum* (*Mcc*), *Mycoplasma leachii*, *Mycoplasma putrefaciens*, *Mycoplasma bovis, Mycoplasma bovigenitalium, Mycoplasma 111rginine, Mycoplasma californicum, Mycoplasma alkalescens, Mycoplasma canis, Acoleplasma laidlawii, Pasteurella multocida* and *Mannheimia haemolytica*. Parameters were set to check for both the strand and the complementary strand on a circular molecule allowing one, five and ten mismatches (fuzznuc -complement -scircular -pmismatch 1, 5 or 10). The output gff file was converted to an Excel sheet by a custom script. The *Mccp* specific oligonucleotides were synthesized by Integrated DNA Technologies (USA) while the probes containing the fluorophore 6-FAM (dT-FAM), a tetrahydrofuran site (THF), a quencher (dT-BHQ1) and a 3' block (C3-spacer) were synthesized by Biosearch Technologies, Inc. (USA).

RPA assays. The RPA reactions were performed using the reagents and protocols from the TwistAmp exo kit (TwistDx, UK). Each 50 µL reaction contained: 29.5 µL rehydration buffer,

12.2/8.2 µL nuclease-free water, 2.1 µL each of forward primer/ reverse primer (10 mM), 0.6 µL

probe (10 mM) and 1.0/5.0 µL template. The reagents were vortexed briefly prior to dissolving

the reaction pellet. To start the reaction, 2.5 µL 280 mM magnesium acetate (MgOAc) was

added to the cap of the tube followed by a brief centrifugation, another vortex and a final brief

centrifugation. The amplifications were read in an ESEQuant Tube Scanner (Qiagen, Germany)

at 42 °C every 30 sec for 30 min in the FAM channel (excitation 470 nm, emission 520 nm).

Initially agitation after four and six minutes was evaluated. To distinguish positive from negative

results a cut-off value was set for every measurement corresponding to three standard deviations

(3SD) of the negative control.

Clinical specimens and optimization of sample preparation. The RPA assay was evaluated

using a panel of pleural fluids (n=5) and lung tissue specimens (n=2), collected from goats

during a recent outbreak of CCPP in Kenya in 2012 (Table 3). Ten-fold dilutions in liquid

medium, as described above, were used to determine the CCU/mL titer in the pleural fluid

samples. Tissue samples were also cultured in liquid media for one week, as above, to determine

the presence of *Mycoplasma*. If needed the initial tissue culture was filtered through a 0.22 µm

filter (Carl Roth, Germany) before diluting the filtered material 1:10 in fresh medium prior to

continued growth.

Different methods for samples preparation, i.e. bacterial lysis, sample dilution and

template volume for the RPA assays were evaluated in duplicates on a subset of the pleural fluid

samples (n=2) and on included lung tissue samples (n=2). Pleural fluid samples were diluted

1:20 or 1:50 in either 10 mM Tris-HCl,pH 8.5 or nuclease-free water for subsequent heat-lysis

(60°C for 10 min) or in 0.2M KOH or nuclease-free water for direct lysis. Lung tissue samples,

five from each animal, were cut into small pieces (≤125mm^3) using a scalpel. The tissues were homogenized manually using a tissue grinder (Rotilabo, Carl Roth, Germany) in either 500 □L of 10 mM Tris-HCl, pH8.5 or 0.2M KOH or nuclease-free water. Samples in nuclease-free water and 0.2M KOH had no additional treatment, while 10mM Tris samples were heated at 60°C for 10 min. One or five μL of the different lysates (pleural fluid/lung tissue homogenate) was used as a template in a final reaction volume of 50 μL for the RPA assays. Once the sample preparation conditions had been optimized, the remainder of the specimens were tested in duplicates using the most suitable method.

Set up of RPA assays-lyophilization for field use. RPA reaction pellets (TwistAmp exo kit) were constituted with buffer, primer, probe and water as described above albeit excluding template (5 μL) and MgOAc. Then, the RPA reactions were frozen at -80°C prior to lyophilization in a LyoQuest (Telstar Life Science Solutions) using a temperature of -55°C and a pressure of 0.400 mBar. Aliquots of MgOAc (2.5 μL) were also lyophilized in separate tubes. Aliquots of lyophilized RPA reactions and MgOAc were either used directly or stored at 4°C and 37°C for at least five days prior to use. To set up the reactions, 5 μL template and 45 μL nuclease-free water was added to the lyophilized MgOAc, which then was added to dissolve the lyophilized RPA reactions prior to immediate reading out in the tube scanner. The sensitivities of the lyophilized reactions were determined as described below using cultured *Mccp* ILRI181 in spiking experiments. Furthermore, the read-out system (ESEQuant Tube Scanner) was tested for field applicability by running the reactions in a car using a 120W Laptop Car power supply (L40BB) connected to the car cigarette lighter.

Determination of specificity of the Mccp primers/probe. The specificity of the *Mccp*

primers/probe was determined by running duplicate RPA reactions using genomic DNA

(corresponding to 10^7 copies of the genome) from 87 total strains (Table 2). Positive controls

using 10^7 genome copies of *Mccp* ILRI181 DNA were included in each run.

Determination of RPA assay sensitivities. Sensitivity of the RPA assay was determined using

genomic DNA (corresponding to 10^0-10^6 genome copies/μL) and lysed bacteria (corresponding

to 10^0-10^5 CCU/mL) of *Mccp* ILRI181 in spiking experiments. Every run was repeated eight

times. The DNA was diluted 10-fold in nuclease-free water while live bacteria was diluted 10-

fold in plasma (resembling pleural fluid) isolated from a healthy goat prior to a 100-fold dilution

in nuclease-free water for direct lysis. Five microliter template was used from each dilution in a

total RPA reaction volume of 50 μL. Nuclease-free water and plasma diluted 1:100 in nuclease-

free water were included in every run as negative controls for the DNA and live bacteria spiking

experiments respectively.

Assessment of RPA performance using pleural fluid. Pleural fluid samples (n=5) were diluted

1:50 in nuclease-free water and 5 μL of this dilution were used as template for the RPA

amplification. The reaction was carried out in duplicate.

Results

In silico specificity of primers and probe for RPA

The primers were designed to amplify a 245 bp region within a single-copy gene in the

genome of *Mccp* strain ILRI181 (locus tag MCCPILRI181_00726) (19). To ensure that the target

sequences were exclusive to *Mccp*, the selected primers and probe were screened *in silico* against the genomes of 14 bacteria causing infections in ruminants (Table 4). No complementary regions were found when allowing one or five sequence mismatches for the primer sequences. Permitting ten mismatches did give rise to sequence matches, however only for one of the two primers used. According to this *in silico* analysis the designed primers fulfilled the requirements to ensure specificity of the RPA technology (22).

Sample preparation optimization

Various methods for sample preparation for the RPA assay were evaluated. Direct lysis of pleural fluid specimens (n=2) in nuclease-free water was sufficient for release of nucleic acids from mycoplasma. There was no significant difference in signal intensity and onset of the RPA reaction between samples lysed in a 1:20 or 1:50 dilution (regardless of the diluent used, e.g. H_2O, Tris-HCl or KOH). However, heat-lysis (60°C for 10 min) in nuclease-free water or Tris-HCl buffer (10 mM, pH 8.5) and alkaline lysis in 0.2M KOH reduced fluorescent signal intensities (mV) and delayed the onset of the reaction (*data not shown*). Subsequently, RPA reactions with pleural fluid samples were performed using direct lysis in nuclease-free water in a dilution of 1:50. Similar methods for bacterial lysis were also evaluated for lung tissue samples (n=2). Five tissue samples from each animal were tested. Great variability was seen in the ability to detect *Mycoplasma* using tissue samples. All five samples from each animal did give a positive result, albeit with large differences in signal intensity. Positive results were obtained within 10 to 14 minutes when bacterial lysis was done using 0.2M KOH and 1 µl lysate was used as template (Table 2). Similar results were obtained when 5µl template was used but the amount of lysate i.e. the amount of added KOH did have a negative impact on the appearance of the

fluorescent intensity curves (*data not shown*). Direct lysis in nuclease-free water and heat-lysis in Tris-HCl buffer did not improve the signal intensity.

Specificity of the RPA reaction

To assess the specificity of the *Mccp* RPA assay, genomic DNA from a panel of *Mycoplasma* spp. and non-*Mycoplasma* spp. ruminant bacterial pathogens were tested (Table 2). As expected, amplification was only obtained when template DNA from *Mccp* strains was used. All 14 *Mccp* strains tested (Table 2) reacted positively. All of the 72 non-*Mccp* strains as well as the negative controls remained negative throughout the 30-minute analysis time. Primers for *M. mycoides* subsp. *mycoides*, while sensitive, were cross-reactive with *M. bovis* (Figures 4-7).

Sensitivity of the RPA reaction

The detection limit of the RPA reaction was assessed using 10-fold dilutions of genomic DNA extracted from *Mccp* ILRI181. Eight reactions were performed for each DNA dilution. An example of the output is shown in Figure 1A. High DNA concentrations (10^6-10^5 genome copies/reaction) were detected within seven minutes of amplification while 500-50 genome copies/reaction could repeatedly be detected within 15 minutes. The overall assay sensitivity was also assessed by running 10-fold dilutions of the cultured *Mccp* strain ILRI181 diluted in goat plasma. Figure 1B depicts an example of an output. At high bacterial concentrations i.e. 500 CCU/reaction an increase in the fluorescent signals (mV) were detected within 10 minutes of amplification while concentrations of 50 CCU were detected within 15 minutes. Bacterial concentrations of 5 CCU were consistently detected albeit at rather low signal intensities. Mixing

of the reaction tube by quickly vortexing after four or six minutes did not improve the sensitivity or signal intensity (*data not shown*).

RPA performance using clinical specimens derived from a recent Kenyan outbreak

The RPA assay was evaluated using pleural fluid samples (n=5) and lung tissue samples (n=2) collected during a CCPP outbreak in Kenya 2012. All clinical specimens were cultured to determine presence of *Mycoplasma* and/or CCU/mL titers. All but one replicate of the tissue cultures had *Mycoplasma.* Bacterial titers ranging between 10^7 to 10^9 CCU/mL were obtained from pleural fluid samples cultured in duplicates (Table 3). All pleural fluid samples were positive within 10 to 13 minutes after initiating the RPA reaction. One example of a sample curve from a pleural fluid sample is depicted in Figure 2.

RPA performance in field setting

In order to test the storage conditions for RPA reagents for field use, RPA reactions and MgOAc were both lyophilized and stored at 4°C and 37°C for at least 5 days. Prior to reactions, lyophilised MgOAc pellets (2.5 µL) were reconstituted with 5µL template (cultured *Mccp*) and 45µl nuclease-free water, which then was, added to the lyophilised RPA reactions. The results showed that 37°C is not suitable for storing both lyophilized RPA reactions and MgOAc. No signal was obtained using lyophilized RPA reactions stored at 37°C even with fresh MgOAc. Similarly, there was no signal using fresh RPA reactions with lyophilized MgOAc stored at 37°C. However, the lyophilized RPA reactions and MgOAc can be stored at 4°C for at least five days with acceptable compromise of the reaction signals compared with fresh RPA reactions (Figure 3). The reaction sensitivity for lyophilized RPA and MgOAc stored at 4°C was

determined by cultured *Mccp* ILRI181 spiking experiments. A detection limit of 5000 CCU was obtained in triplicate runs, which is about 10-fold less than the fresh RPA reactions. Furthermore, the read-out system (ESEQuant Tube Scanner) was successfully powered using a 120W Laptop Car power supply (L40BB) once connected to the car cigarette lighter.

Discussion

Successful surveillance and control of livestock diseases requires specific, sensitive and rapid diagnosis of pathogens. In developed countries, laboratory-confined tests such as ELISA and real-time PCR are suitable for prompt diagnosis due to an efficient network of diagnostic laboratories, correct storage of specimens/samples, and a courier system that guarantees fast transport of specimens to the laboratory. However, the absence of such systems in many regions of sub-Saharan Africa limits the feasibility of these tests. Furthermore, lack of basic infrastructure, e.g. reliable power and refrigeration, also complicates the development of field-adapted assays. In this study we have developed a novel diagnostic assay based on recombinase polymerase amplification (RPA) and assessed its applicability to rapidly and robustly diagnose contagious caprine pleuropneumonia (CCPP), one of the major diseases affecting small ruminants in developing countries.

The assay was able to detect the pathogen *Mycoplasma capricolum* subsp. *capripneumoniae* (*Mccp*) specifically and sensitively from laboratory samples (purified genomic DNA) and cultured bacteria, as well as from field samples (pleural fluid and lung tissue from a CCPP outbreak). Correct differentiation between *Mccp* and other closely related mycoplasmas and bacterial pathogens causing CCPP-like symptoms is of paramount importance for a diagnostic method.

The specificity of this assay was achieved by engineering the RPA assay to reduce off-target binding and cross-reactivity with other closely related *Mycoplasmas*. No amplification was obtained from a large panel of DNA from closely related mycoplasmas and other bacteria causing infections in ruminants while DNA from the 14 *Mccp* strains were correctly identified (Table 2), thus demonstrating a high degree of specificity. Amplification was detected within a few minutes when using DNA extracted by conventional methods, and DNA concentrations as low as 50 genome copies were repeatedly detected. This demonstrates that RPA can be used for diagnostic detection of *Mccp* in extracted DNA. However, to circumvent the need for nucleic acid recovery we further evaluated simple methods for sample preparation, including direct bacterial lysis with nuclease-free water, heat lysis, and alkaline lysis (0.2M KOH). The two latter alternatives did not result in sufficient recovery from cultured material and pleural fluid as demonstrated by a delayed rise and lower intensity of the fluorescent signal (*data not shown*). Direct bacterial lysis with nuclease-free water, however, was sufficient. Similar sensitivities were thus obtained from cultured bacteria that had been spiked in plasma as compared to extracted DNA (Figure 1B).

CCPP is known for its very high morbidity, high mortality and a great proportion of animals that develop pathomorphologic lesions exhibiting effusions with high concentration of *Mycoplasma*, as in our case 10^7-10^9 CCU/mL. Direct isolation and cultivation of the causative bacteria from infected lung tissues or pleural fluid taken post mortem is still the gold standard for diagnosis of CCPP. Cultivation requires specialized nutrient media, takes several days, and is often unsuccessful due to the fastidious nature of the organism. Currently available molecular diagnostic tools employing PCR either require a well-equipped laboratory or extensive sample preparation, thus limiting its application in the field. Here we have surpassed these limitations by

demonstrating the ability to detect *Mccp* directly from pleural fluid samples collected from infected animals using RPA. With a simple dilution in nuclease-free water, bacteria could be detected within 15 min (Figure 2). Thus, inclusive of sample preparation time, a definite diagnosis of CCPP presence in a herd can be achieved in less than 45 min.

In addition, we tested the field applicability of the method by lyophilizing the RPA reactions and MgOAc and storing them at 4°C and 37°C. The fresh lyophilized RPA reagents had similar sensitivity as the fresh RPA reactions (*data not shown*). However, after storage at 37°C for a few days, the reagents lost activity completely. The lyophilized RPA reagents can be stored at 4°C for at least five days and still maintain a detection limit of 5000 CCU. The pleural fluid from CCPP positive animals has a range of 10^7 to 10^9 CCU/mL; therefore, the lyophilized RPA reactions should be able to detect *Mccp* easily from pleural fluid. Moreover, the device used to quantify the fluorescence from the RPA reaction, the ESEQuant Tube Scanner, is portable and can be powered by a car battery. Thus, this method is suitable for rapid CCPP diagnosis in the field. The RPA reagents can be lyophilized in the lab and transported to the field with ice pack, then the reaction can be performed in a car with a definite result within 45 min. Our results indicate that RPA assays are robust, rapid, specific, sensitive and suitable for application at the POC. However, the use of other specimens such as broncholavage and nasal swabs needs to be evaluated and optimized.

The assay developed here is based on a single-copy target region in *Mccp*. Ruminant *Mycoplasma* have been reported to have minimal genomes that are able to exchange DNA via a conjugation like process driven by integrative conjugative elements (ICE) (23, 24). Therefore, it would be advisable to have multiple target regions, thus increasing the sensitivity of the method. Additionally, the use of multi-copy targets should be considered.

This CCPP assay provides an excellent foundation for the development of a novel POC diagnostic tool because of the low temperature required for RPA technology. The reaction can be transferred to a microfluidics platform powered by a smartphone which provides the required temperature for the assay and reads out florescence, hence the test result. We are currently working on such systems. Future mobile phone-driven diagnostic point-of-care tests will allow the easy implementation of national disease early warning systems via dissemination of test results through mobile phone apps.

Acknowledgments

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Tables

Table 1: Oligonucleotide primers and probe used in this study

Oligonucleotide	Sequence (5' - 3')
Mccp-F	AATCGGTTTATCAAGCCATTCGACATTCTATAAAAT
Mccp_R	GAAAATTAAACTTTGAAAGAAATAGAATTTAGTTT
Mccp_P	CTCTCTTTTATCACTAACAAAATTCAAAAAGA[FAM-dT][THF][BHQ1-dT]CCTTTAAGTCATAAAA[3'-block]

F-forward primer; R-reverse primer; P-probe; FAM-dT-thymidine nucleotide carrying

fluorescein; THF-tetra hydrofuran spacer; BHQ1-dT-thymidine nucleotide carrying Black Hole

Quencher 1, 3'-block

Table 2: Bacterial strains used in this study.

Species	Strain designation	Country of origin	Year of isolation	Host species	RPA result
Mycoplasma capricolum capripneumoniae	ILRI181	Kenya	2012	Goat	pos
Mccp	8789	Chad	1987	Goat	pos
Mccp	9231-Abomaso	Ethiopia	1982	Goat	pos
Mccp	4/2LC	Oman	1988	Goat	pos
Mccp	M79/93	Uganda	1993	Goat	pos
Mccp	Gabes	Tunisia	1980	Goat	pos
Mccp	M85/98	Tanzania	1998	Goat	pos
Mccp	91106/66011	Dubai	1991	Goat	pos
Mccp	95043	Niger	1995	Goat	pos
Mccp	F38	Kenya	1976	Goat	pos
Mccp	87F05	Turkey	2005	Goat	pos
Mccp	GL97P	Tunisia	1980	Goat	pos
Mccp/M.leachii	D1800/04-2	Dubai	2004	Gazelle	pos
Mccp/M.ovipneumoniae	3535	Qatar	2005	Mouflon	pos
M. capricolum capricolum	California kid= R48 [NCTC 10154]	USA	1955	Goat	neg
Mcc	8086-1=039/10	France	1980	Goat	neg
Mcc	6443			Goat	neg
Mcc	1400 0024			Human	neg
Mcc	4146=329/97	France	1997	Goat	neg
Mcc	7714=404/97	France	1967	Goat	neg
Mcc	C47=29/92	Germany	<1992	Sheep	neg

Mmc	153/93=385/94	Canary Islands	1993	Goat	neg
Mmc	7730=377/94	France	1994	Goat	neg
Mmc	7302=374/94	Portugal	<1994	Goat	neg
Mmc	G1255.94=209/94	Germany	1994	Barbary sheep	neg
Mmc	G1313.94=211/94	Germany	1994	Barbary sheep	neg
Mmc	My-325=64/97	Croatia	1986	Goat	neg
Mmc	PG3	Turkey	1950	Goat	neg
Mmc	CapriL=402/97	France	1975	Goat	neg
Mmc	Y-goat=R57	Australia	1956	Goat	neg
Mmc	95010=403/97	France	1995	Goat	neg
Mmc	Wi18079=369/09	Germany	2009	Goat	neg
Mmc	D2482=395/97	Switzerland	1991	Goat	neg
M. mycoides mycoides	L2=008/06	Italy	1993	Cattle	neg
Mmm	Gladysdale	Australia	1953	Cattle	neg
Mmm	Afadé	Cameroon	1968	Cattle	neg
Mmm	B66	Kenya	2000	Cattle	neg
Mmm	C11=010/06	Chad	1962	Cattle	neg
Mmm	Fatick=009/06	Senegal	1968	Cattle	neg
Mmm	Mandigwan=640/08	Namibia	2001	Cattle	neg

Mmm	PO2=007/06	France	1980	Cattle	neg
Mmm	Matapi=639/08	Namibia	2004	Cattle	neg
Mmm	Tan8=637/08	Tanzania	1998	Cattle	neg
Mmm	PG1	unknown	unknown	Cattle	neg
M. leachii	B144P	USA	1956	Cattle	neg
Ml	CP291	Portugal	1987	Goat	neg
Ml	PG50 [NCTC 10133]	Australia	1963	Cattle	neg
Ml	PAD3186	India	1993	Goat	neg
Ml	FRD424=326/93	India	1993	Goat	neg
M. bovis	Donetta PG45	USA	1962	Cattle	neg
Mb	12DD0691	Germany	2012	Cattle	neg
Mb	12DD0690	Germany	2012	Cattle	neg
Mb	11DD0669	Germany	2011	Cattle	neg
Mb	DL018/91	Germany	1991	Cattle	neg
Mb	DL012/92	Germany	1992	Cattle	neg
Mb	DL981/84	Germany	1984	Cattle	neg
Mb	DL097/81	Germany	1981	Calf	neg
Mb	DL778/80	Germany	1980	Cattle	neg
Mb	DL997/79	Germany	1979	Calf	neg
Mb	DL598/78	Germany	1978	Calf	neg

Mb					
Mb	PG45 [NCTC 10131]	USA	1962	Cattle	neg
M. feriruminatoris	G5847	Germany	1993	Alpine Ibex	neg
M. ferriminatoris	8756-C13	USA	< 1987	Rocky Mountain Goat	neg
M. dispar	462/2	Germany	1982	Cattle	neg
M. buteonis	407/97	Israel	2011	Buzzard	neg
M. gallisepticum	PG31	UK	1977	Poultry	neg
M. bovirhinis	PG43 [NCTC 10118]	UK	1967	Cattle	neg
M. bovoculi	M165/69	UK	1972	Cattle	neg
M. ovipneumoniae	Y98	UK	1973	Sheep, Goat	neg
M. calfornicum	St-6 [NCTC 10189]	USA	1981	Cattle	neg
M. bovigenitalium	PG11 [NCTC 10122]	UK	1947	Cattle	neg
M. putefaciens	KS1 [NCTC 10155]	USA	1955	Goat	neg
M. arginini	G230 [NCTC 10129]	USA	1968	Mouse	neg
M. canadense	275C [NCTC 10152]	Canada	1974	Cattle	neg
M. verecundum	107 [NCTC 10145]	UK	1974	Cattle	neg
M. alkalescense	PG51 [NCTC 10135]	Australia	1961	Cattle	neg
M. canis	PG14 [NCTC 10146]	UK	1951	Dog	neg
M. pneumoniae	FH	UK	1974	Human	neg
Acoleplasma axanthum	S743 [NCTC 10138]	UK	1970	Tissue culture	neg
A. laidlawii	PG8 NCTC 10116	UK	1967	unknown	neg
Pastaurella multocida	ATCC 43137	Canada	1962	pig	neg

Table 3: Pleural fluid and tissue samples collected during a recent CCPP outbreak in Kenya (2012).

	Pleural fluid		Lung tissue	
Sample ID	CCU/mL	RPA	Culture	RPA
Goat 092	10^7-10^8	Pos.	Pos.	Pos.
Goat 181	10^9	Pos.	Pos.	Pos.
Goat DOD 7-8/10	10^8-10^9	Pos.	n.d.	n.d.
Goat F	10^7-10^8	Pos.	n.d.	n.d.
Goat Y	10^9	Pos.	n.d.	n.d.

n.d.- not determined; Pos.- positive; CCU/mL - colony forming units per milliliter; RPA- recombinase polymerase amplification

Table 4: Results of the bioinformatics analysis to test the specificity of the RPA primers and probe against 14 bacterial genomes of ruminant pathogens.

up to 1 mismatch	F-primer	R-primer	Probe
	5'AATCGGTTTATCAAGCCATTCGACATTCTATAAAAT-3'	5'GAAAATTAAACTTTGAAAGAAATAGAATTTAGTTT-3'	5'CTCTCTTTTATCACTAACAAAATTCAAAAAGATATCCTTTAAGTCATAAAA-3'
Mccp **ILRI181**	745351 745386	745561 745595	745404 745454
Mccp **F38**	745144 745179	745354 745388	745197 745247
Mcc **ATCC 27343**	no match	no match	no match
Mmm **PG1**	no match	no match	no match
Mmc **GM12**	no match	no match	no match
M.bovis **PG45**	no match	no match	no match
A.laidlawii **PG8A**	no match	no match	no match
P.multocida **PM70**	no match	no match	no match

M.haemolytica S185	no match	no match	no match
M.leachii PG50	no match	no match	no match
M.californicum ST6	no match	no match	no match
M.putrefaciens KS1	no match	no match	no match
M.bovigenitalium 51080	no match	no match	no match
M.arginini 7264	no match	no match	no match
M.alkalescens 14918	no match	no match	no match
M.canis UF31	no match	no match	no match

up to 5 mismatches	F-primer	R-primer	Probe
	5'AATCGGTTTATCAAGCCATTCGACATTCTATAAAAT-3'	5'GAAAATTAAACTTTGAAAGAAATAGAATTTAGTTT-3'	5'CTCTCTTTTATCACTAACAAAATTCAAAAAGATATCCTTTAAGTCATAAAA-3'
Mccp ILRI181	745351 745386	745561 745595	745404 745454
Mccp F38	745144 745179	745354 745388	745197 745247
Mcc ATCC 27343	no match	no match	no match
Mmm PG1	no match	no match	no match
Mmc GM12	no match	no match	no match
M.bovis PG45	no match	no match	no match
A.laidlawii PG8A	no match	no match	no match
P.multocida PM70	no match	no match	no match
M.haemolytica S185	no match	no match	no match
M.leachii PG50	no match	no match	no match
M.californicum ST6	no match	no match	no match
M.putrefaciens KS1	no match	no match	no match
M.bovigenitalium 51080	no match	no match	no match
M.arginini 7264	no match	no match	no match

M.alkalescens 14918	no match	no match	no match
M.canis UF31	no match	no match	no match

up to 10 mismatches	F-primer	R-primer	Probe
	5'AATCGGTTTA TCAAGCCATTC GACATTCTATA AAAT-3'	5'GAAAATTAA ACTTTGAAAGA AATAGAATTTA GTTT-3'	5'CTCTCTTTTA TCACTAACAAA ATTCAAAAAG ATATCCTTTAA GTCATAAAA-3'
Mccp ILRI181	745351 745386	many matches	745404 745454
Mccp F38	745144 745179	many matches	745197 745247
Mcc ATCC 27343	no match	many matches	no match
Mmm PG1	no match	many matches	no match
Mmc GM12	no match	many matches	no match
M.bovis PG45	no match	many matches	no match
A.laidlawii PG8A	no match	many matches	no match
P.multocida PM70	1125582 1125617	no match	no match
M.haemolytica S185	no match	1224217 1224251	no match
M.leachii PG50	no match	many matches	no match
M.californicum ST6	no match	342412 342446	no match
M.putrefaciens KS1	no match	many matches	no match
M.bovigenitalium 51080	no match	many matches	no match
M.arginini 7264	no match	58971 59005	no match
M.alkalescens 14918	no match	many matches	no match
M.canis UF31	no match	many matches	no match

Figures

Figure 1: Graph depicting the RPA amplification (development of fluorescence, mV) over time (minutes). A) *Mccp* DNA ($5x10^6$-0 copy/reaction) diluted in nuclease-free water. B) *Mccp* CFU ($5x10^5$-0 CCU/reaction) spiked in plasma from a healthy goat.
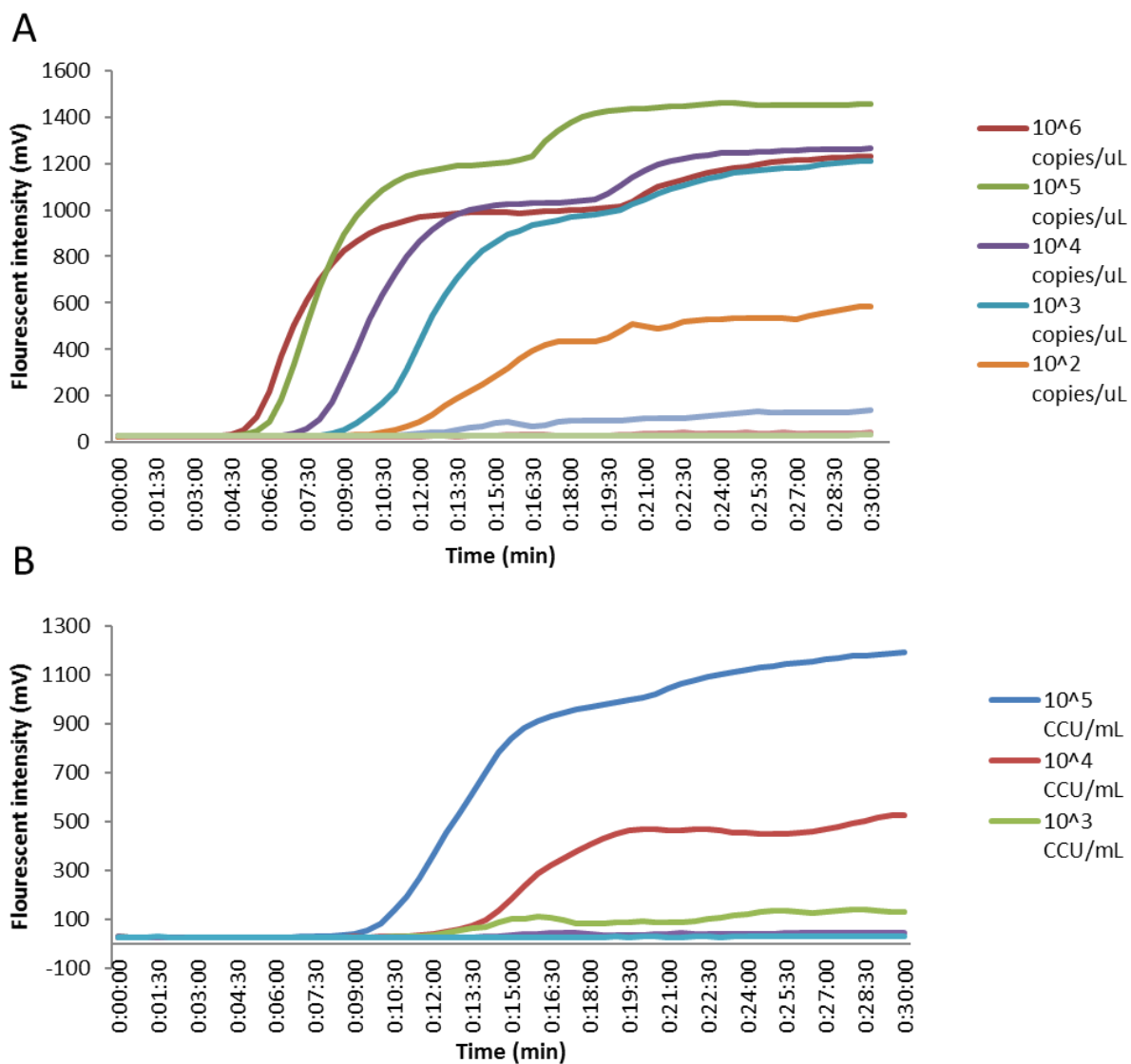
Figure 1.

Figure 2: Graph depicting the amplification from pleural fluid samples from a CCPP infected animals (Goat 181) in duplicate. Negative control; nuclease-free water.
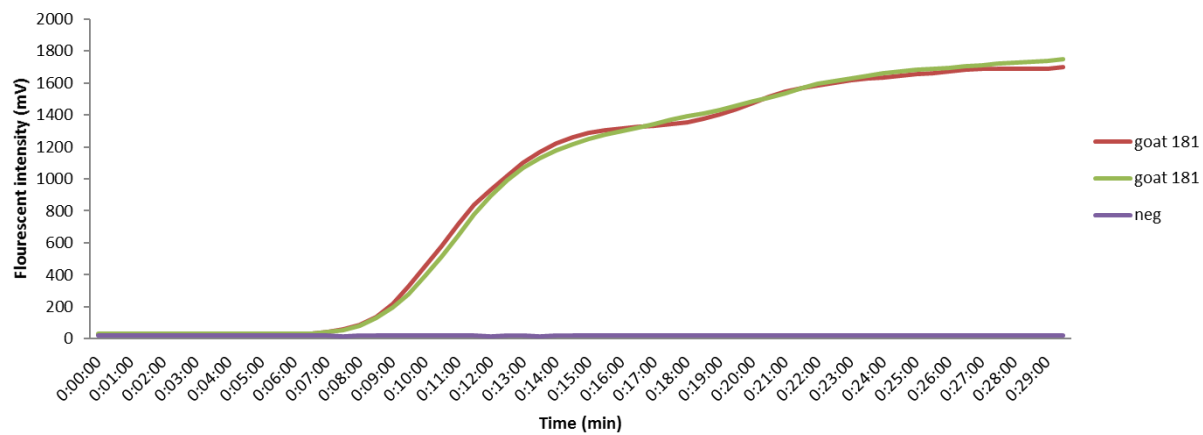
Figure 2.

Figure 3: Graph depicting the amplification from cultured bacteria (10^5-10^3 CCU/reaction in plasma) using fresh lyophilized and stored (4°C) lyophilized reaction pellets. Negative control; plasma from a healthy goat.
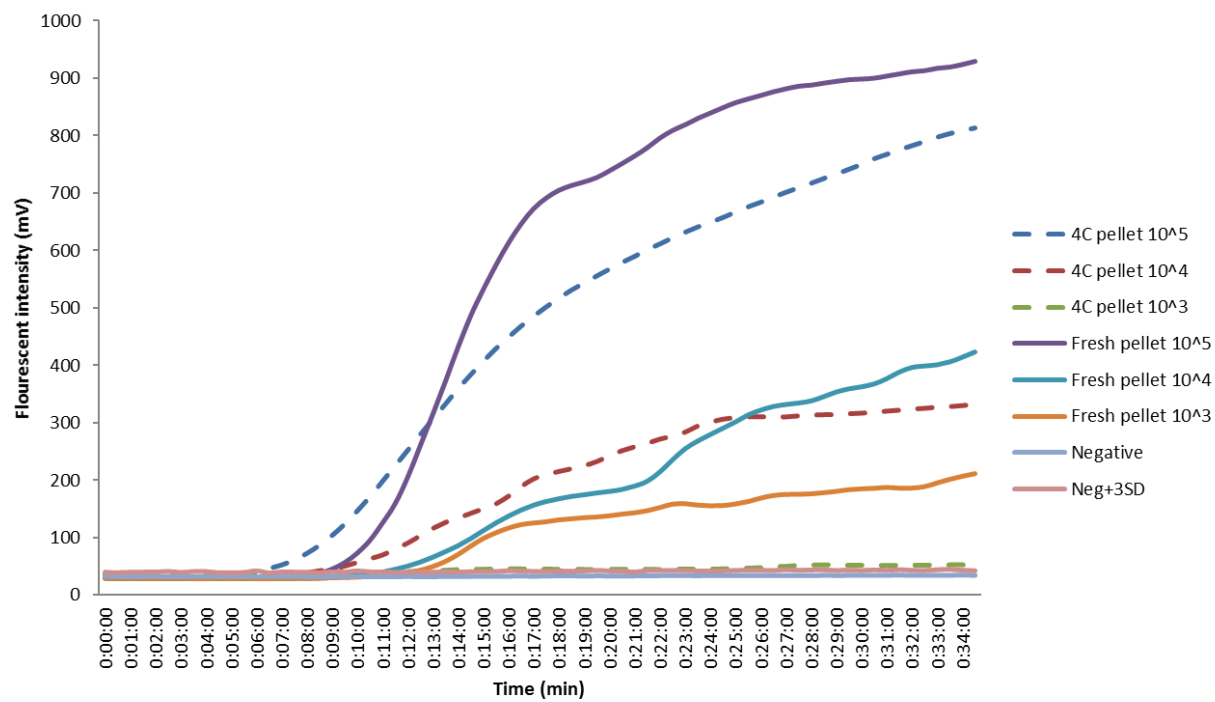
Figure 3.

Figure 4: *Mmm* primers and probes distinguish between *Mmm*, *Mmc*, and *Mcc*. RPA was performed using purified DNA from *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*), *M. mycoides* subsp. *capri* (*Mmc*), and *M. capricolum* subsp. *capricolum*. [DNA] = 10 ng/μL.
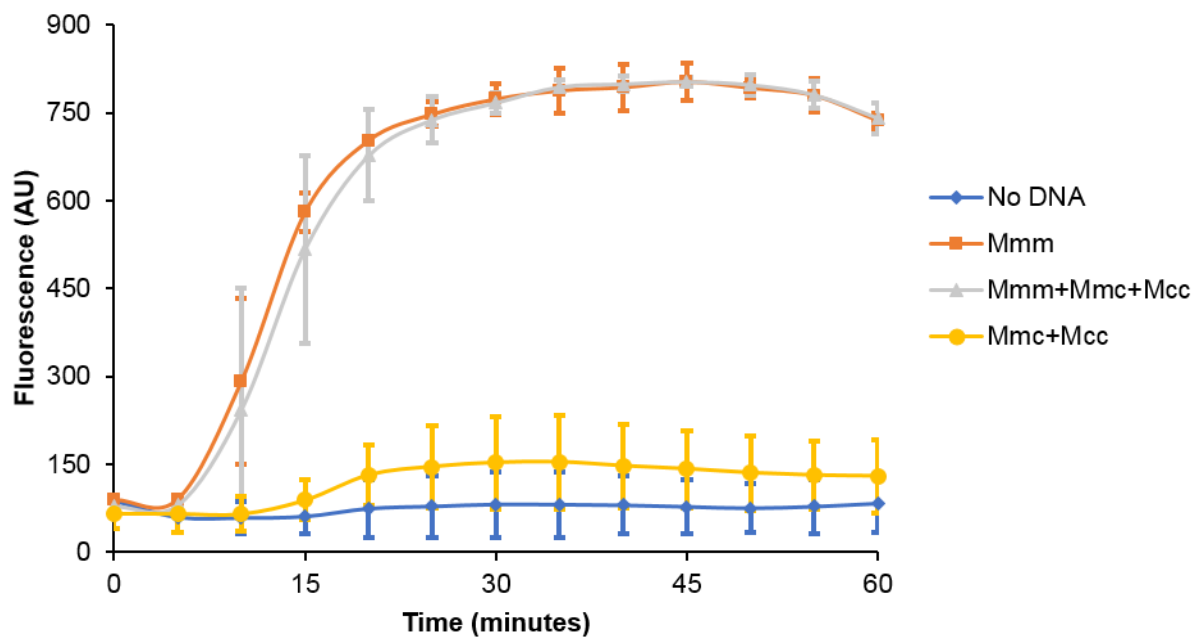
Figure 4.

Figure 5: RPA has a low detection limit for *Mmm*. RPA was performed using purified *Mmm*

DNA (0.02 pg *Mmm* genomic DNA is ~10 copies).
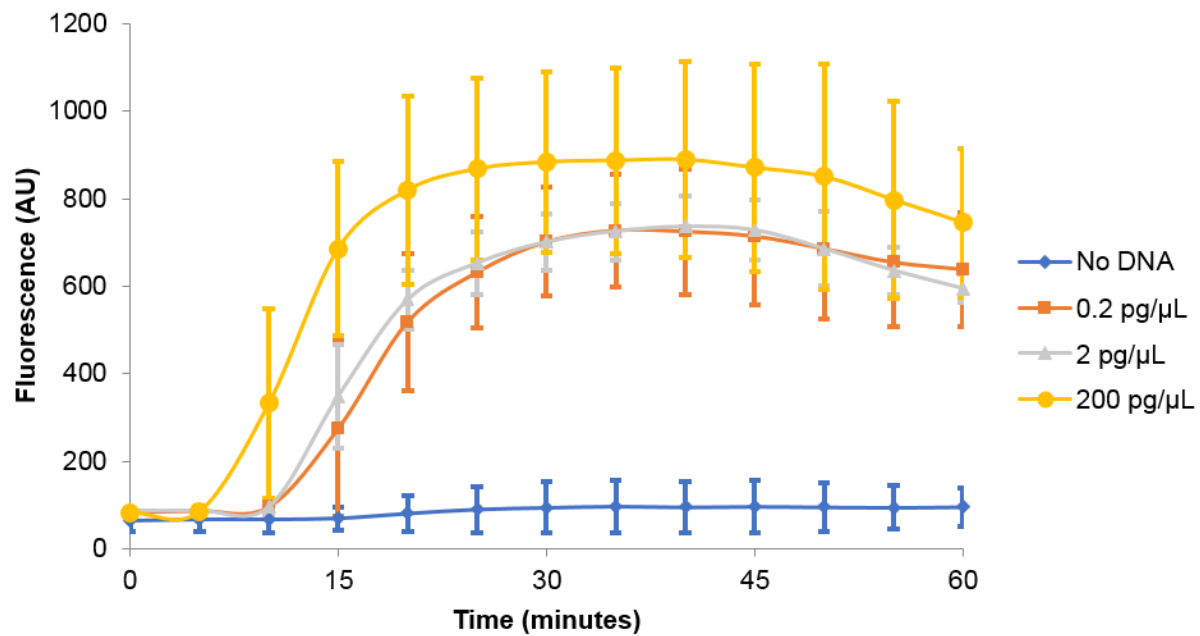
Figure 5.

Figure 6: Bovine serum does not inhibit RPA. *Mmm* genomic DNA was spiked into water,

PPLO growth medium, or bovine serum to a final concentration of 10 ng/μL and fluorescence

after 1 h.
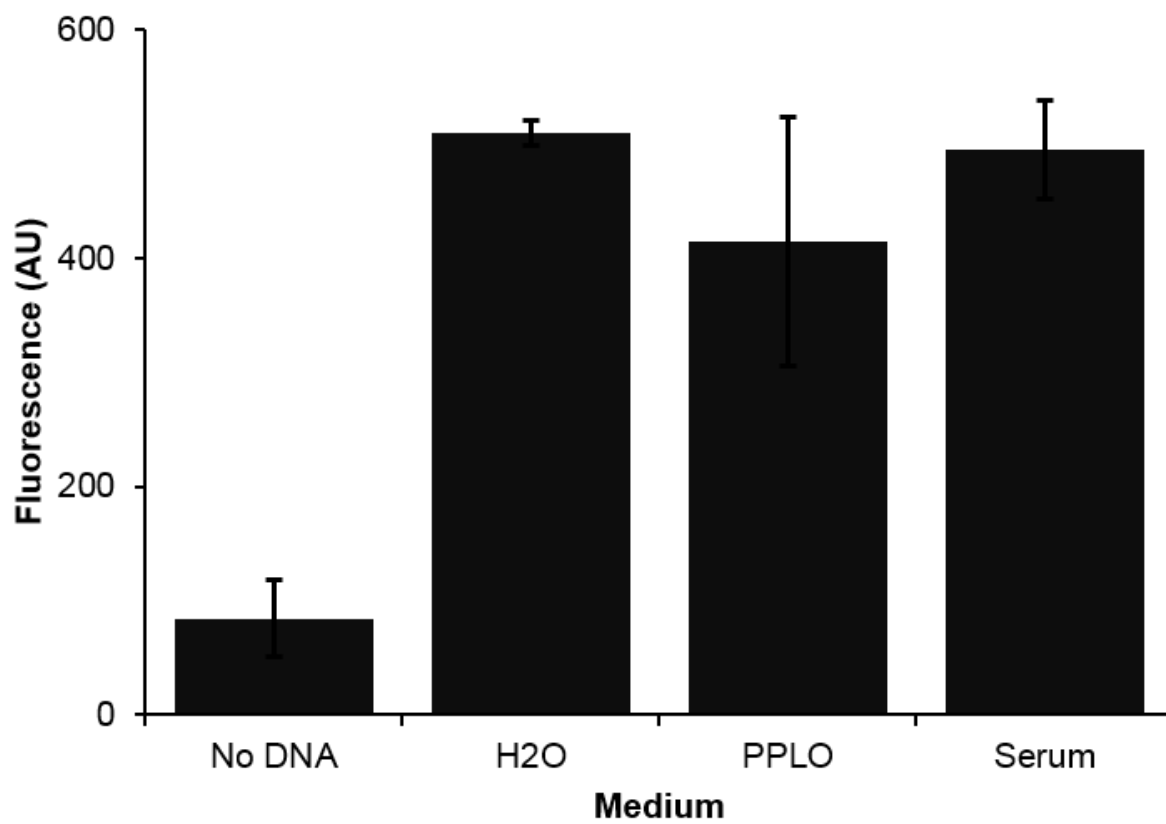
Figure 6.

Figure 7: RPA detects unpurified DNA from lysed cells. RPA was performed using *Mmm*

cells lysed by heating at 95°C and diluted in bovine serum. Final reaction volume was 25 μL.

Fluorescence was measured after 1 h.
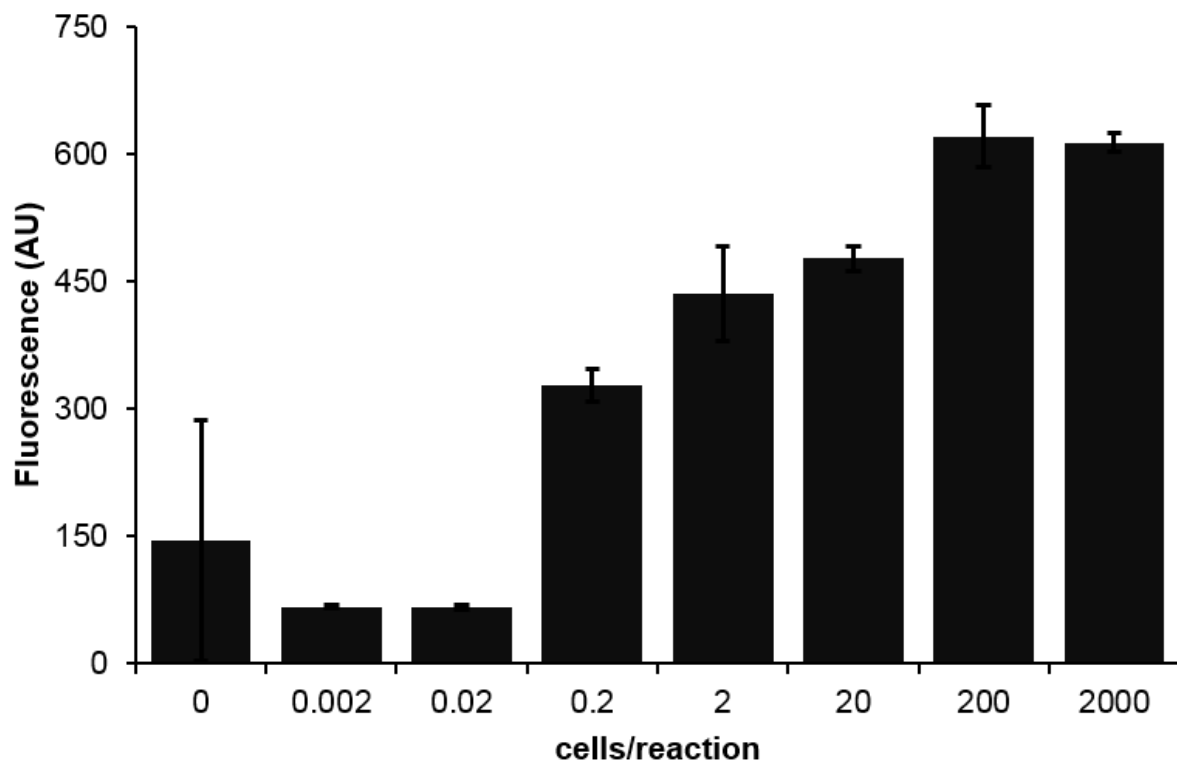
Figure 7.

CHAPTER 4

Adapting microfluidic studies of microbial behavior for educational use

Adapted from

Stilwell MD*, Nepper JF*, Clawson ED, Blair V, Tangen T, & Weibel DB. 2017. "Exploring predatory nematode chemotaxis using low-cost and easy-to-use microfluidics." *Am Biol Teach* **79**(9):753-762. (*denotes equal contribution)

and

Clawson ED, Blair V, Nepper JF, Stilwell MD, Tangen T, Weibel DB (2017). "Laboratory activity using accessible microfluidics to study nematode behavior in an electrical field." *J Microbiol Biol Ed*, (*in review*).

For the first paper, JFN and MDS designed and conducted experiments, analyzed data, tested experiments with students, and wrote the manuscript. For the second paper, JFN and MDS tested the experiments with students and wrote the manuscript.

Abstract

Symbiosis is a fascinating phenomenon that takes many forms. It is important to study symbiosis

when attempting to understand ecology, as it helps us understand relationships between

organisms and provides insight into co-evolution, mutualism, adaptation, and survival.

Ecological studies are challenging to implement in K-12 classrooms because they often require

multiple organisms and complex environments. It is difficult to accurately replicate the many

variables (e.g., soil composition, temperature, pH, and humidity) of an ecosystem, and the

organisms themselves are often very different in size. These factors can make it difficult to study

and quantify ecosystem dynamics in a particular ecological niche. We developed two

inexpensive, quantitative experiments for classrooms that highlight important aspects of

microbial symbiosis, pathogenesis, and ecology, and fill the experimental/demonstration void in

this area of education. These experiments are low-cost, designed for K-12 teachers, use common

materials found in classrooms, and teach students about the exciting relationship between

bacteria, worms, and insects.

Introduction

Symbiosis—the interaction between different species—is found in every ecosystem studied to date (1). To fully understand an ecosystem, one must look at all of the interacting partners to see how the actions of one species affect another. Most well-known examples of these interactions—e.g., sea anemones and clown fish (2, 3), or oxpecker and rhinoceros (4)—are difficult to bring into classrooms. Microbes, on the other hand, are usually inexpensive and easy to acquire and grow. However, the small size of microbes (typically 1-50 microns) can make them difficult to study in a classroom, especially when microscopes are not available. Nematodes are large enough to see with the naked eye (~1 mm) and are inexpensive and easy to grow. They also are involved in several interesting interactions with other organisms, making them ideal for studying symbiosis in the classroom.

Nematodes have adapted to almost every ecological niche on the planet, and are so abundant that they represent four out of five animals on the planet (5). Nematodes form mutually beneficial relationships with bacteria to acquire nutrients. While numerous species are animal or plant parasites, many nematodes and their mutualistic bacterial symbionts are useful for pest control in agriculture, making them excellent model organisms for academic studies of symbiosis (6, 7). For example, the soil-dwelling, entomopathogenic (insect-eating) nematode *Steinernema feltiae* forms a symbiotic relationship with the bacterium *Xenorhabdus bovienii*, which helps it kill and digest its insect prey (see Box 1) (8).

Steinernema nematodes respond to environmental cues, such as volatile organic compounds and carbon dioxide, which makes it possible for them to find prey (9, 10). We use microfluidic devices to study the chemotaxis and electrotaxis—movement in response to chemical signals or an electrical field, respectively—of *Steinernema feltiae* nematodes. *S. feltiae*

navigates through soil towards its prey by responding to chemical signals, or chemoeffectors, released by their insect prey, such as α-pinene and furan (Fig. 1)(11).

Different species of entomopathogenic nematodes respond differently to electrical fields, with some moving towards the cathode, some moving towards the anode, and others displaying no net response (12, 13). It has been previously shown that *S. feltiae* shows a preference for the cathode (13). The techniques presented in this paper demonstrate how students can use simple and reusable microfluidic devices to study nematode behavior in electrical fields. As the reason for the movement of nematodes in electric fields is not currently known, this activity provides an opportunity for students to propose hypotheses and potentially test them in subsequent experiments.

It is easiest to study nematodes when their movement is confined. We use small microfluidic channels to accomplish this. These channels are shallow enough that their motion is essentially limited to two dimensions, making them easier to observe (14). Microfluidics experiments use very small volumes of fluids (as little as 10^{-18} liters) in channels that have a width or height that has dimensions of micrometers (1 micrometer is one millionth of a meter) (15). Scientists and engineers use microfluidic technologies to reduce the size, cost, and materials needed for experiments ranging from chemical synthesis to DNA sequencing (16-18). The constraint imposed by microfluidic channels creates a phenomenon referred to as laminar flow (see goo.gl/BxIHNf for a demonstration of this property). Fluids exhibiting laminar flow move in 'sheets' and mix only by diffusion, which differs from the turbulent flow that is often observed in mixing fluids on a larger scale (Fig. 2) (19). Laminar flow can be used to manipulate fluids very precisely (15).

Microfluidics is a powerful method to study nematode behavior, but traditional methods for creating microfluidic channels are often very expensive and require resources that are not commonly available (20). We use a technique that requires only inexpensive office supplies (i.e., double-sided tape and transparency sheets) and a craft cutter (21). Using these microfluidic channels, we demonstrate an easy way to measure the movement of nematodes in response to various chemical and electrical signals. Using this approach, students can. This gives them the tools to answer fundamental ecological questions about nematode behavior; in this case, how the nematode *S. feltiae* identifies and hunts insect prey.

In creating this approach, we focused on materials and techniques to quickly create and test microfluidic device designs. We created microfluidic devices by sandwiching double-sided adhesive craft paper containing patterns of channels between two flat sheets of plastic transparency. Using open source vector graphic design software, we created layouts of microfluidic channels for different experiments and cut them into the adhesive craft paper using a desktop craft cutter. Cutting and assembling a single device takes ~5 min. Students tested the microfluidic devices in outreach activities to ask whether nematodes swim toward or away from different stimuli—including those produced by insects. We successfully cleaned and reused these microfluidic devices multiple times. This approach creates opportunities for K-12 teachers to use microfluidic devices in a wide range of classroom investigations, including: physics (e.g., studies of fluid flows at low Reynold's numbers), chemistry (e.g., chemical gradients), and biology (e.g., biological behavior of microorganisms) (22) . Using the devices to study nematode behavior is an excellent way to explore key biological concepts, such as: biological systems and models and making predictions about organism behavior (NGSS 4).

Materials and Methods

Nematode preparation from sand

Place a piece of cheesecloth (2-4 layers) over a shallow dish. Place a square of delicate task wipe over the cheesecloth (most paper towels are too thick for this, though a strong facial tissue could be used if delicate task wipes are not available). Center the wipe over the dish (Fig. 3A). Scoop out ~1 cm^3 of the nematode-containing sand and place into the center of the wipe (Fig. 3B). Add water to the sand until all of the sand is dissolved (Fig. 3C). Let sit for 10-15 min, making sure sand does not dry out. Carefully gather the corners and edges of the wipe and cheesecloth and gently squeeze all the water out of the sand (Fig. 3D). Tilt the dish up so the water sits at one end of the dish (Fig. 3E). Let sit for 10-15 min. The nematodes will swim down to the bottom of the dish.

Concentrate the nematodes by centrifuging 900 μL taken from the bottom of the dish above for about 10 sec. in a microcentrifuge. If a microcentrifuge is not available, the nematodes will migrate down to the bottom of the tube if left sitting for 20-30 min. Remove 600 μL of the supernatant and resuspend the nematodes in the remaining 300 μL.

Nematode preparation from sponge

Place the nematode-soaked sponge in room temperature tap water in a shallow container (Fig. 3F). Allow nematodes to swim out of the sponge without squeezing the sponge. Squeezing the sponge may release the sand they are packaged with, which can clog the microfluidic channels.

Chemotaxis device assembly

Assemble the microfluidic channels as described in (21). Cut the channels out of the double-sided adhesive, as well as the top and bottom out of the transparency sheet. Remove the paper backing from the double-sided adhesive and attach to the smaller transparency. Ensure holes are cut out of the transparency either by the craft cutter or manually using the 3 mm hole punch. Remove the paper backing from the other side of the adhesive and adhere to the larger transparency (Fig. 4B). Push all layers together from end-to-end to avoid creases.

To create the chemoeffector well and syringe adapter, cut two 0.5-0.75 cm pieces of tubing (we use Tygon PVC tubing, SAE, 3/16"; inside diameter, ¼" outside diameter, 1/32" wall). Hot glue one piece of tubing to one inlet to create a well to hold the chemoeffector (Fig. 4C). Attach the other piece of tubing to the tip of the syringe to make pulling the solutions into the device easier. Use caution with the hot glue to avoid burns. Avoid sealing the access holes to the microfluidic device.

Chemoeffector preparation

To prepare the attractant (waxworm extract), use the mortar and pestle to grind five (5) frozen waxworms (freeze waxworms while they are fresh). Suspend the waxworm mash in 3 mL of 70% isopropanol. Prepare a twenty-fold dilution from this solution using water. Add food coloring to visualize the liquid. (Note: if sacrificing insects is not acceptable for the experiment, chemically pure chemoeffectors can be purchased from scientific supply companies.)

To prepare the repellant (acetic acid), make a solution of 5% acetic acid in water (or use white vinegar, which is ~5% acetic acid in water) and add a second color of food coloring to visualize the liquid.

To prepare the control effector, add a third color of food coloring to visualize the liquid. Adding different colors to the solutions will make it easier to see the laminar flow profile and clarify that the device is working as expected.

Chemotaxis device loading

Melt a 2% agar solution and keep warm in a water bath (approximately 55 °C). For each group of students, prepare three microfluidic channels, one for attractant, repellant, and control, as outlined above and in (21). Provide each group of students with assembled microfluidic channels (or have them assemble their own), along with the 1 mL syringe connected to the extra piece of tubing and some sort of pipet. One student loads a chemoeffector solution into the inlet tubing well (as in Fig. 5b). Meanwhile, a second student mixes nematode suspension with the 2% agar solution in a 3:1 ratio, so that the final agar concentration is 0.5% (e.g. 0.5 mL of the nematode suspension with 1.5 mL of the 2% agar solution). Immediately after mixing, the second student pipets the nematode-agar mixture onto the other inlet hole (as in Fig. 5c). As soon as the nematode solution covers the inlet hole, the first student quickly and gently draws the two solutions into the channel using the tubing-tipped syringe placed at the outlet hole (as in Fig. 5d). Note that the agar solution cools quickly, so the nematode loading step must be performed quickly.

Chemotaxis experiment

Image the channels using a microscope. Record the number of nematodes in each side of the device over time. To quantify the nematode response to the chemoeffector, use the following equation to calculate the "chemotaxis index" (CI):

$$CI = \frac{\#\ nematodes\ on\ chemoeffector\ side}{total\ \#\ of\ nematodes\ in\ device}$$

A chemotaxis index of 1 indicates that the chemical was a strong chemoattractant and a value of 0 indicates a strong chemorepellant.

Electrotaxis device assembly and loading

Download Nematode Electrotaxis Device (see Supplementary Materials), or design your own. Make and assemble device as described above (Fig. 6A-B). Prepare a 2% agar solution with 0.01% sodium chloride. The salt increases the electrical conductivity of the agar. Aliquot into smaller volumes for ease of use (Fig. 6C). Keep the agar in a liquid state at 50°C in a heat block or water bath. We were able to keep the agar sufficiently melted in hot tap water (Fig. 6D). This temperature is low enough to not cause nematode distress, but high enough to keep the agar melted.

Make electrode leads out of the titanium wire by bending a 5 cm piece of wire in half, and twisting to create a very small loop at one end that will fit into the inlet/outlet holes of the device. Twist all the way to the end of the wire, then bend the free end over to create another, larger loop to use as an attachment for the alligator clips. Secure the ends by twisting them down tightly (Fig. 7A).

Immediately prior to loading the devices, mix 3 parts nematode preparation to 1 part agar. We used 300 μL nematodes to 100 μL agar. Mix quickly and thoroughly by pipetting. Promptly load the microfluidic device by placing a drop of the nematode/agar mixture on the inlet hole of the device (Fig. 7B). Before the agar cools and solidifies, use the syringe to pull the nematodes into the device. Take care not to pull all the nematodes out the other end. (Fig. 7C)

After loading the devices, carefully insert the small, single-wire width loop of the titanium electrode leads into each of the inlet/outlet holes in the device, such that part of the small loop is partially out of the hole (Fig. 7D). Use a small piece of masking tape to tape the leads to the device (Fig. 7E).

Electrotaxis experiment

Students prepare channels, load them with nematodes suspended in a solution of agar, place them under a microscope, and tape the device to the microscope stage to keep it in place (Fig. 7F). Students count the number of nematodes in each zone in the channels at time=0 (Fig. 8); students should choose how to count nematodes that may be positioned on the lines between zones, deciding to define them as either belonging to the zone to the right or to the left of the line, and using this counting approach consistently throughout experiments. Next, students attach the titanium electrodes to a 9V battery using alligator clips (Fig. 9). Be sure to attach the right lead to the negative terminal, and the left lead to the positive terminal. After connecting the battery, students immediately start a timer and count and record the number of nematodes in each zone every min for a total of 5-10 minutes.

Notes:

1. We measured the voltage across the channels to be 1.7-4.0 V with electrodes connected to a 9 V battery and channels filled with agar as described in Appendix 1.

2. Bubbles which may accumulate at the end of the channel connected to the negative terminal of the battery may disrupt the electric field and alter the directional movement of nematodes.

Cleanup and disposal

Microfluidic devices and syringes can be easily cleaned and reused. To do so, flush the channel with hot water by pressing a water stream up to one of the inlet/outlet holes or soak the device in hot water, loosen and extract the agar with the syringe, and then flush with plain water. Syringes should also be flushed with hot water to remove any agar. Note that the agar/nematode mixture cannot be re-melted once it has solidified without killing the nematodes. The 2% agar solution with 0.01% sodium chloride can be saved and reheated. We recommend only mixing small amounts of agar and nematodes as needed. The nematodes used here are naturally occurring, soil-dwelling nematodes and are not harmful to humans. They can be disposed of down the drain or in regular trash.

Results and Discussion

Microscopic organisms move differently from larger ones (23). *Steinernema* nematodes move by pushing their body against solid objects, which makes it difficult to observe nematodes swimming in water (24). We filled channels with an agar solution, which produces a transparent gel in which the worms can swim (25). To form the gel, the agar solution must be boiled before allowing it to cool. We found the nematodes could survive brief exposure to the high temperature of the solution of melted agar, and after the gel set the nematodes could easily swim through the gel. The agar solution cools and solidifies rapidly, so filling the channels may require a level of coordination that is too challenging for elementary school students.

For chemotaxis studies, we designed a microfluidic system consisting of two straight inlet channels (with inlet holes) intersecting with a single central channel and ending in an outlet hole (the channel system looked like the letter 'Y') (Fig. 4). We used three layers of material to form the channels: 1) a layer of double-sided adhesive tape with the channels cut into it; 2) a top

layer of transparency forming the channel 'ceiling' with the shape and dimensions of the adhesive tape (this layer contained the inlet and outlet holes); and 3) a bottom layer of transparency forming the channel 'floor,' with the shape of the adhesive tape but slightly longer to provide a handle. We used a craft cutter to cut the channels out of the double-sided adhesive and to cut the top and bottom layers out of transparency sheets. We assembled the system by pressing a transparency layer on each side of the adhesive, then hot gluing a small piece of Tygon tubing to one inlet (Fig. 4). A microscope slide can be used instead of transparency for the bottom layer of the device if it makes visualization easier. For a very detailed description of the process for making the microfluidic system, a step-by-step video can be found at https://youtu.be/BDFWlELvzJo.

The tubing acted as a reservoir for the chemoeffector, to ensure the channels would not become filled with air. We filled the tubing reservoir with our chemoeffector of interest while preparing for subsequent steps (note: if the tubing prevents the device from fitting onto a microscope, after filling the channels, the tubing can be removed by pulling it off or using scissors). After soaking the sponge containing nematodes in water, we mixed the nematode solution with the warm agar solution, pipetted it on the other inlet hole (without tubing), and drew the two solutions through the device using a syringe positioned at the outlet (Fig. 5). To create a better seal, we put a small piece of tubing on the tip of the syringe as well. After a few minutes, the device was moved to the microscope to visualize nematodes.

Our microfluidic chemotaxis system differs from those reported previously in a seemingly small but important way: we do not load channels entirely with a solution of agar. Instead, we dissolve chemoeffectors in water (instead of in an agar solution) and use laminar flow to fill one half of the main channel with chemoeffector and one half with agar and

nematodes. After the agar sets, half of the channel contains the agar gel and the other half of the

channel contains the chemoeffector solution. There are two primary advantages to this approach.

First, the channels are much easier for students to load when agar is added to only one of the two

inlets, as it cools and solidifies rapidly. We found this task to be difficult, so younger students

would certainly have trouble correctly loading the channels. Our second reason for loading

chemoeffectors in water is that the nematodes swim well in the agar but not in the liquid, so

chemotaxing nematodes are essentially trapped if they move to the chemoeffector side. This

makes counting the worms easier. We have not observed any nematodes swimming into the

chemoeffector area (water side) when a repellant is present, so we believe false positives are

unlikely.

To test our system, we compared the nematodes' responses to an attractant (waxworm

extract), a repellant (acetic acid: i.e., vinegar), and water (a control). In each experiment, we

observed 4-12 nematodes in the main channel (note that the amount of water used to soak the

nematode sponge can be adjusted to increase or decrease the number of worms suspended in

liquid in each channel). To quantify the nematode response to each chemical stimulus, we

calculated a "chemotaxis index" (CI) using the following equation:

$$CI = \frac{\#\ nematodes\ on\ chemoeffector\ side}{total\ \#\ of\ nematodes\ in\ device}$$

According to this equation, a perfect repellant will create a CI of 0 and a perfect attractant

will yield a CI of 1. After performing the experiment in triplicate, we found that the waxworm

extract yielded a CI of ~0.71, the vinegar yielded a CI of ~0.03, and the water yielded a CI of

~0.14 (see Table 1 for individual experimental values, or Fig. 10 for a photo of the channels and

quantification of the nematode response). We found that adding food coloring to the

chemoeffector solution greatly helped us visualize the boundary between the agar and the

chemoeffector, and made it easier to see that both solutions were loaded into the channel. We also saw that the laminar flow profile in channels containing both the warm agar solution and the waxworm extract was not always evenly distributed, most likely due to the evaporation of the isopropanol in the waxworm extract. An occasional 'wavy' boundary between the two solutions will not affect the outcome of the experiment.

We tested the chemotaxis experiment with 76 middle school students lacking formal biology training. The electrotaxis experiment was tested with 40 middle school students. The students selected these workshops from a number of other field trip options. To assess the students' enjoyment and engagement in the activity, we asked them to complete a short survey after the workshops. Our survey was based on the learning activation surveys developed by Activation Lab (activationlab.org). Because no identifying information was collected from the students and all responses were aggregated, an Institutional Review Board deemed these evaluative measures to be exempt from IRB approval.

For brevity, following the chemotaxis experiment we aggregated responses from several questions addressing a similar concept (collaborative practices of scientists) into a single measure. (Fig. 11a). When asked in anonymous surveys what they enjoyed most about the chemotaxis activity, more than half the students said "everything" or "we learned by doing things instead of listening the whole time", while the rest "enjoyed being able to see the ways the nematodes reacted." We found this activity to be a rich platform for collaboration. We observed students talking about what they were doing among themselves and working together to do the activity successfully, in much the same way that academic scientists collaborate in the research lab. Additionally, over 90% of the students reported that they asked questions, tried out new ideas, and discussed the experiment with their mentors and peers to aid their comprehension

(Fig. 11a). 80-95% of the students surveyed seemed to be actively engaged and reported that they had learned something about science (Fig. 11b). Similarly, students were highly engaged in the electrotaxis experiment (Fig. 12). We found these activities to be appropriate for students as young as 6th grade and can be adapted for students in high school and college by exploring the key concepts more deeply.

Conclusions

Using simple, inexpensive, easy-to-make microfluidic channels, we demonstrate how students can identify and study signals that alter the behavior of predatory nematodes, such as the chemicals that nematodes use in nature to track their prey and avoid danger. An important aspect of these activities is the engagement of students in the design of authentic scientific investigations. We often think of ecology on the scale of large animals and ecosystems, however our activities reinforce the importance of ecosystems of small animals and bacteria and enable teachers to investigate a complex and important ecological web using tools that are widely accessible and quantitative.

These activities can be expanded to fit within existing biology and environmental science curricula, as different aspects of symbiosis and ecology are reflected in the experiments: e.g., the mutualistic relationship between the worms and their symbiotic bacteria, as well as the pathogenic relationship between the worms and their insect prey. These concepts could fit in a curriculum studying bacteria, pathogens, chemical ecology, soil science, and insects. This activity can be modified to use different chemicals, temperatures, microfluidic channel shapes/designs, or species of nematode, and enables students to make predictions about the response of nematodes to those conditions.

Microfluidics makes possible the investigations described here because it imposes dimensions on liquids that bring out the unique laminar behavior of fluids. Traditional methods for making microfluidic channels are tricky to incorporate into school activities because they require materials and facilities that are expensive and difficult to access. Our method uses inexpensive office materials, a craft cutter to pattern the double-sided sticky tape and transparency sheets, and a simple method of assembly and introducing fluids. The ease of this method enables students to create and test new channel designs, and makes it possible to incorporate perspectives on engineering into lessons.

Educators will be able to incorporate the Educators Evaluating the Quality of Instructional Products (EQuIP) Rubric to measure the alignment to the Next Generation Science Standards (NGSS) to help determine which elements from these investigations connect to the science and engineering practices (SEP), disciplinary core ideas (DCI), and/or crosscutting concepts (CCC) of NGSS. The embedded tasks expected of students in these activities may demonstrate their proficiency of one or more performance expectations. For example, students develop and use SEPS, DCIs, and CCCs that fit within the EQuIP three dimensions framework. Teachers will need to identify the key CCCs relevant to their instructional framework so that students can translate specific information to general principles.

Looking deeper into the EQuIP (v3.0) rubric are intentional opportunities for students to explain phenomena and design solutions that are integrated into life science DCIs (MS-LS2-1,2,4 & HS-LS2-8) & and SEPs (MS/HS-ETS1). In particular the evidence statements reflected in NGSS HS-LS2-8 connect very well to the potential for students to develop a causal explanation on the group behavior dynamics of chemotaxing nematodes in microfluidic channels. (http://www.nextgenscience.org/sites/default/files/evidence_statement/black_white/HS-LS2-

8%20Evidence%20Statements%20June%202015%20asterisks.pdf) Students will use science and engineering design practices in these activities and other potential investigations of ecosystems and inter-organism interactions.

Acknowledgements

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Tables

Table 1: Nematode chemotaxis results using the microfluidic channels.

	Number of nematodes in agar side	Number of nematodes in chemical side	Chemotaxis Index
Waxworm Extract	3	1	0.25
	1	8	0.889
	0	4	1
Vinegar (5% acetic acid)	10	0	0
	10	1	0.091
	7	0	0
Water	9	2	0.182
	6	0	0
	10	3	0.231

Figures

Figure 1: Nematodes respond to chemical signals released by prey insects. Each prey insect produces and secretes organic compounds that nematodes chemotax towards and use to hunt the insect. Wavy lines indicate production of volatile compounds. Compounds shown are (from top left, clockwise): α-pinene (waxworm), dimethylsulfone (house crickets), furan (earwig), and acetone (pillbug).
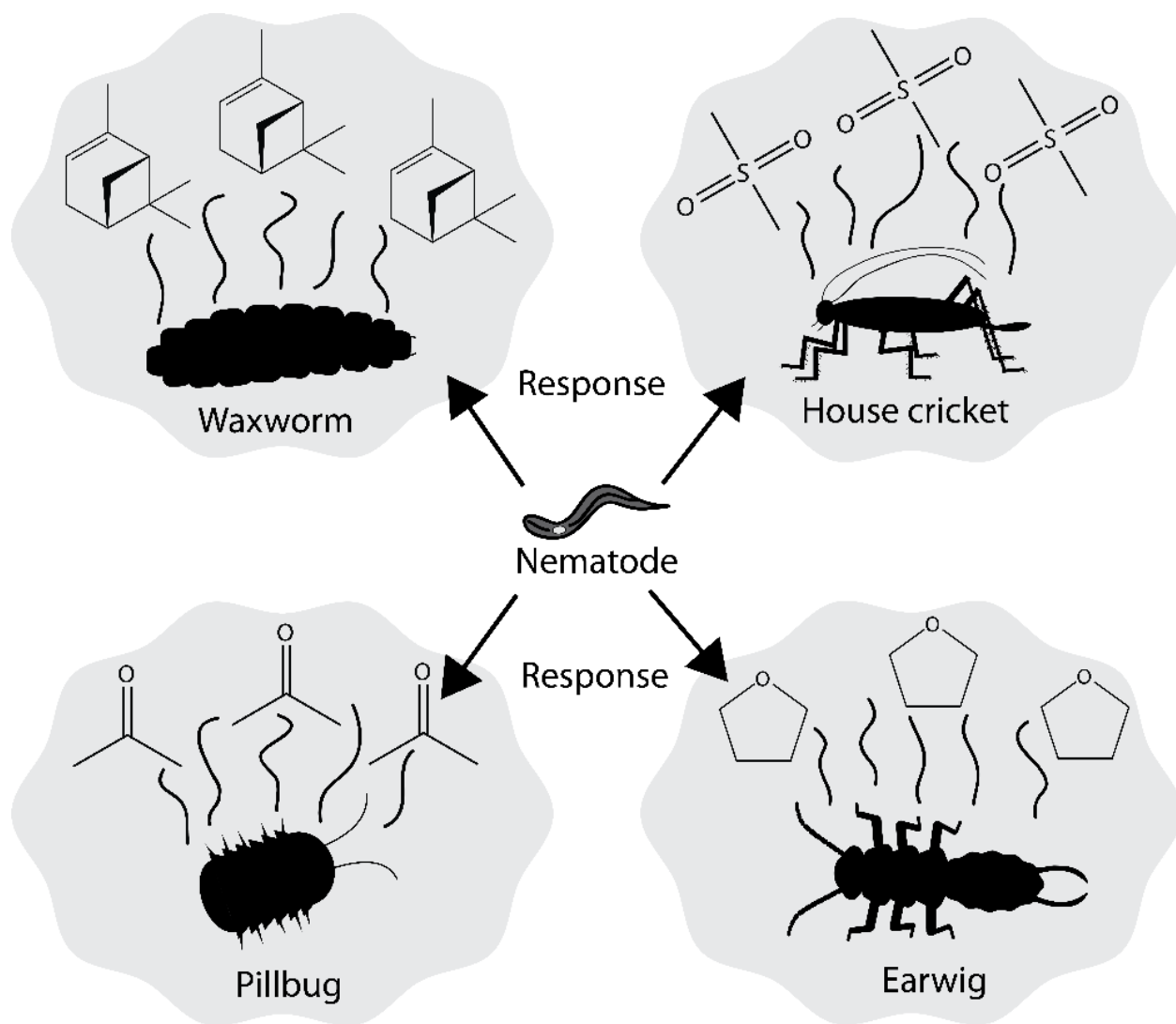
Figure 1.

Figure 2: Laminar flow versus turbulent flow. A) Laminar flow causes fluids to move in sheets such that the fluids only mix by diffusion. B) Turbulent flow causes fluids to readily mix and is the state we commonly observe when watching liquids. A convenient way to adjust between laminar and turbulent flow is by adjusting the diameter of a channel, 'd'.
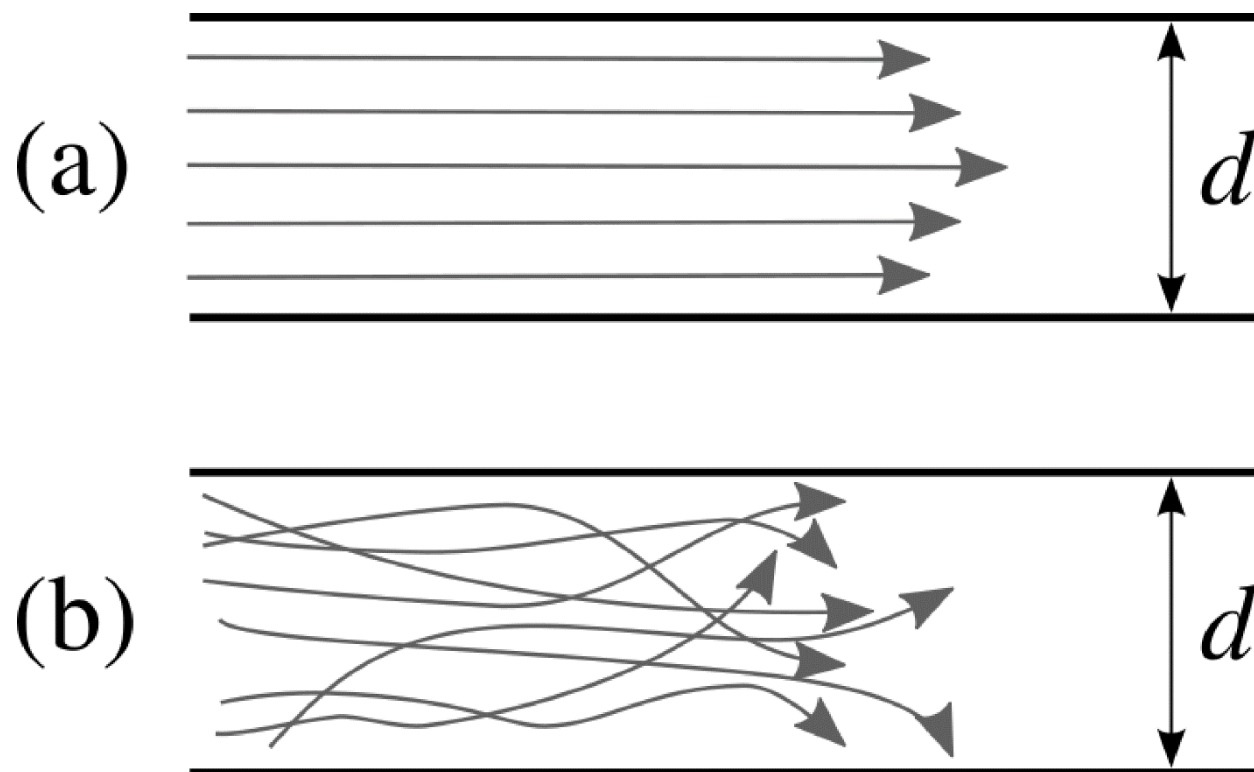
Figure 2.

Figure 3: Nematode extraction. (A) Delicate task wipe on top of cheesecloth in shallow dish.

(B) Nematode-containing sand on wipe and cheesecloth in dish. (C) Sand suspended in water.

(D) Squeezing water out of cheesecloth. (E) Tilted dish to collect nematodes. (F) Nematode-

containing sponge in shallow dish.
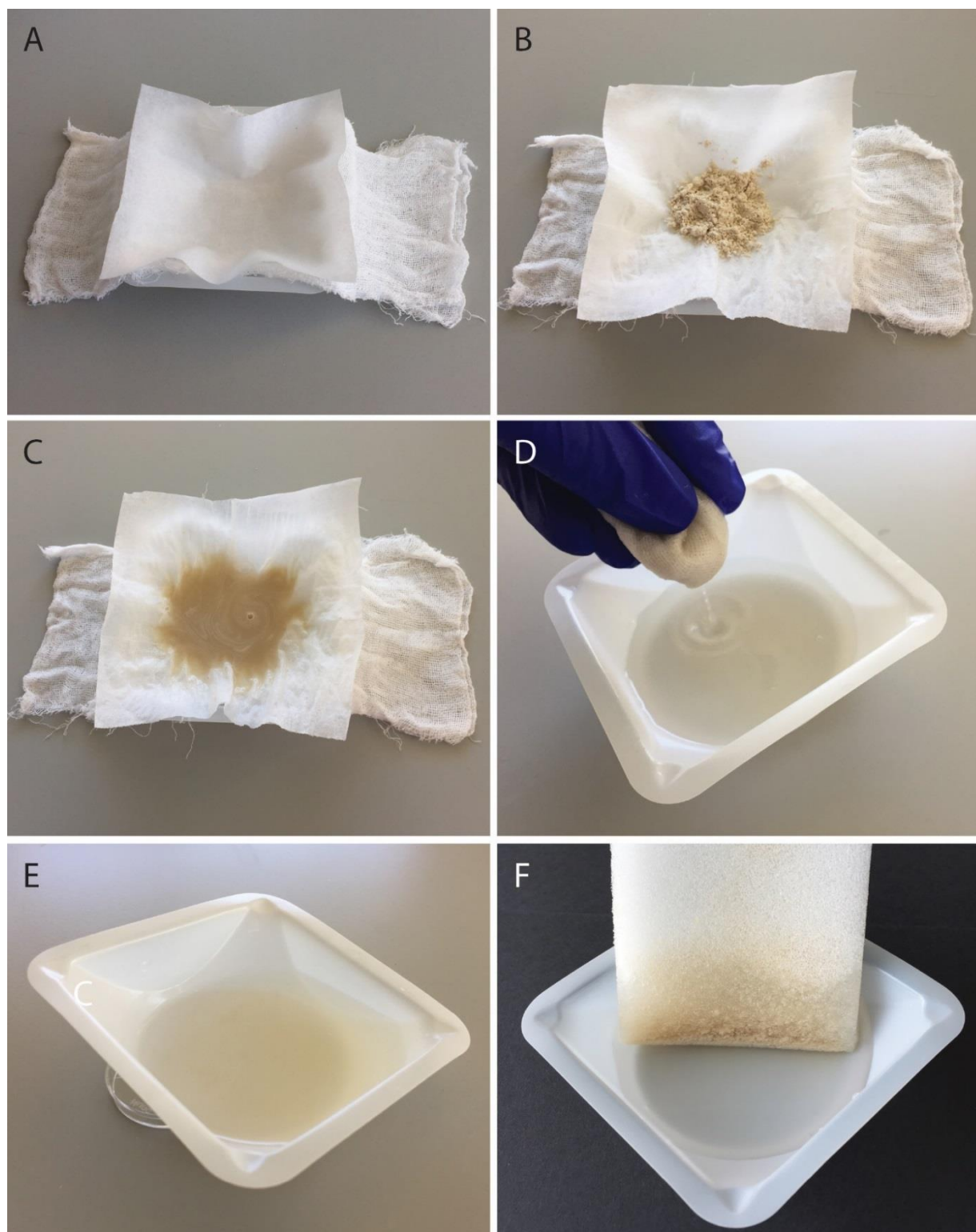
Figure 3.

Figure 4: A sequence of photos showing the structure of the microfluidic channels and their assembly. A) The components of a single device. B) Carefully aligning the adhesive with the smaller piece of transparency. C) After attaching the second transparency, the plastic tubing is hot glued onto only one inlet. D) The finished device.
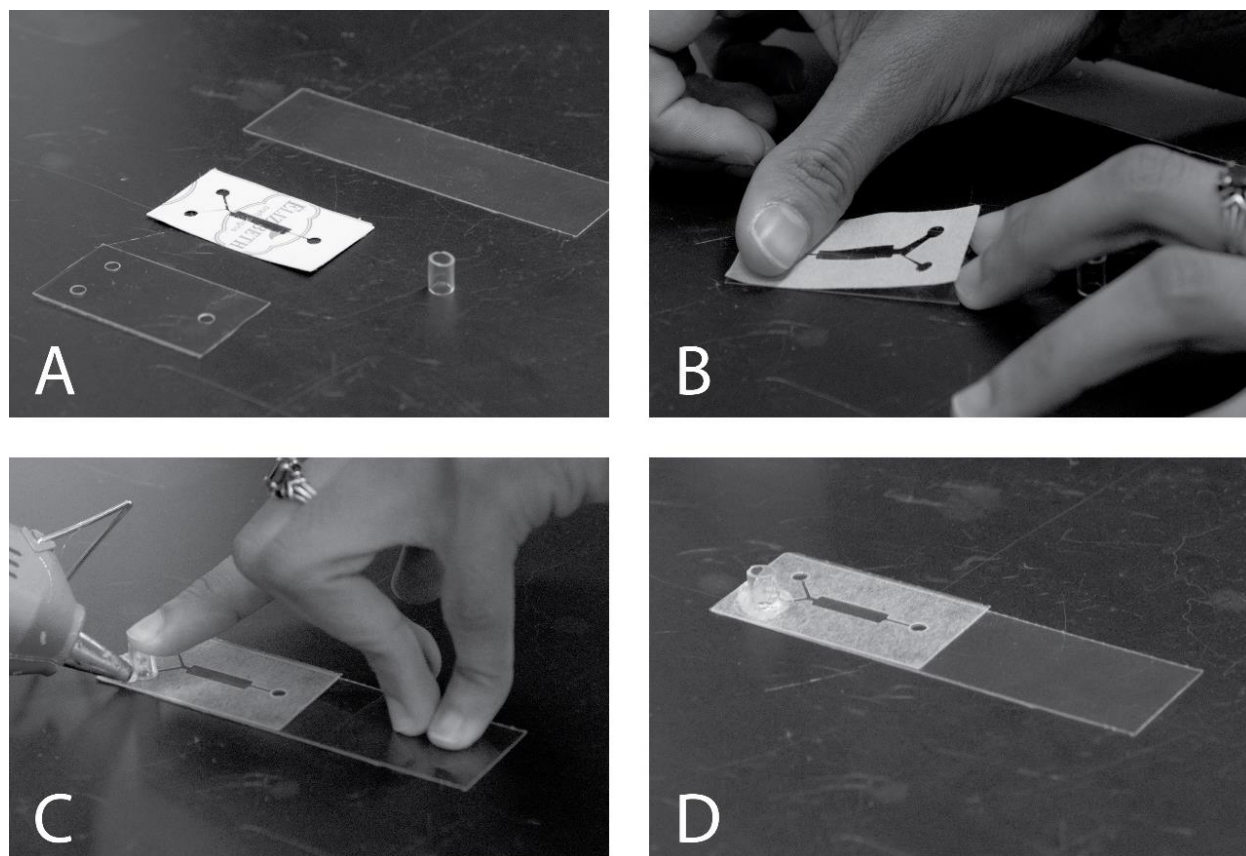
Figure 4.

Figure 5: Creating a laminar flow profile. A) An image depicting all of the materials required for this experiment. B) The tubing at one inlet is filled with chemoeffector. C) The agar is loaded into the other inlet. D) The fluids are drawn into the microfluidic channels using a tubing-tipped syringe. E) An image of the laminar flow profile.
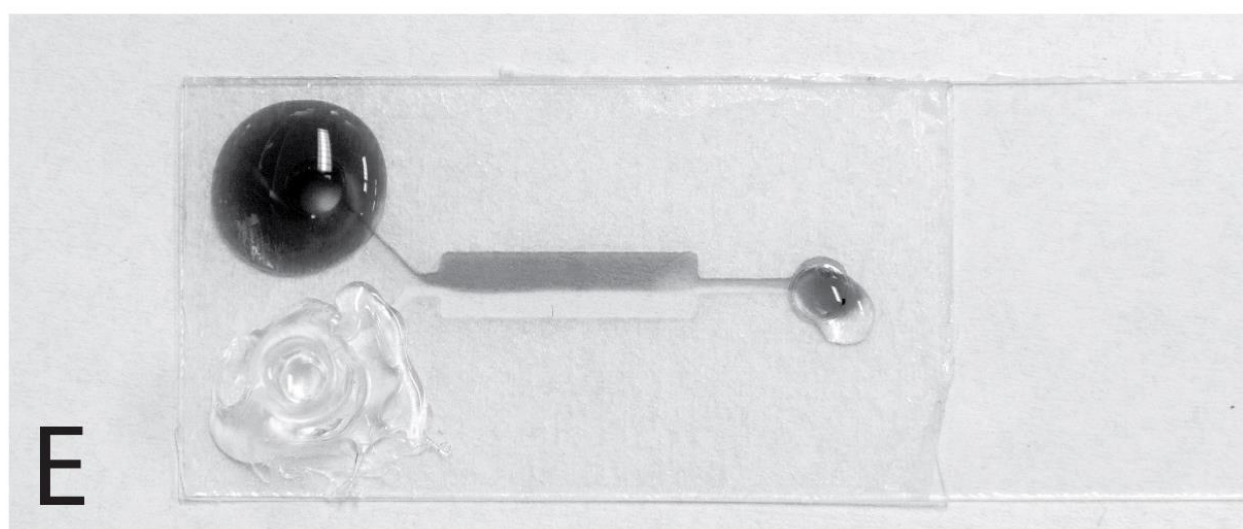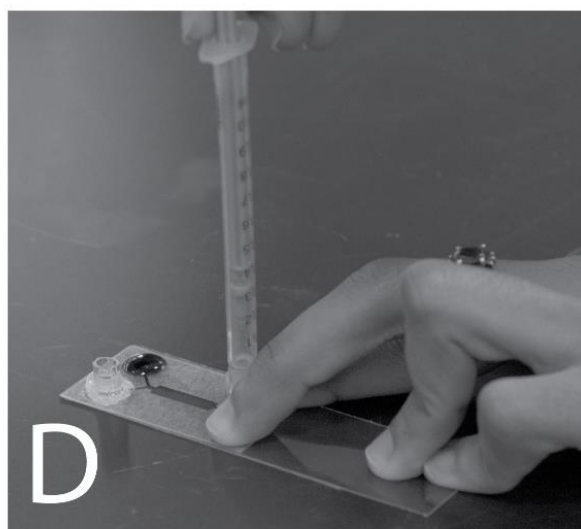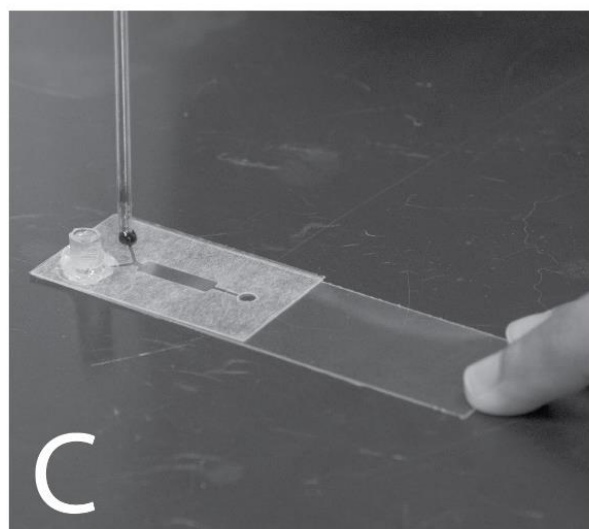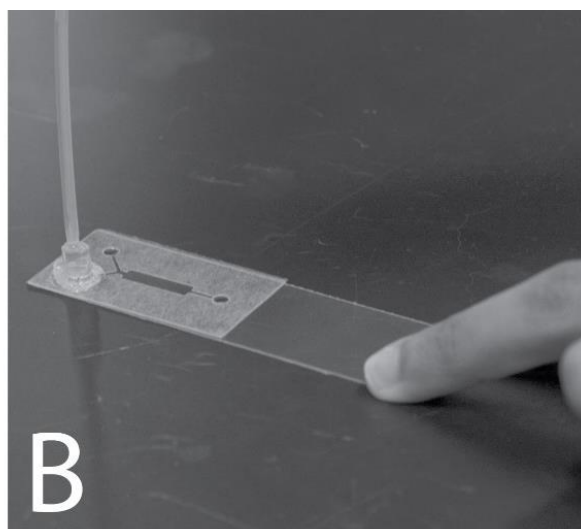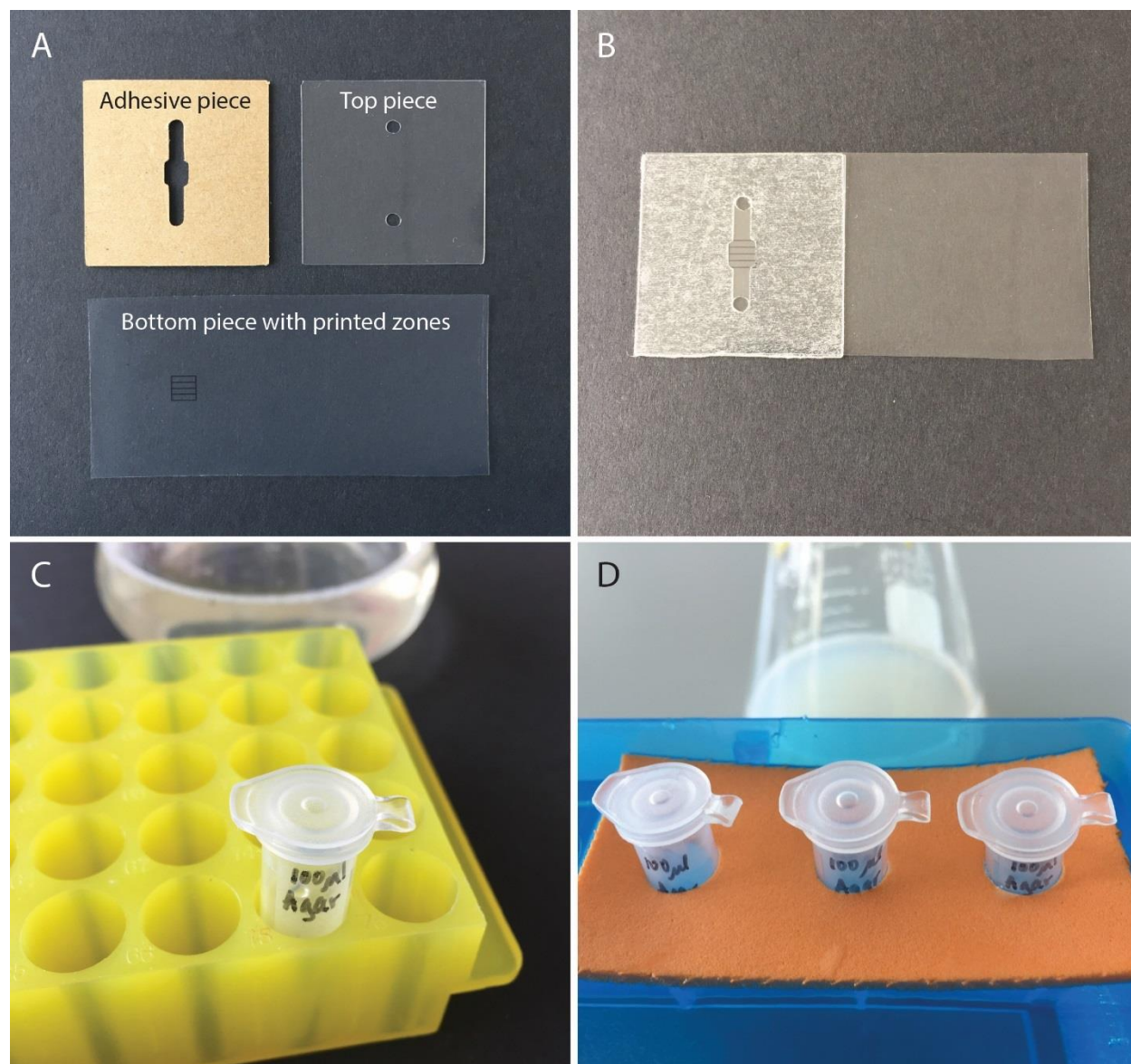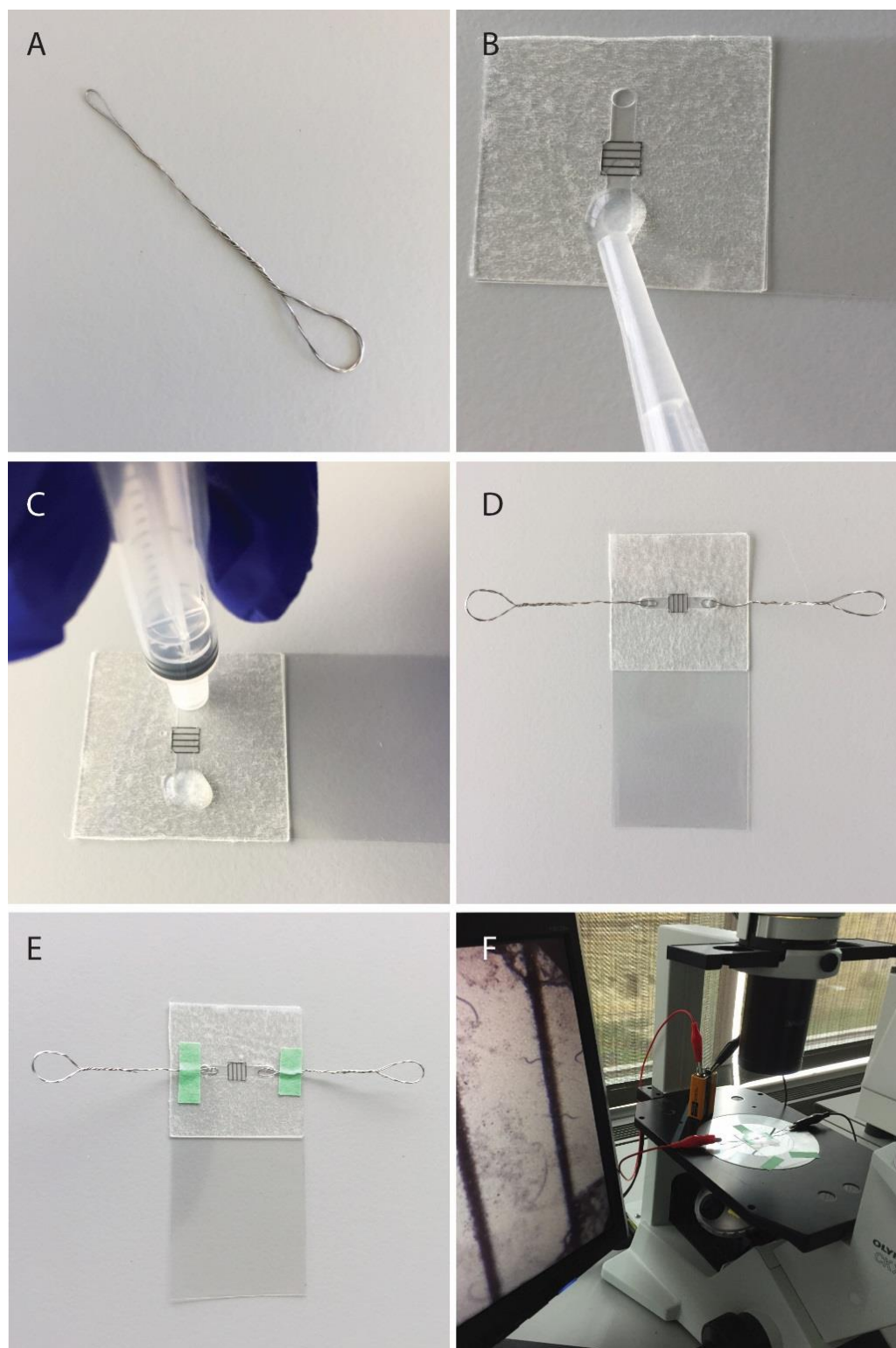
Figure 5.

Figure 6: Material preparations for electrotaxis activity. (A) Electrotaxis device pieces. (B) Assembled electrotaxis device. C) Agar solution and aliquot in microcentrifuge tube. (D) Agar solution aliquots in craft foam float in the hot water bath.

Figure 6.

Figure 7: Loading electrotaxis device. (A) Titanium electrode. (B) Drop of nematode preparation onto device inlet hole. (C) Pulling nematodes into microfluidic device using syringe with tubing on the end. (D) Titanium electrodes inserted into inlet/outlet holes of microfluidic device. (E) Electrodes taped down onto microfluidic device. (F) Loaded microfluidic device taped to microscope platform with alligator clips attached to a 9V battery. Screen to the left of the microscope shows magnification with 10X objective.

Figure 7.

Figure 8: Diagram of nematodes in zones under microscope. Nematodes respond to an

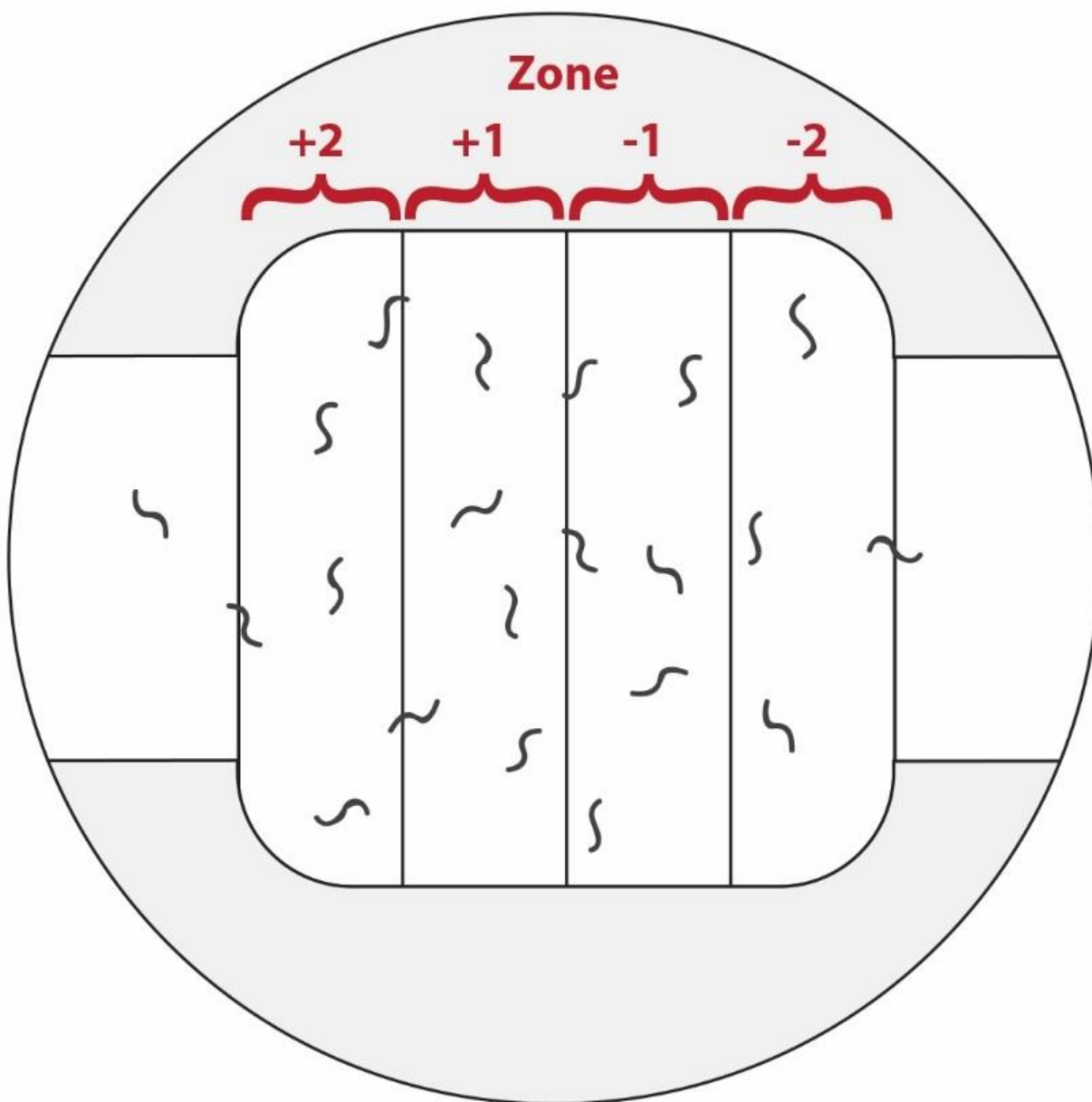applied electric field by moving through different zones of a microfluidic channel.
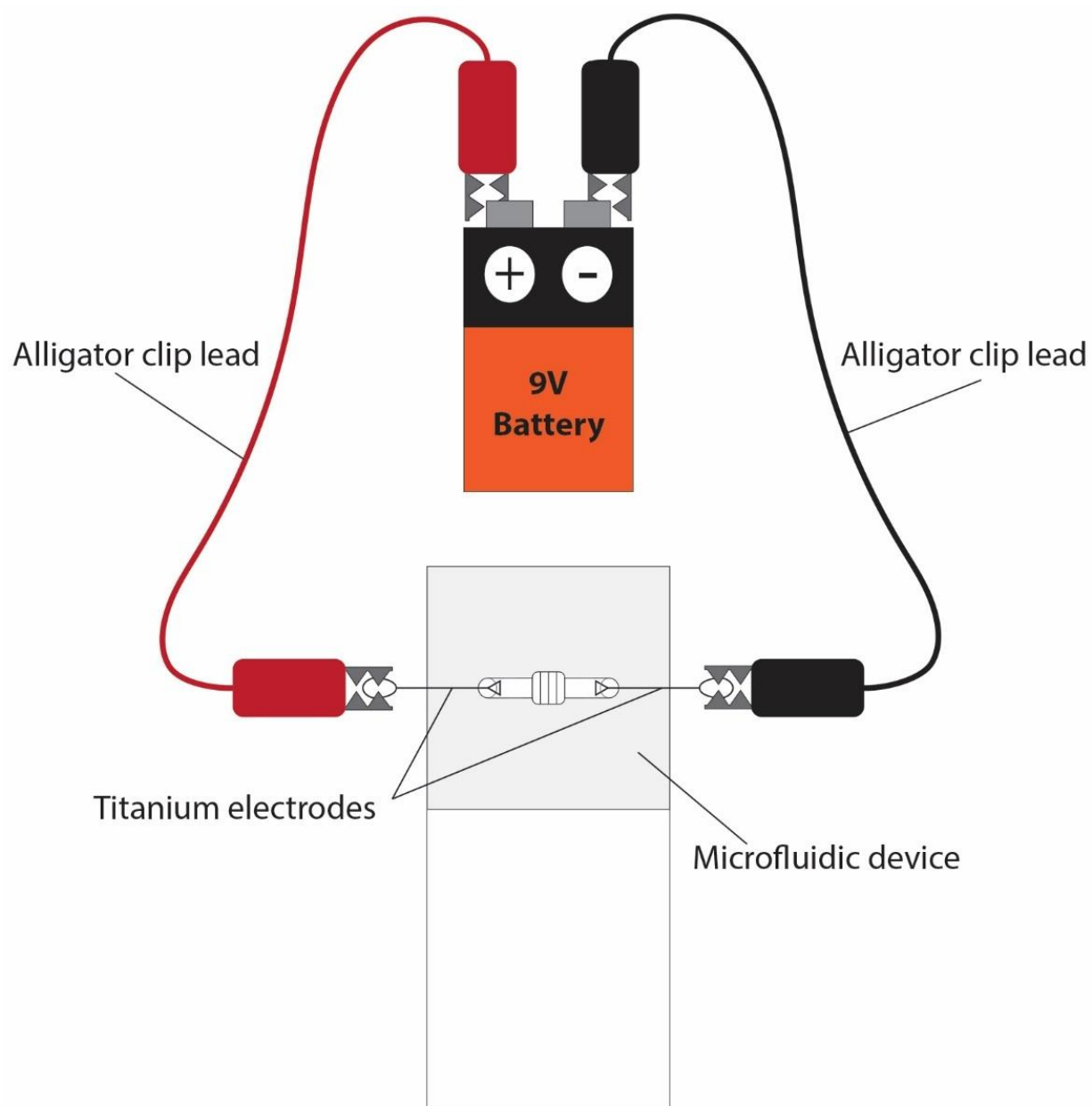
Figure 8.

Figure 9: Microfluidic setup for the electrotaxis activity. A 9V battery supplies an electric field across a microfluidic channel.

Alligator clip lead

Alligator clip lead

9V Battery

Titanium electrodes

Microfluidic device

Figure 9.

Figure 10: A) An image of 5 nematodes in a channel loaded with waxworm extract; the image was taken ~5 minutes after loading the chemoattractant using an LG® G3 smartphone and a platform microscope outfitted with the lens from a laser pointer. Arrows indicate live nematodes. B) Chemotaxis indices calculated from 3 separate experiments.
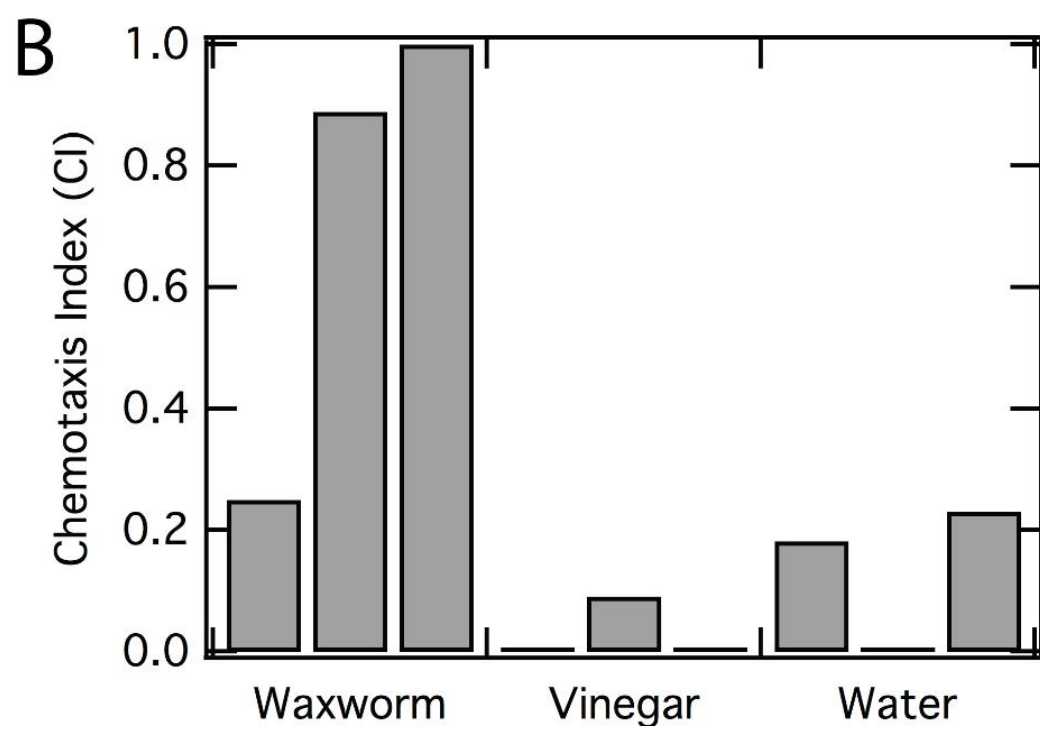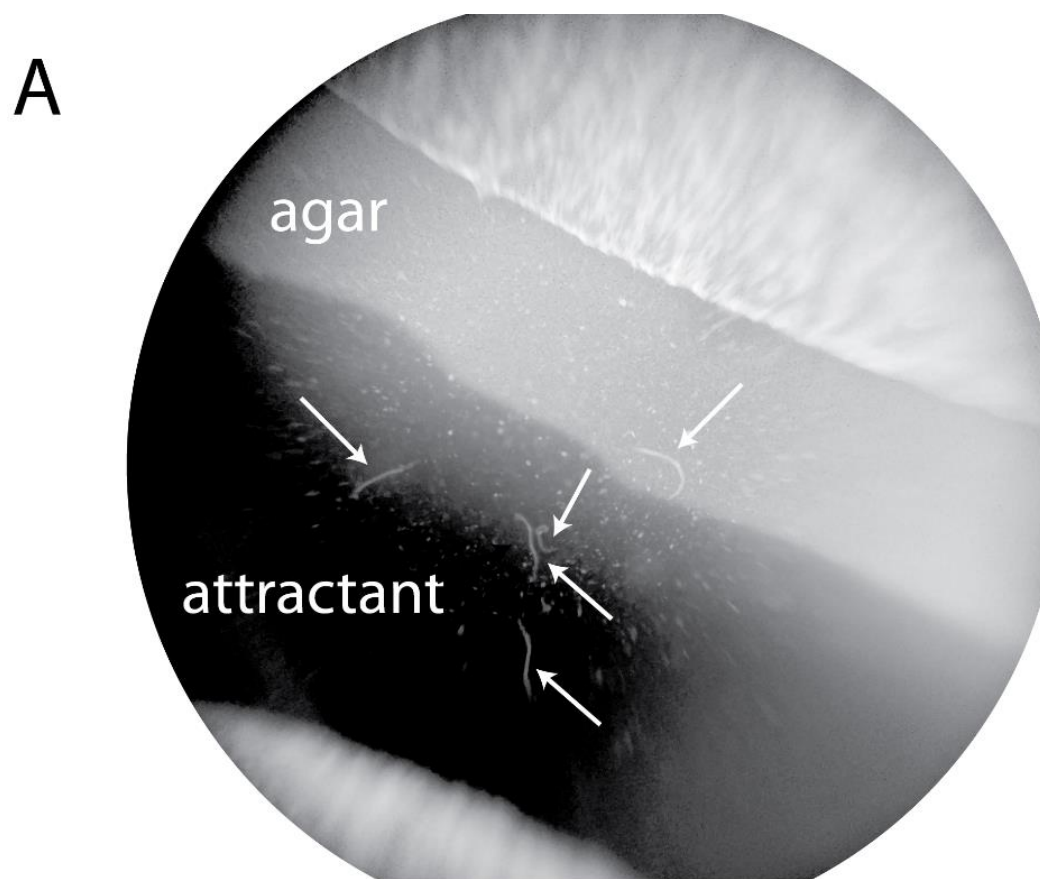
Figure 10.

Figure 11: Student responses to survey questions regarding the activity. A) Student responses to questions concerning collaboration and exploration. $N = 76$. B) The majority of students participated in the activity because they wanted to, not because they were instructed to. N = 76. Most of the students felt they learned something about science and were actively checking their own understanding of the experiment. $N = 22$.

A

Collaborative Practices of Scientists

B
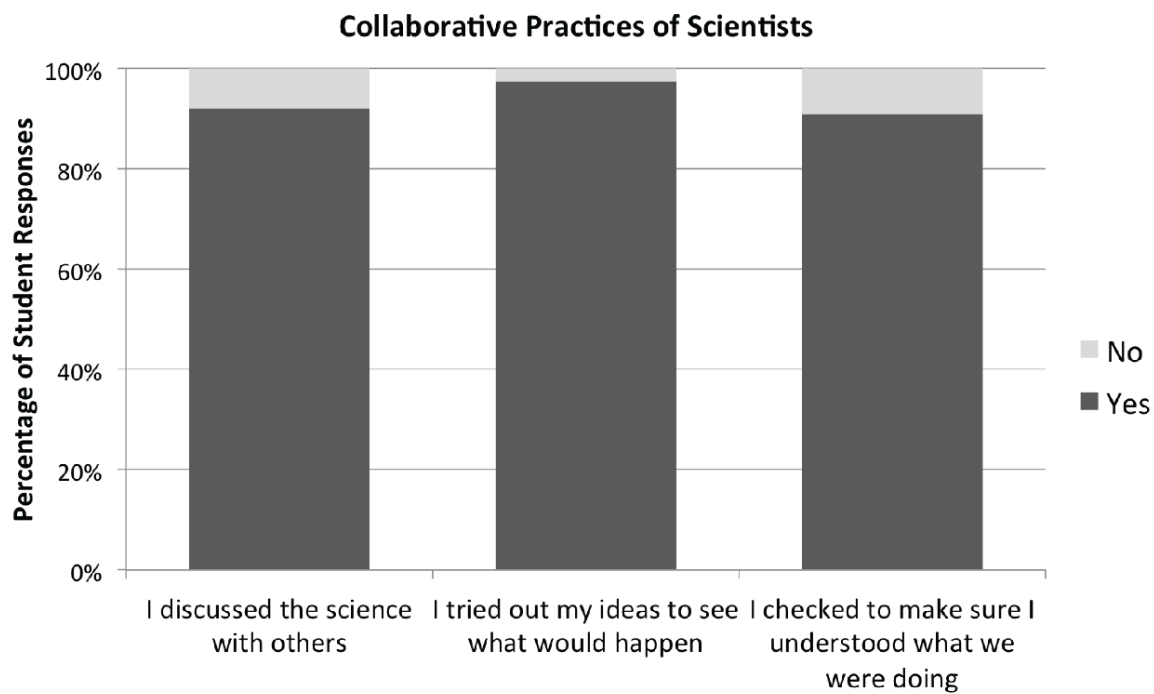
Student Engagement
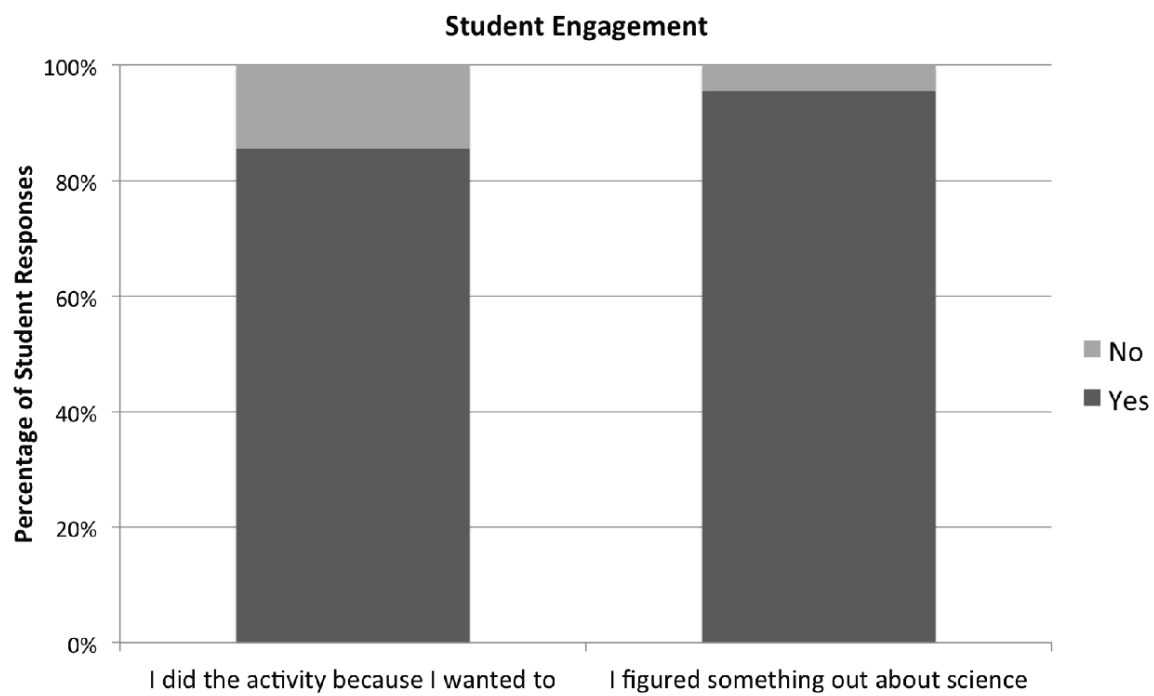

Figure 11.

Figure 12: Analysis of student engagement (N=40). An engagement score of 3 or above is a
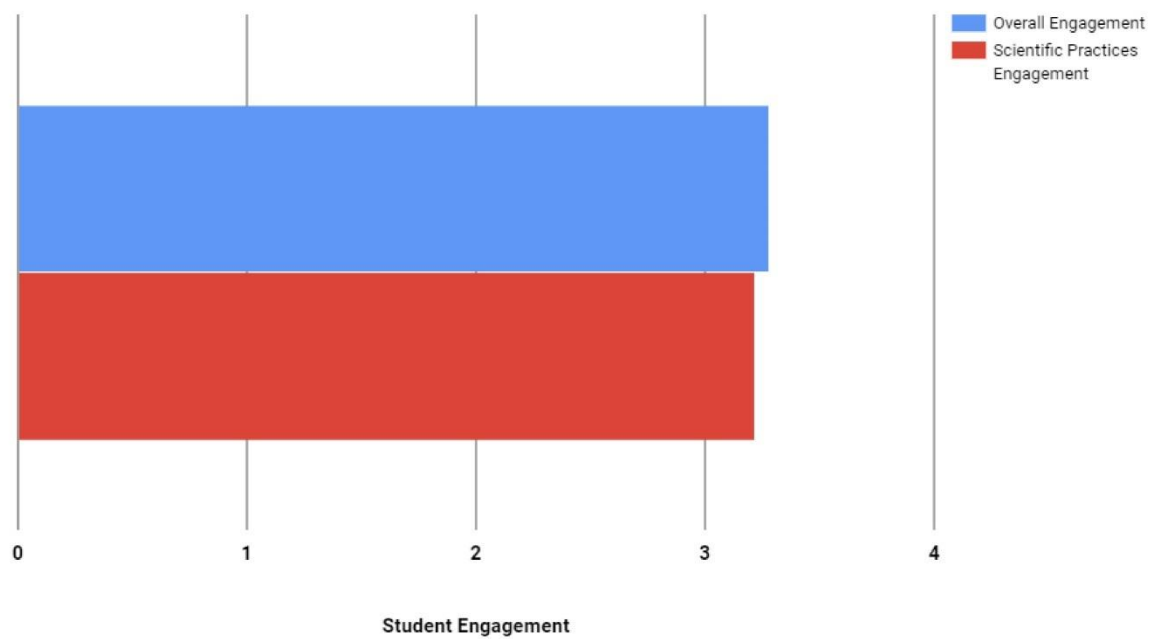
high level of engagement based on the survey.

Figure 12.

CHAPTER 5

Conclusions, Significance, and Future Directions

Physiological roles of cardiolipin in *E. coli*

Few studies have addressed the importance of phospholipid metabolism in the process of bacterial biofilm formation. A recent paper (1) demonstrates that depleting cardiolipin impacts biofilm development in *Escherichia coli*; the molecular connection between these two phenotypes was not elucidated. In Chapter 2, I demonstrate that in the absence of cardiolipin, early stages of biofilm formation are inhibited due to the activation of the Rcs envelope stress response. Further, cardiolipin depletion impairs protein translocation across the inner membrane. Protein translocation is crucial for outer membrane protein maturation, which is monitored by the Rcs signaling system through the lipoprotein RcsF. My study provides empirical evidence of the role of anionic phospholipids in biofilm development via regulation of protein secretion, and highlights modulation of the membrane composition as a potential method of altering bacterial phenotypes related to bacterial cell adaptation and survival.

CL depletion also affects other membrane processes, such as ATP production (1, 2). I found that Δ*cls* mutants produced significantly less ATP than WT, even in rich medium (Fig. 1A). This may be an indirect effect of general protein translocation deficiency. Although no bacterial *cls* inhibitors have been described, inhibitors of other phospholipase D (PLD)-type enzymes exist for eukaryotic proteins (3, 4). As reconstituting ATPase *in vitro* in liposomes was unfeasible, I attempted to reduce membrane CL content *in vivo* with ML299, one such PLD inhibitor, but was unsuccessful (Fig. 1B). A logical next step would be optimizing a system utilizing inducible protein expression to fine-tune CL synthesis and determine how ATP production varies as a function of CL concentration.

As part of future studies, I will characterize a motile Δ*clsA* mutant which I isolated from a swim assay (Fig. 2A, inset). This mutant, unlike the original Δ*clsA* strain, produces flagella

(Fig. 2B). It also has enhanced biofilm formation relative to the non-motile parent (Fig. 2A). Whole genome sequencing of this spontaneous mutant will reveal suppressors of CL-null phenotypes, providing insight into the role of CL in these regulatory processes.

By growing biofilms in microfluidic flow cells, it is possible to (a) non-invasively interrogate the physiology of the microbial community and (b) observe more advanced developmental stages of biofilm growth (5, 6). The continuous replenishment of nutrients enables the biofilm to develop for an extended time, and by applying different flow rates one may gain insight into the structural properties of the biofilm. I found that CL-deficient biofilms grown in Stovall flow cells were thicker after 24 h than WT (Table 1, Fig. 3). Although the Rcs pathway is activated in the absence of CL, this did not in fact result in greater production of colanic acid or other polysaccharides by *cls* mutants (Fig. 4). Further, *cls* mutant biofilms grown under flow accumulated more biomass than WT biofilms, suggesting some yet-undescribed mechanism that causes dysregulation of community structure.

Expression of *cls* varies depending on growth phase (Fig. 5) (7, 8). In future work, I will establish an expression profile for each *cls* gene over the course of biofilm development. This will further our understanding of the role of CL and each individual synthase in the process biofilm formation.

Isothermal PCR diagnostics

Contagious caprine pleuropneumonia (CCPP) is a highly contagious disease affecting goats in the Middle East, Africa, and Asia, with mortality rates of up to 60% (9). Successful containment and control of livestock diseases requires effective diagnostic tools. Diseases caused by *Mycoplasma* species are particularly challenging to address from a diagnostic standpoint, due

to the very high genetic similarity between these organisms. In Chapter 3, I describe the development of a recombinase polymerase amplification (RPA) to quickly and accurately identify *Mycoplasma capricolum* subsp. *capripneumoniae* (*Mccp*), etiological agent of CCPP. This is a novel assay which can be used to diagnose one of the major diseases which affects small ruminants in developing countries (10).

Isothermal PCR techniques like RPA and loop-mediated isothermal amplification (LAMP), while powerful, still require trained technicians to perform. This reality also introduces significant potential for human error into the diagnostic process. We sought to overcome these barriers by using a microfluidic platform for conducting assays. Microfluidic systems offer many advantages over traditional diagnostic methods: ease of fabrication, portability, reduced use of reagents, and low cost per assay.

We developed the "QuickChip", a low-cost, portable, rapid, and simplified field assay for detecting bacterial and viral pathogens (Fig. 6). QuickChip is a degas-driven microfluidic device that can run dozens of isothermal PCR assays simultaneously to detect pathogens using a small sample volume (11, 12). The device consists of a disposable plastic cartridge for detecting organisms to simplify liquid handling steps and minimize contamination. It interfaces with an incubation/detection system consisting of inexpensive heat control and optical systems, contained within a 3D-printed housing (Fig. 7). The utility of this chip has already been demonstrated in detection of ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) pathogens using RPA (13).

In the future, we will develop this technology to be applicable for identification of viral pathogens of yam (*Dioscorea* spp.), sweet potato (*Ipomoea batatas*), and cassava (*Manihot*

esculenta), three major starch crops of Africa and South America. One of the main constraints to stable production of starch crops in developing countries is the presence of viral disease, and crop sanitation is of particular importance for these crops as they are propagated by vegetative material (14). We will adapt already existing RPA and LAMP assays for detection of viral pathogens of these crops for use with our microfluidic platform in the field.

Classroom-accessible microfluidic explorations of predatory nematode behavior

Microfluidics enables researchers to shrink experiments to a small scale and overcome limitations of cost, accessibility, and time (15). Unfortunately, most commercial microfluidic devices are too expensive for classrooms, and DIY techniques to create them are imprecise or require materials that are uncommon in classrooms, such as pressure-sensitive adhesives or transparent silicone casts of channel molds (15-17). In Chapter 4, I describe a simple activity for students to learn about nematode behavior through active learning, using. a quick, simple, and low-cost technique for creating machine-cut, reusable microfluidic devices with readily available craft supplies found in many schools. We tested this activity successfully in several informal science lab activities for middle and high school students through Discovery Outreach Programs held at the Discovery Building at the University of Wisconsin-Madison. When asked to describe what they learned after the activity, select student responses mentioned nematode behavior, e.g. "I learned what nematodes are and how they react to certain variables," and "I learned about the biology of a specific parasite". Our activity is well suited for teachers at all educational levels to make connections to ecology-focused objectives in science curricula.

Entomopathogenic nematodes form relationships with bacteria that enable nematodes to infect and digest their insect prey. *S. feltiae* nematodes work with *X. bovienii* bacteria to obtain

nutrients from prey insects. A potential extension of our activity is to have students create "aposymbiotic nematodes" (ASN)—worms that lack bacterial symbionts—as described in (18), and study their behavior to learn more about the mutualistic relationship between *X. bovienii* and *S. feltiae*. To confirm that the ASNs do not contain *Xenorhabdus*, students can grind up nematodes and inoculate them onto lysogeny broth (LB) media plates as outlined by (19) and observe bacterial growth (20). After obtaining ASNs, students can compare the ability of aposymbiotic worms to infect and kill prey insects versus bacteria-colonized nematodes (as outlined in (18)).

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Tables

Table 1: Structural parameters of biofilms grown 24 hours at 30°C with flow (~0.25 mL min^{-1}).

	Roughness (R_a)*	% substrate coverage	Avg. thickness (µm)	Max. thickness (µm)	Biomass ($µm^3/ µm^2$)
MG1655	0.50 ± 0.20	64.8 ± 22.1	3.14 ± 0.71	5.85 ± 1.25	1.61 ± 0.49
$\Delta clsA$	0.43 ± 0.33	51.5 ± 25.4	8.32 ± 5.01	20.62 ± 2.79	3.30 ± 2.19
$\Delta clsB$	0.51 ± 0.14	44.4 ± 6.5	2.97 ± 1.93	7.25 ± 0.53	1.03 ± 0.47
$\Delta clsC$	0.83 ± 0.16	29.5 ± 5.1	2.52 ± 0.40	7.32 ± 1.40	0.49 ± 0.21
$\Delta clsABC$	0.16 ± 0.06	68.4 ± 25.8	9.34 ± 1.67	11.64 ± 2.71	4.34 ± 1.29

$*R_a = \frac{1}{N}\sum_{i=1}^{N}\frac{|L_{fi}-\bar{L}_f|}{\bar{L}_f}; L_{fi} = i^{th}\ thickness; \bar{L}_f = mean\ thickness; N = \#\ of\ measures$ (21)
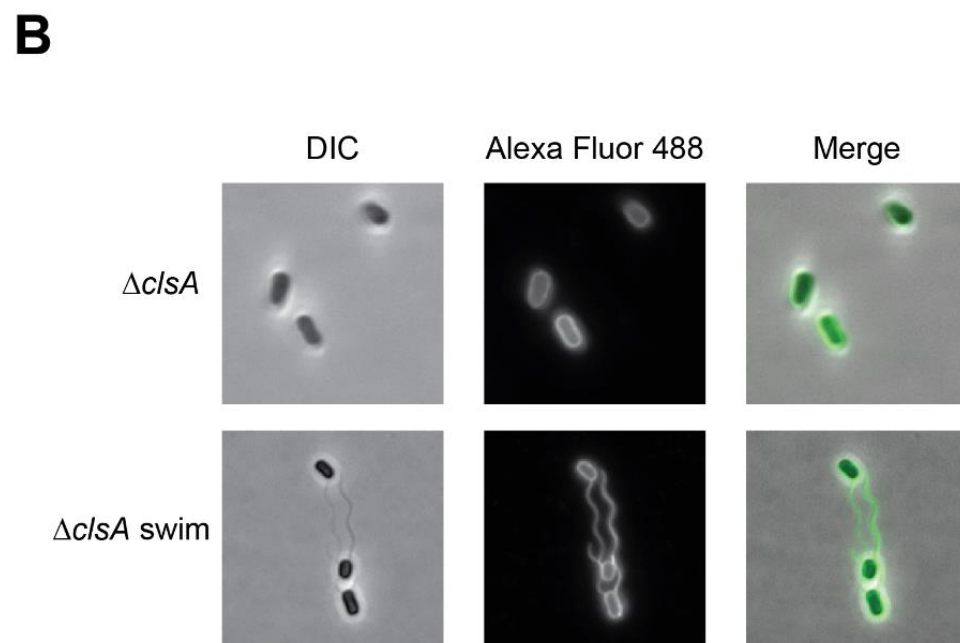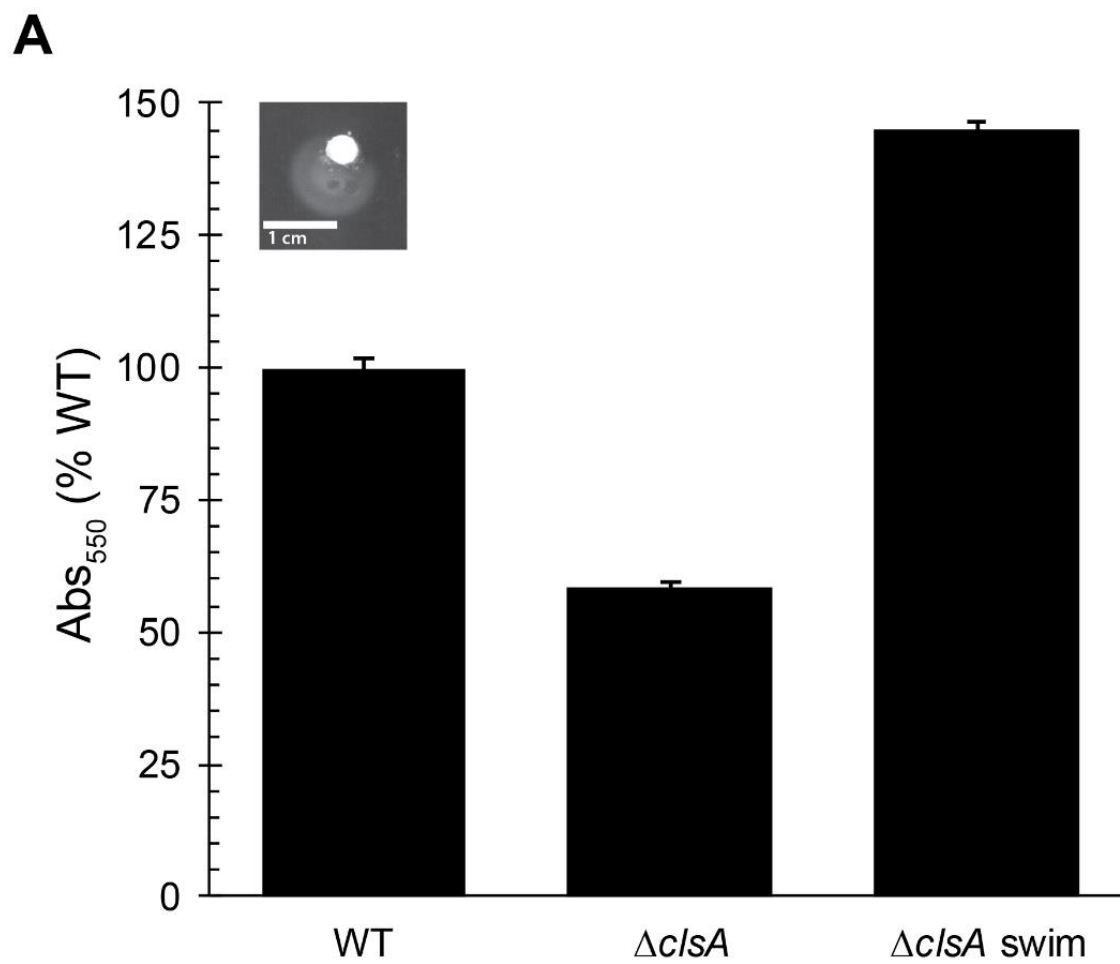
Figures

Figure 1: ATP production is decreased in CL-deficient mutants. (A) The Promega BacTiter Glo assay was used to quantify relative ATP production in *E. coli* cells grown at 37°C in lysogeny broth (LB) in the exponential and stationary phases of growth. Luminescence was quantified using a Tecan M200 platereader and AU values were normalized to colony forming units per mL (CFU/mL). AU/CFU/mL values were normalized to that obtained for wild-type (MG1655) in exponential growth phase. At least three independent replicates were analyzed for each condition. *p<0.05, Student's *t*-test compared to WT. (B) Total membrane lipids were isolated from MG1655 cells grown in lysogeny broth (LB) with DMSO or 100 μM of the PLD inhibitor ML299 for 24 h. After separation by TLC, phospholipids were visualized by fluorescence imaging following treatment with cupric sulfate. ImageJ was used to quantitate lipid spots. Error bars indicate standard deviation of 3 biological replicates.
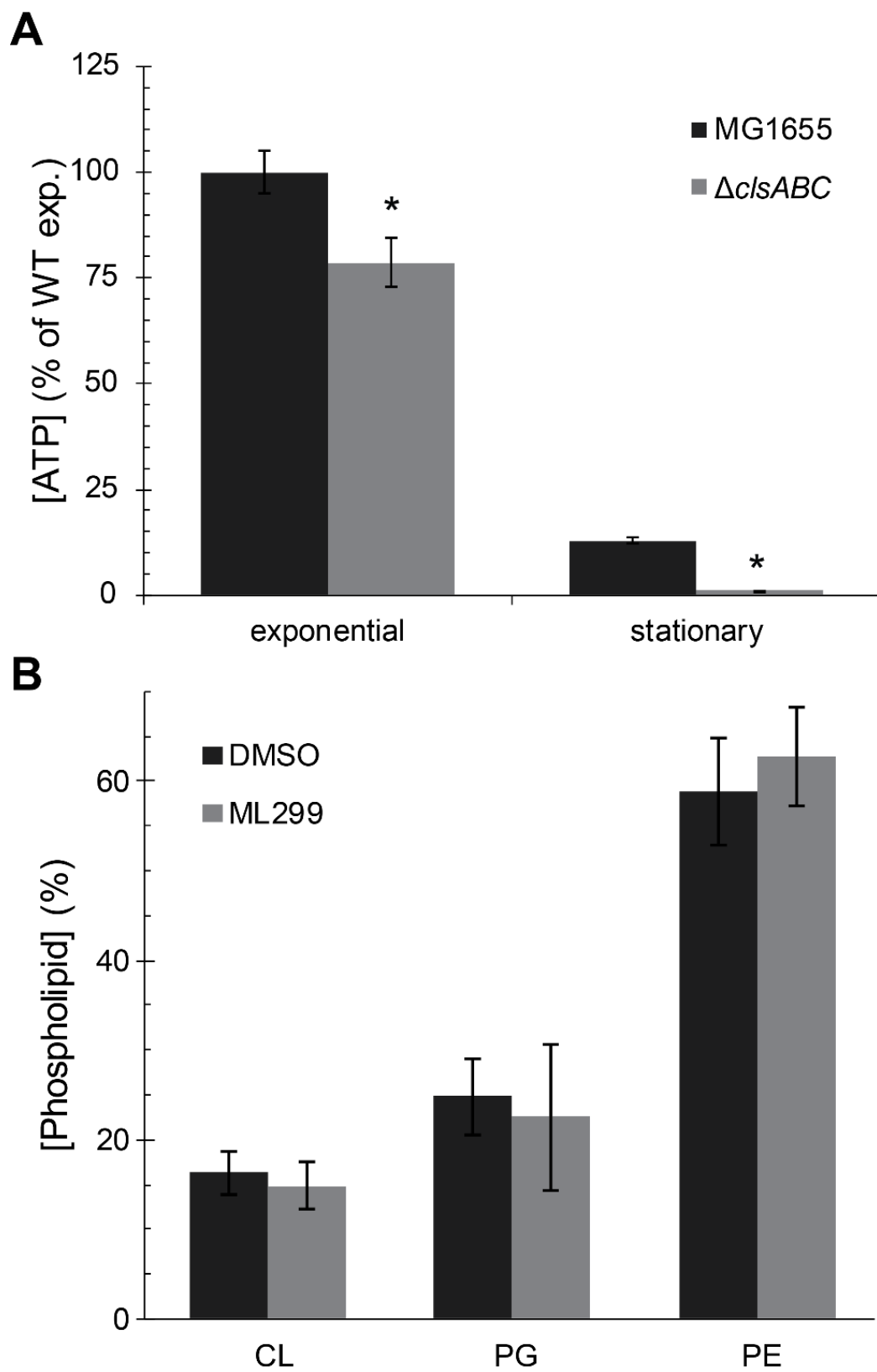
Figure 1.

Figure 2: A motile spontaneous mutant of Δ*clsA*. (A) Cells were grown in microtiter plates for 24 h at 30°C without shaking. Adherent cells were stained with crystal violet (CV), and CV absorbance was measured at 550 nm. Error bars indicate standard error (N ≥ 8). *Inset:* Swim plates were prepared and inoculated as described in Materials and Methods, Chapter 3. (B) I used an antibody raised against FliC to immunostain cells in the late exponential phase of growth.

A

B

Figure 2.

Figure 3: CL-deficient biofilms grow thicker than WT under shear stress. Confocal microscopy images of 24 h old (A) MG1655 and (B) MG1655 Δ*clsABC* biofilms constituitively expressing yellow fluorescent protein (YFP), grown at a flow rate of ~0.25 mL/min at 30°C in M9 supplemented with complete amino acids and 0.4% glucose. The stochastic nature of YFP expression causes some cells to be brighter than others. (C) The MATLAB program COMSTAT (21) was used to quantify biomass of confocal image stacks. Error bars indicate standard deviation of at least 3 independent biological replicates.
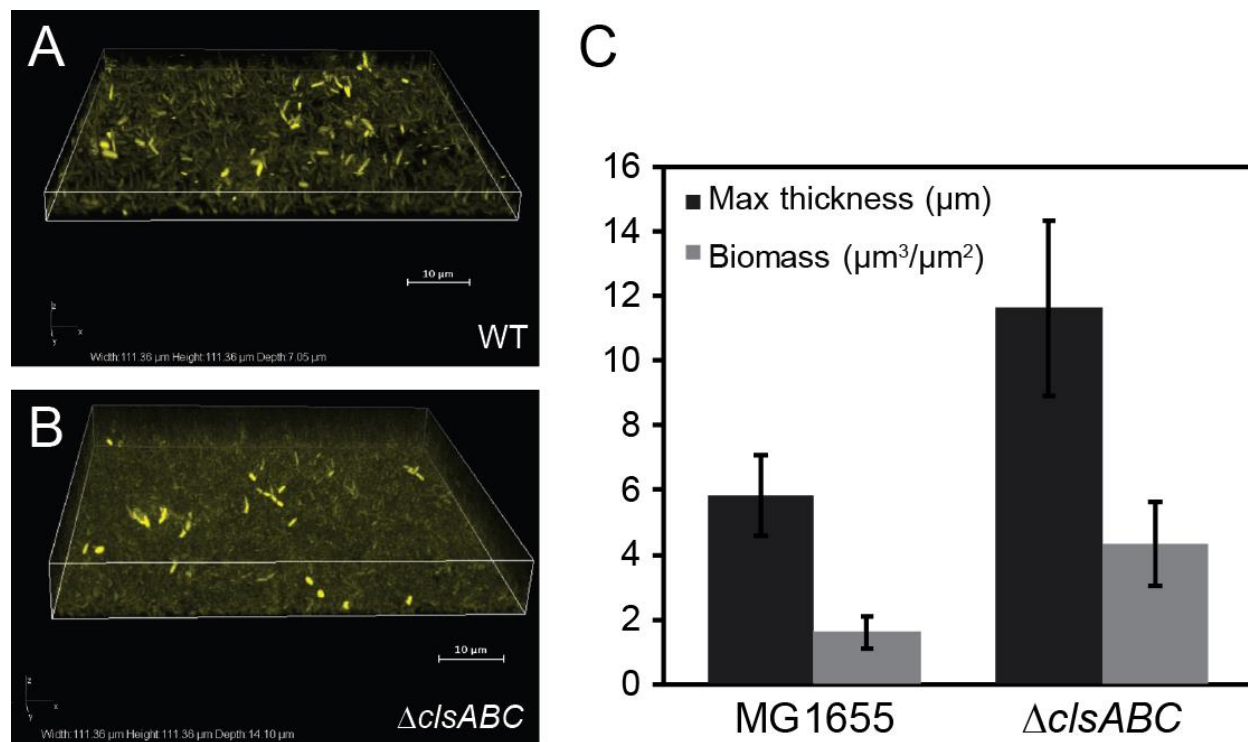
Figure 3.

Figure 4: CL-deficient mutants do not overproduce polysaccharides. Extracellular polysaccharides were isolated from biofilms grown in polystyrene microplates in minimal medium at 30°C for 24 h. Colanic acid was quantified by determining the concentration of L-fucose. *$p<0.05$, as determined by a Student's *t*-test.
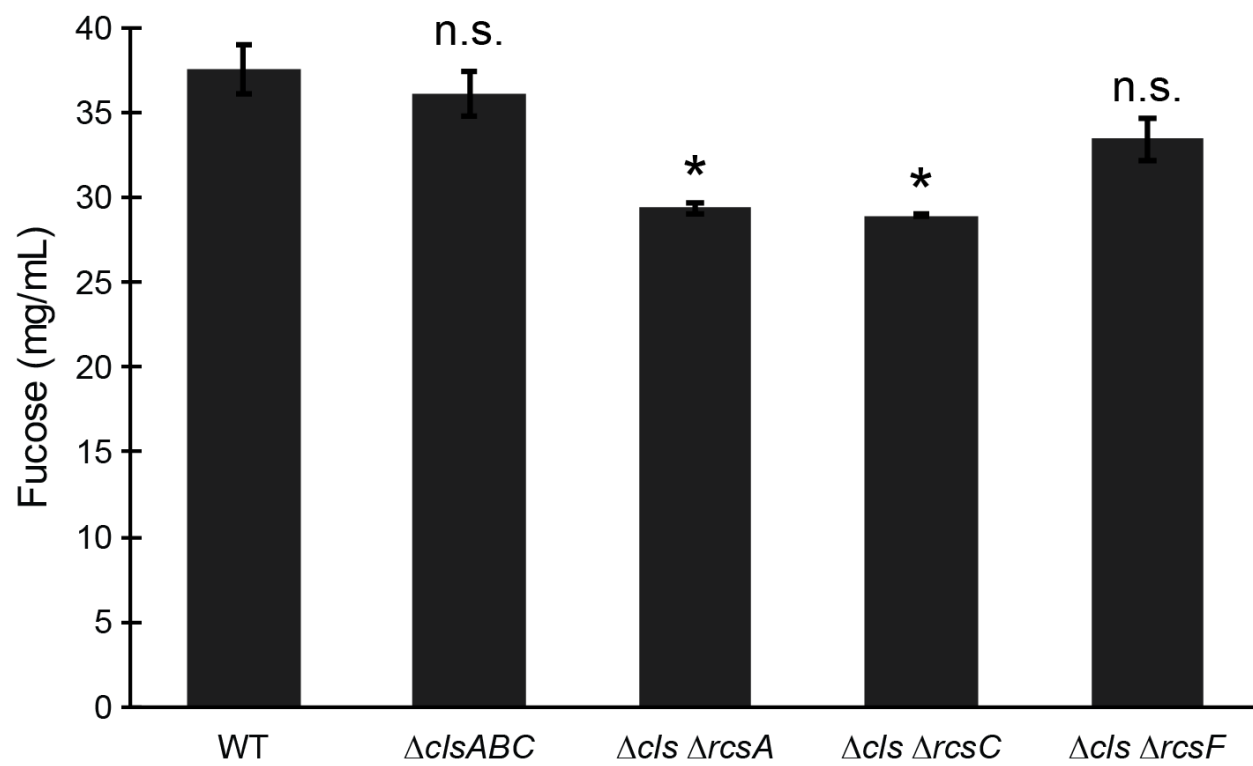
Figure 4.

Figure 5: Promoter activity of *clsA* and and *ymdB* increases as cells enter stationary phase.

Putative *cls* promoter regions were fused to GFP and fluorescence was monitored in cells growing at 37°C in M9 supplemented with 0.4% glucose. Values were normalized to the empty vector (pUA66) control. Shaded regions represent standard deviation of 3 technical replicates.
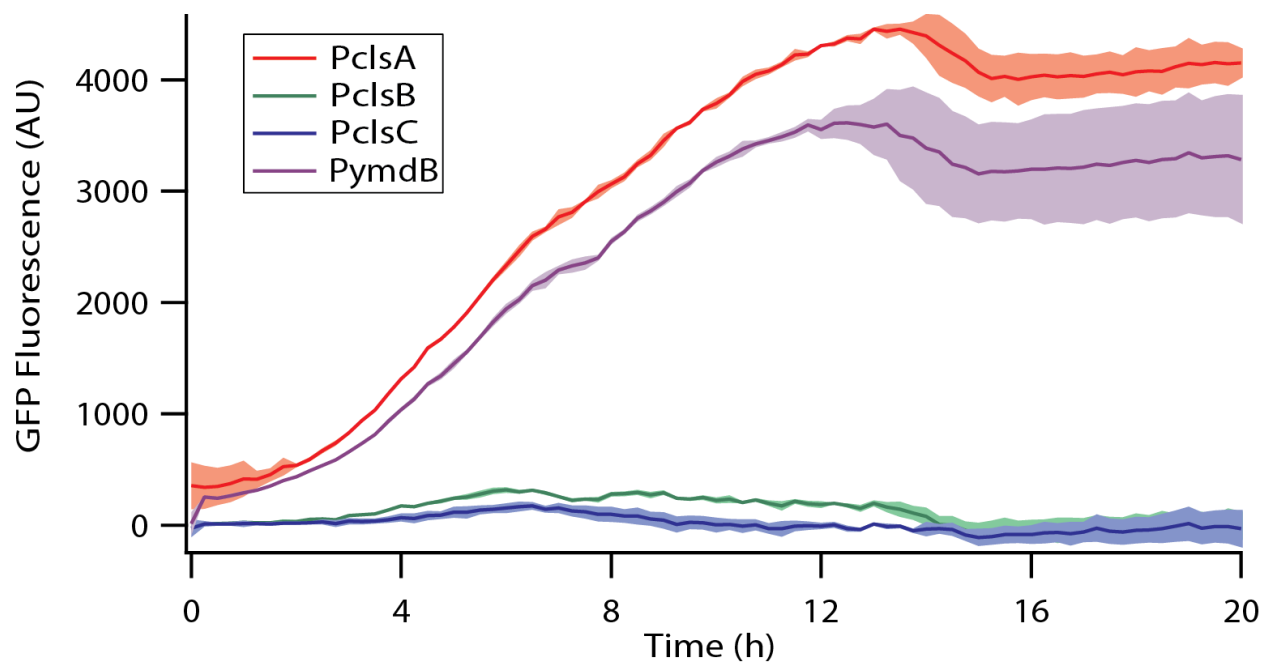
Figure 5.

Figure 6: The QuickChip. (A) A schematic of the chip. (B) A fully assembled chip. Dime is shown for scale.

Figure 6.

Figure 7: The QuickChip reader. The reader is 3D printed, with a simple user interface and a slide-out tray for microfluidic cartridges. It contains an excitation source, camera, heater, fluorescence filters, and control software.

Figure 7.
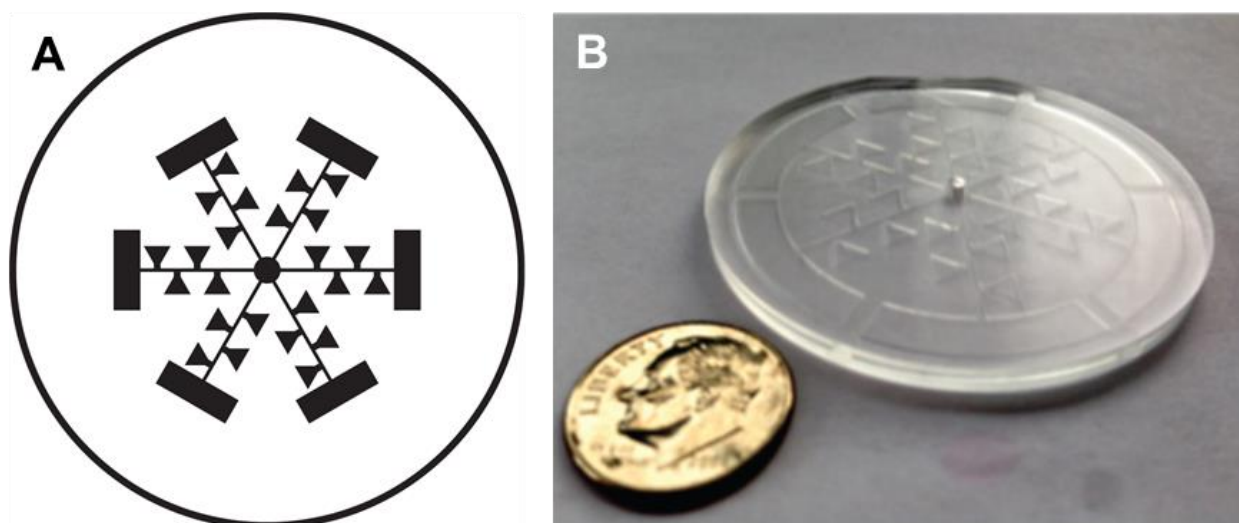
APPENDIX

Examining graduate students' engagement in and attitudes towards science communication

Abstract

Public science engagement is important for maintaining dialogue between the public and the scientific community. Much research focuses on the impact of engagement on the public, and recent efforts have aimed to both understand and increase scientists' participation in public communication. These efforts have largely focused on the activities of scientists in professional settings such as industry scientists and academic faculty. While these studies are informative, the field lacks information on science graduate students. This report is based on a 2017 survey of University of Wisconsin-Madison graduate students, and provides information on attitudes towards and participation in public engagement by scientists-in-training at a large American R1 university. In general, this study finds that graduate students' perceptions of the value and usefulness of public science communication are favorable. This work highlights factors that may affect these perceptions and will prove useful in developing strategies for increasing competency and engagement in science communication across researchers at multiple levels of their academic career.

Introduction

Emerging technologies, environmental threats, and scientific controversies are becoming more and more important for everyone from single working-class parents to venerated researchers to policy makers. To address these issues effectively and democratically, it is imperative that all parties have access to relevant information and are able to understand this information. Multiple studies have attempted to discover what leads scientists to engage in public science communication (PSC) and investigated ways to improve communication efforts. Many studies focus on the roles and perceptions of faculty members, often excluding researchers outside of that category.

Less than 20% of individuals with graduate degrees will go on to become tenured research professors, but these individuals still consider themselves scientists and will often pursue research-related careers outside of academia (1). It is important to understand how this population, which will arguably compose the majority of the next generation of "scientists", thinks about public communication. I used data from graduate students at a highly regarded U.S. institution to improve our understanding of PSC engagement in this demographic.

The relationship between science and the public

Science and technology play ever larger roles in modern life. Our society has grown so dependent on innovations like the smartphone that these devices have begun to affect our biology—"smartphone separation anxiety" is a phenomenon has caught the attention of psychologists and media outlets (2). Greater knowledge of scientific topics has a positive effect on attitudes towards science in general (3), which is becoming increasingly important for fruitful

discussions of culturally and politically relevant topics, as these topics become ever more rooted in scientific developments.

A century ago, "de-extinction"—the resurrection of an extinct species—could not even be conceived of. Now the possibility of bringing woolly mammoths or gastric breeding frogs back from the dead is real and present (4, 5). We as a society must carefully consider not only the technical challenges of such pursuits, but also the ecological and ethical implications of these endeavors. De-extinction, colonization of Mars, cancer research, children conceived without a female, and so many more topics have massive implications for our culture and the future of our species.

However, many Americans are neither interested in nor knowledgeable about science; according to the National Science Foundation (NSF) Americans only answer 65% of factual knowledge questions correctly on average ([Science and Engineering Indicators 2014](#)). This disconnect has been the subject of much study in the past few decades, e.g. Bensaude-Vincent (6), who explored the history of the gap between science and society and the basis of its formation, and Tlili and Dawson (7), who took a more modern look at policy developments and shifts in perception relating to the interaction of scientists and the public. Recent outbreaks of non-compliance with childhood vaccination schedules, despite copious evidence that vaccines are safe and effective, are an excellent example of this rift (8, 9).

Scientists typically place little emphasis on public communication for a variety of reasons (10, 11). Much of this has to do with the average scientist's view of "the public"; scientists tend to follow a deficit model and view the public as largely homogeneous, unknowledgeable, and uninterested in learning (12-14). Scientists also believe that the public is irrational and excessively self-interested, leading them to assume that any engagement on their part will be in

vain and leave them feeling marginalized (15, 16). Despite the fact that their own personal interactions with the news media are mostly positive, scientists often blame journalists for perpetuating the public's scientific misconceptions (17, 18). This trend of non-communication is changing, however.

Improving scientists' public communication

The scientific community, particularly in the academic and government sectors, has been making significant efforts to improve public perception and understanding of science (17-19). Major funding agencies such as the NSF now require "broader impacts"—detailed explanations of how the research will be communicated and shared with the public (The National Science Foundation proposal and award policies and procedures guide, 2014). Universities are offering classes and workshops that are centered around communicating science. For example, in 2013 graduate students at Harvard University and the Massachusetts Institute of Technology initiated "ComSciCon," a science communication workshop for students. The Alan Alda Center for Communicating Science at Stony Brook University and the American Association for the Advancement of Science offer workshops for scientists at all levels. At the University of Wisconsin-Madison (UW-Madison), students can take classes like "Science and Social Media" and "Communication of Science and Technology" in the Life Sciences Communication department. Professional societies like the American Physical Society and the American Society for Microbiology also encourage their members to be active in community outreach.

There have also been positive shifts in perceptions of PSC by scientists (20). Scientists are interacting more with journalists, and use of social media platforms such as Twitter is also on the rise (17, 21-23). Twitter is particularly popular among social scientists; according to Michael

Macy, a professor of sociology at Cornell University, "Human interactions are what social scientists are really all about" (24). Scientists in general are likely beginning to realize the increasing importance of social media in the scientific world, as internet denizens like Retraction Watch are on the lookout for subpar articles and posting their scathing opinions of such within days of publication (25). Still, engagement in PSC seems to be the exception rather than the norm.

Predicting scientists' engagement in public communication

Scholars are trying to build an understanding of what factors make a scientist more likely to engage in PSC in order to devise more effective interventions. Variables such as age, gender, and discipline do not seem to be important determining factors in a scientist's level of engagement (11). Factors that do predict engagement are centered more on the personal views of the scientist in question (11).

Feelings of efficacy are one indicator of civic engagement; scientists are more likely to participate in PSC if they feel that they have the skills to do so, and that the public will respond favorably as a result of their efforts (26, 27). Not surprisingly, scientists are also more likely to engage with the public if they have positive perceptions of PSC and if they believe that their colleagues are participating in PSC (perceived norms), and less likely to do so if they feel that they will be judged negatively as a result of their participation (21, 26). One of the strongest predictors of future public engagement is past public engagement (11, 28). The biggest motivations for PSC by scientists, regardless of engagement predictors, is a desire to educate the public about science and technology and to improve the public's attitude towards science (17, 18).

Who are the targets of public communication programs?

As mentioned above, PSC by junior scientists is not well studied. It is important to understand how scientists view and engage in PSC, yet this large segment of the scientist population remains unaddressed (11). To successfully target science communication training efforts to young scientists, we must (a) make students aware of these opportunities, (b) encourage them to take advantage of such opportunities, and (c) understand what makes scientists-in-training want to communicate science with the public. Some have advocated for requiring training in science communication in graduate curriculums, but in order to be effective, these programs must be carefully crafted to suit the needs of the junior scientist demographic.

Research Questions

Because so many scientists have attended graduate school, these early stages of career development are ideal for targeted interventions in public science communication advocacy. Little is known about junior scientists in the context of PSC, relative to their more senior counterparts. To remedy this dearth of empirical information, I will use the following research questions to guide this exploration:

RQ1: Do graduate students' perceptions of science communication activities differ from those of faculty?

RQ2a: What is the level of engagement of graduate students in public science communication?

RQ2b: What is the level of engagement of graduate students in public science communication training?

RQ3: What are predictors of engagement in science communication by graduate students?

Methods

Survey distribution. In order to obtain a sample representative of graduate students at a large research institution, I requested an email list of all currently enrolled graduate students from the University of Wisconsin-Madison registrar's office. Students that had placed a hold on release of their contact information were excluded (<10%). I identified 8,264 graduate students for participation in the survey. The survey distribution and collection were administered by the University of Wisconsin Qualtrics Survey Hosting Service.

The survey was fielded from January 20 through February 20, 2017 using a multiple wave contact procedure (29). The first contact was an emailed link to the survey with a brief summary of its purpose. A second email was sent to non-responders 1 week later, and a final email sent to any remaining non-responders 1 week after the second. The final response rate was 16.6%, resulting in a final sample size of *N* = 1374.

Survey design—operationalizations. The survey encompassed seven major concepts: PSC engagement, attitudes, media familiarity, self-efficacy, campus climate, formal training, and demographics.

Familiarity with media. To address students' media exposure, they were asked how frequently they used common social media platforms (Facebook, Twitter, reddit, etc.) for science-related purposes. These responses were aggregated into four categories: Never (1), rarely (2, less than once a month), occasionally (3, a few times a month), and often (4, once to a few

times a week; and 4, every day), and combined into a single measure for "Media Familiarity" ($\alpha = 0.79$) (Question 2).

   PSC Engagement. To determine levels of PSC engagement on social media, we aggregated responses to Question 3 (A-D) and Question 4 (A-E) ($\alpha = 0.87$). To determine levels of engagement in PSC outside of social media platforms, participants were asked, "Thinking about your public outreach activities, how often do you engage in public outreach efforts related to your field of research/science in general?" (Appendix, Question 7A-B). Five response possibilities were combined into four: Never (1), less than once a year (2), a few times a year (3), and more than every few months (4 and 5). Responses to these questions were combined into a single measure for "Public Outreach Engagement" ($\alpha = 0.85$).

   Attitudes. Graduate students responded to several questions designed to assess their attitudes towards public communication, public communicators, and social media as a platform for scientific discourse. Respondents were asked to indicate "how much do you disagree or agree with the following [statements]?" on a scale from 1 (strongly disagree) to 5 (strongly agree) with statements expressing positive or negative views. Only attitudes towards PSC could be combined into a single measure with reasonable reliability ($\alpha = 0.78$) (Question 8C, 8E).

   Campus climate. Support from advisors and department faculty is important for students seeking to engage in PSC. "Campus Climate" was determined based on how strongly students agreed with statements such as "My research advisor is supportive of my non-academic pursuits" ($\alpha = 0.75$) (Question 9A-C, 9E-F).

   Formal training. UW-Madison offers a large number of workshops, seminars, classes, and other opportunities for training in PSC. Survey participants were asked the number and nature of the formal PSC training resources they had utilized (Question 10).

Self-efficacy. Students were asked questions designed to interrogate their self-efficacy with regard to getting training for PSC and for engaging in PSC. Responses to Question 11A-C were combined to measure self-efficacy for obtaining PSC training ($\alpha = 0.78$). Despite a significant correlation between the questions addressing self-efficacy for PSC, Cronbach's alpha for a combined measure was low (Question 8A-B). Thus, they were compared to other variables individually.

Demographics. Given previous research indicating that women are more likely to be involved in popularization of science (30), gender was added as a variable (Question 17). Male was coded as 1 and female coded as 2. Respondents were also asked to indicate their race (white = 1, nonwhite = 2) (Question 19). Religiosity was measured based on respondents' answer to the question, "How much guidance does religion provide in your everyday life?" on a 10-point Likert scale from 1 (no guidance at all) to 10 (great deal of guidance) (Question 21). Graduate students were asked to indicate their level of conservatism in relation to economic (Question 20A) and social (Question 20B) issues, and responses were aggregated into a measure for Conservatism ($\alpha = 0.68$).

Analysis. IBM SPSS Statistics version 22 was used for all data analysis.

Limitations. My study only considers individuals at a single university, which will limit the use of any results in creating a general model of junior scientist behavior. Participant responses may be skewed by subjects misremembering or forgetting past events. The responses may be skewed even further because I am a member of two of the departments I am studying (Biophysics and Biochemistry), which may affect response rates as well as the actual responses. Additionally,

because this study is observational, rather than experimental, there is less certainty that interventions conceived of based on these results will actually be effective. Despite these limitations, the results of this investigation will guide future explorations into this very important topic.

Results

Of those who responded, 42.0% were male and 56.3% were female (Table 1). Caucasians represented 72.8% of respondents. The mean age of respondents was 28 years (SD = 6.1 years), with a median of 26 years. On a 6-point Likert scale ranging from 0 = 'Very liberal' to 5 = 'Very conservative,' the mean ideology of our sample was 1.82 (SD = 1.23) for economic issues and 1.08 (SD = 1.11) for social issues (Table 1).

Of the UW-Madison graduate students surveyed, 95% reported that they use social media (*N* = 1036). Figure 1 shows students' use of these and traditional media platforms, such as television and public radio, for "science-related purposes". Similarly to scientists at this institution, wikis (e.g. Wikipedia) were the most commonly used among social media sources (72% of graduate students at least once a month vs. 77% of scientists). General social networks and YouTube were reported to be the next most frequently used (79% and 76%, respectively), followed by blogs, professional social networks, and podcasts (65%, 62%, and 56%, respectively). Over two-thirds (68%) of students never used reddit for science-related purposes. Compared with UW-Madison scientists, graduate students used reddit and general social networks more (15% vs. 33% and 43% vs. 65%, respectively), but did not differ greatly in their use of other social media platforms for science-related purposes.

Looking deeper into graduate student social media use for science purposes, almost 3/4 (74%) reported using social media to post or comment on content related to general scientific topics, compared with less than half (48%) of scientists (Fig. 2). Students engagement in online discussion was lower when related with their own work (53%), similar to reports by scientists (48%).

Many graduate students did not have strong opinions regarding the impact of social media on scientific credibility. Just over half did not believe that using social media negatively impacted their reputation (51%), but a third were ambivalent (37%) (Fig. 3). Only 19% of students thought that social media use would increase their academic impact, a very slight increase from reports by scientists (17%). However, in response to **RQ1,** 43% of students think social media is effective at engaging with peers, compared with only 24% of scientists.

Looking at social media's potential for public engagement rather than engagement with peers, and further investigating **RQ1,** graduate students' views were generally (1) favorable, and (2) more favorable than those of scientists. Only 40% of students believed social media was too time-consuming, a nearly 20% drop compared to scientists (58%) (Fig. 4). Most students indicated that they thought there were interested lay audiences on social media (59%), and an overwhelming majority believed scientists should actively take part in discussions about controversial scientific issues (88%).

RQ2a asked about students' engagement in PSC. UW-Madison graduate students are actively engaged in public outreach, but less so than UW scientists. Over a quarter of graduate students (27%) reported never participating in public outreach, while less than 7% of scientists never engaged in outreach (Fig. 5). Most students indicated engaging in public outreach a few times a year or less (59%).

Graduate students' views of their departmental/program climate were generally positive. Only 12% of participants reported that their research advisor was not supportive of their non-academic pursuits, and 56% indicated that others in their department encouraged them to use science training opportunities (Fig. 6). Approximately half of students (46%) knew active science communicators in their department and/or program, and did not associate being active in science communication with low scientific ability (96%).

Turning to intrinsic factors on public engagement, most graduate students felt that they were able to contribute to public science communication efforts, and that these efforts effected public attitudes toward science (72% and 84%, respectively) (Fig. 7). However, many students are unaware of opportunities to participate in science communication or improve their skills in this area, as well as how to find opportunities for science communication training (60%, 54%, and 60%, respectively).

Over half of graduate students reported engaging in at least one type of science communication training (56%, Fig. 8), addressing **RQ2b**. Of these students, most attended short workshops or seminars, and half attended workshops or seminars at conferences (73% and 48%, respectively) (Fig. 9). Many students took advantage of other training opportunities on the UW-Madison campus, with 13% having taken classes in the department of Life Sciences Communication and 24% taking courses through the Delta program.

Considering the professional utility of public engagement, most graduate students thought that participation in science communication was important to be able to obtain funding, and did not believe that science communication skills would be irrelevant to their future careers (62% and 80%, respectively) (Fig. 10*A*). In general, students' opinion on whether science communication skills were relevant for their career goals was not correlated with their career

aspirations. However, there was a small but significant correlation between this opinion and an interest in careers in industry research or science policy (Pearson's correlation coefficients of -0.107 and -0.143, respectively; $p < 0.01$).

Looking at UW-Madison graduate students' perceptions of lay audiences, 75% believe that lay audiences can provide valuable insights about scientific research, 54% agree that scientists should be aware of public interests, and only 20% agree that scientists know what is best for the public (Fig. 11). Conversely, 65% of respondents think that scientists' opinions are more important than those of the public regarding decisions on ethical implications of research. Most students did not think that new research of public interest should be communicated immediately, but many agreed that scientists should comment on published findings through social media (76% and 38%, respectively).

Among students in STEM (Science, Technology, Engineering, and Math) fields, PSC engagement on social media was significantly positively correlated with media familiarity, formal training levels, campus climate, attitudes towards PSC, and self-efficacy in terms of contributing to PSC and explaining difficult scientific concepts (Table 2). Engagement in public outreach was positively correlated with engagement on social media in addition to these factors, as well as self-efficacy for finding PSC training resources. In general, the majority of these variables were positively correlated with one another on a significant level.

As other studies have shown, gender played a role in public outreach engagement, with females in STEM being more likely to engage ($p < 0.001$) (Table 3). This trend did not hold for engagement on social media. Interestingly, race was correlated with social media engagement, with nonwhites being more likely to participate in this form of PSC. Conservatism and religiosity were not significantly associated with engagement in any dimension.

Significance. This study will be one of the first to address the role of junior scientists in PSC. US institutions alone grant almost 200,000 master's and doctoral degrees in science and engineering fields per year, yet very few of these individuals will end up as a tenured faculty member at a research institution (31). Therefore, it is crucial to understand the role of these scientists. Understanding factors that influence junior scientist engagement in PSC will inform future policy changes to improve both the level and quality of scientists' communication with the public. This in turn will advance public discourse on socially relevant topics in science and technology.

Discussion

Overall, the UW-Madison graduate students surveyed were active in public outreach, and believe that these efforts are both important and effective. More graduate students are using social media regularly for scientific engagement than previous generations, though opinions on the scientific credibility of social media have largely remained the same. Students generally reported positive departmental climates regarding public engagement. Although most students feel they are capable of contributing to public science communication efforts, many are unaware of opportunities for training in science communication. Students agree that lay audiences should be informed about new scientific developments and that the public's wishes should be considered, but remain skeptical of the value of public opinion on scientific ethics.

With respect to **RQ3,** it seems that a number of factors are significant predictors of PSC engagement. Due to the relatively high number of missing values for many questions, I was unable to fit a regression model to the data. Yet some useful conclusions may still be drawn. It is

unsurprising that self-efficacy and familiarity with the subject matter are likely to increase students' engagement in PSC, and that positive attitudes from superiors would improve student attitudes and participation. Thus, it is reasonable to conclude that efforts to bolster faculty and institutional support for PSC-related activities would be quite helpful for increasing graduate students' engagement in PSC and PSC training. By continuing to expand offerings for PSC training and ensuring the presence of a positive atmosphere for PSC activities, UW-Madison will positively impact graduate students' feelings of self-efficacy around PSC, their attitudes and familiarity for it, and actual meaningful contributions to public discourse both on and offline.

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Tables

Table 1. Gender and discipline comparisons for scientists from the American Academy for the Advancement of Science (AAAS), National Science Foundation (NSF), and University of Wisconsin-Madison (UW-Madison). AAAS is the national organization whose members' social media habits were surveyed and reported in 2015 by Pew. UW-Madison science graduate students closely match the demographics of NSF scientists with respect to discipline, but there is a smaller proportion of male trainee scientists at UW-Madison. Compared with the overall science graduate population[4], survey respondents in scientific disciplines overrepresented biological sciences and underrepresented physical science ($N = 746$). Males were slightly underrepresented.

	AAAS[2]	NSF[1]	UW-Madison		
			Scientists[3]	Grad students[4]	Survey respondents
Gender Male	71.0%	64.5%	66.5%	59.2%	51.9%
Discipline Biological Sciences	48.1%	32.6%	36.2%	29.5%	43.2%
Physical Sciences	27.4%	32.5%	29.9%	36.2%	20.8%
Social Sciences	8.9%	34.8%	29.9%	34.3%	36.0%
Other/Multiple	15.6%		4.0%		

[1]National Science Foundation, National Center for Science and Engineering Statistics, *Survey of Doctorate Recipients* (2013). http://ncsesdata.nsf.gov/doctoratework/2013/.

[2]Pew Research Center (February 15, 2015), *How Scientists Engage the Public*. Available at: http://www.pewinternet.org/2015/02/15/how-scientists-engage-public/.

[3]Science, Media, and the Public Research Group (SCIMEP). (2015). *Scientists and Social Media*. University of Wisconsin-Madison. Madison, WI: Department of Life Sciences Communication. Available from: http://scimep.wisc.edu/projects/reports/.

[4]Office of the Registrar, *Registrar's Enrollment Report, Spring 2015-2016* (March 22, 2016). University of Wisconsin-Madison. Madison, WI. Available from: https://registrar.wisc.edu/semester_enrollments.htm

Table 2. Correlations between variables for respondents in STEM fields.

	Self-efficacy (explain science)	Self-efficacy (contribute to PSC)	Attitudes (PSC)	Campus climate	Formal training	Self-efficacy (Training)	Media Familiarity	Engagement (Public Outreach)	Engagement (Social Media)
Engagement (Social Media)	.115**	.186***	.223***	.128**	.260***	.020	.566***	.321***	1
Engagement (Public Outreach)	.221***	.481***	.456***	.236***	.415***	.184***	.293***	1	
Media familiarity	.117**	.158***	.155***	.121**	.230***	.081*	1		
Self-efficacy (Training)	.100*	.192***	.150***	.326***	.270***	1			
Formal training	.133**	.260***	.289***	.227***	1				
Campus climate	.082*	.201***	.215***	1					
Attitudes (PSC)	.411***	.544***	1						
Self-efficacy (contribute to PSC)	.404***	1							
Self-efficacy (explain science)	1								

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Pearson's correlation coefficient). N ≥ 613.

Table 3. Correlations between control variables and engagement variables for respondents in STEM fields. For gender, male was coded as 1 and female as 2; for race, white was coded as 1 and nonwhite as 2.

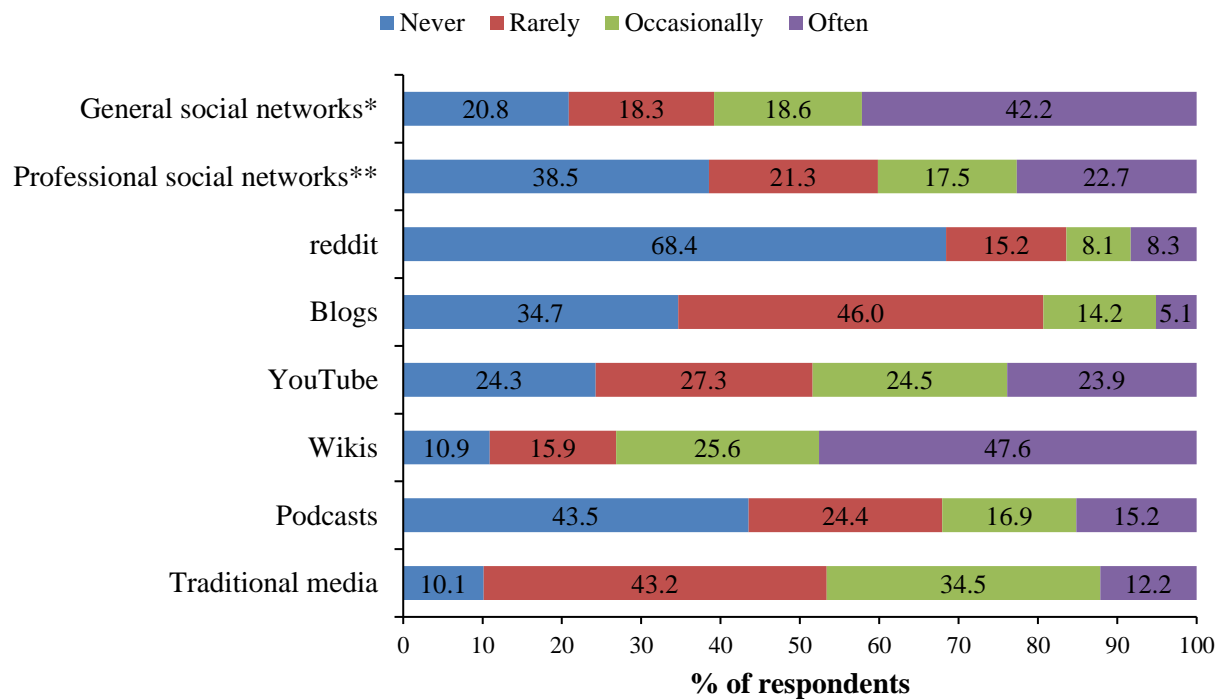|	Engagement (Social Media)	Engagement (Public Outreach)	Gender	Race	Conservatism	Religiosity
Engagement (Social Media)	1					
Engagement (Public Outreach)	.321***	1				
Gender	.035	.148***	1			
Race	.116**	.063	-.045	1		
Conservatism	-.054	-.055	-.138**	.131**	1	
Religiosity	-.004	-.050	.039	.021	.367***	1

**p < 0.01, ***p < 0.001 (Pearson's correlation coefficient). N ≥ 502.

Figures

Figure 1. Use of media sources for "science-related purposes" by UW-Madison graduate students who use social media. Traditional media sources (e.g. newspapers, public radio, TV) were reported to be the most used, while reddit was the least used source. (5 point scale, with 1='Never,' 2='Less than once a month' (Rarely), 3='A few times a month' (Occasionally), 4='Once to a few times a week,' and 5='Every day.' 'Once to a few times a week' and 'Every day' were combined into 'Often.' Missing values excluded.)

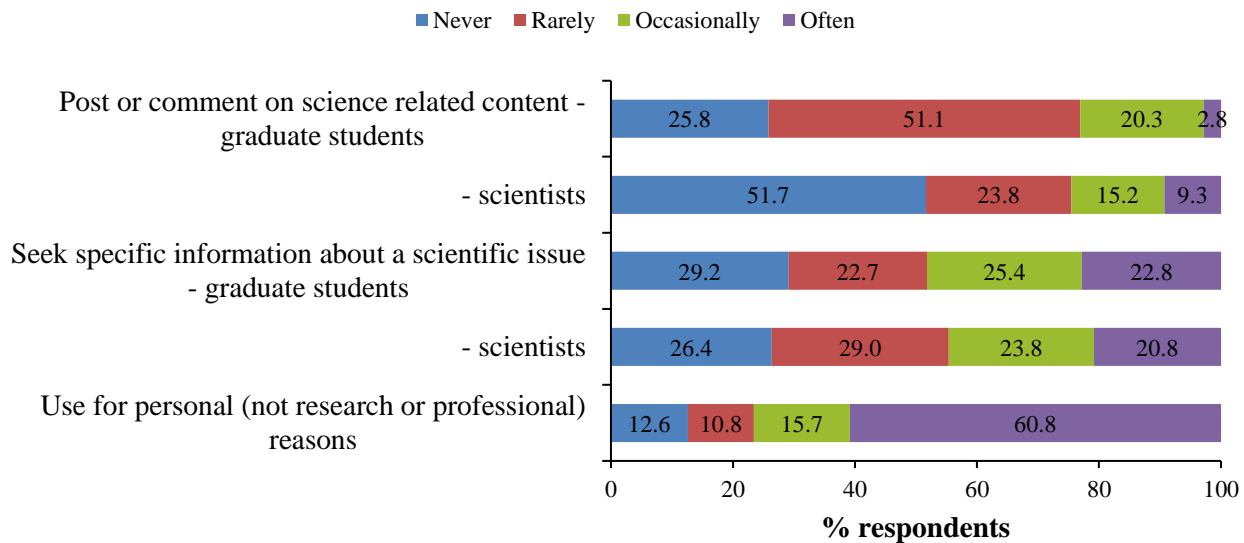# Media sources used by UW-Madison graduate students for science-related purposes

Figure 1.

Figure 2. UW-Madison graduate students' use of social media for personal use or scientific research not related to their own field ($N = 1036$). More than 70% of students reported using social media to seek information or engage in discussion on science-related content. (5 point scale, with 1 = 'Never,' 2 = 'Less than once a month' (Rarely), 3 = 'A few times a month' (Occasionally), 4 = 'Once to a few times a week,' and 5 = 'Every day.' 'Once to a few times a week' and 'Every day' were combined into 'Often.' Missing values excluded.)

Figure 2.

Figure 3. UW-Madison graduate students' use of social media for personal use or scientific research not related to their own field ($N = 1036$). More than 70% of students reported using social media to seek information or engage in discussion on science-related content. (5 point scale, with 1 = 'Never,' 2 = 'Less than once a month' (Rarely), 3 = 'A few times a month' (Occasionally), 4 = 'Once to a few times a week,' and 5 = 'Every day.' 'Once to a few times a week' and 'Every day' were combined into 'Often.' Missing values excluded.)
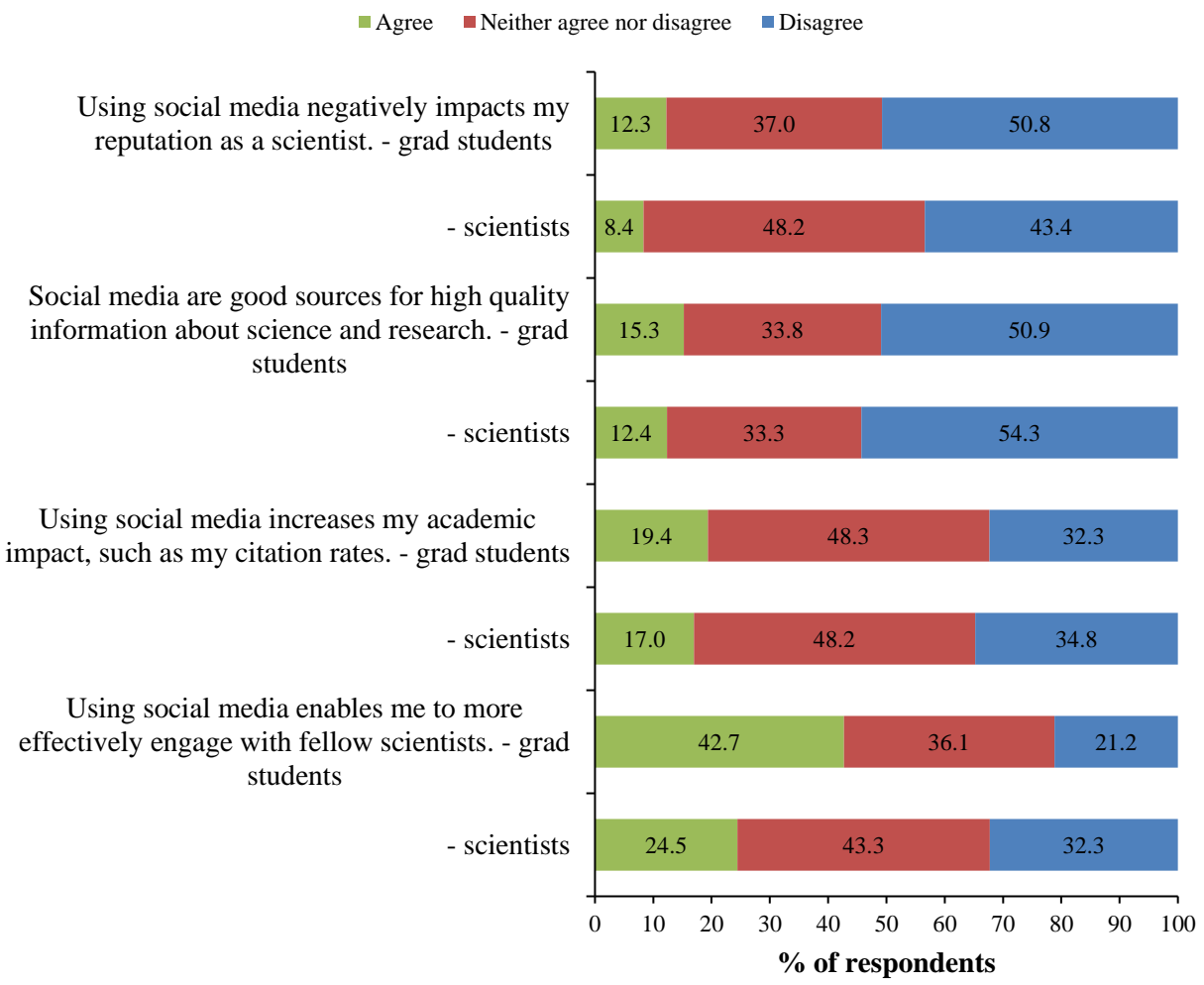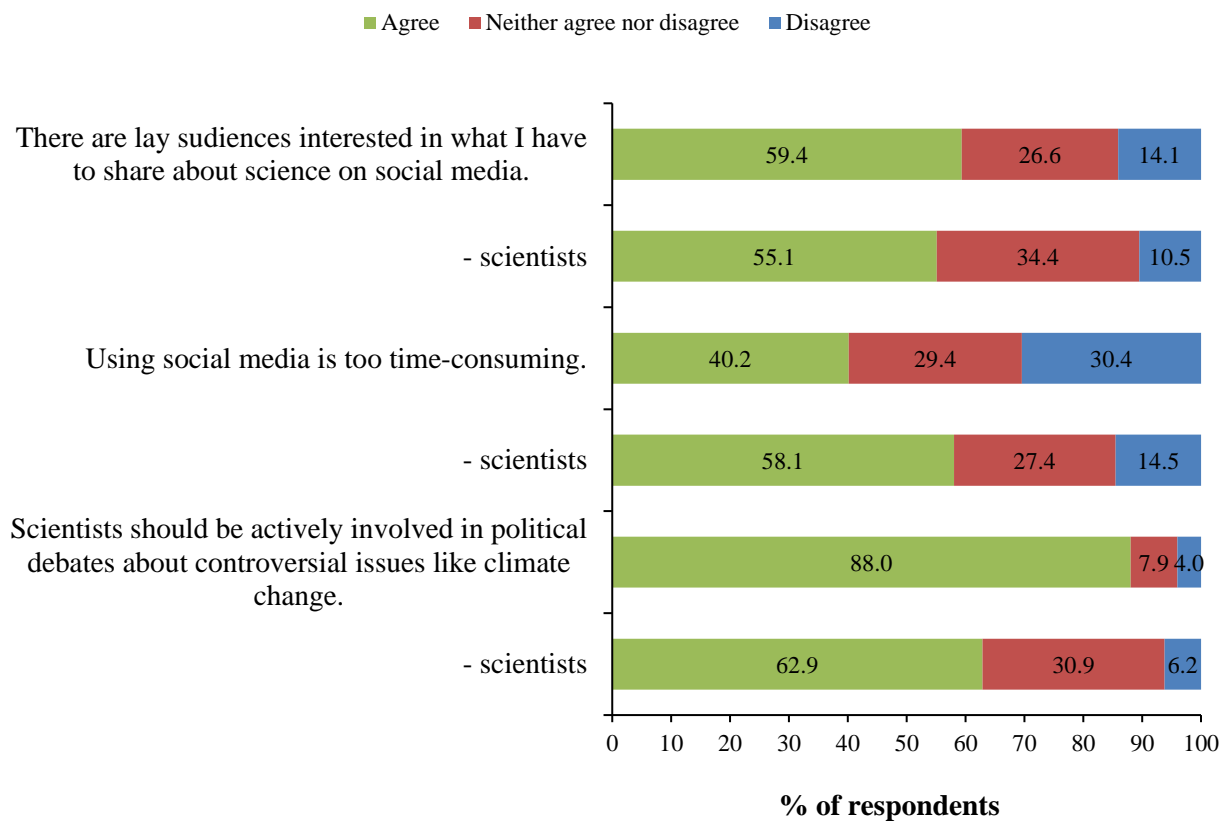
Figure 3.

Figure 4. UW-Madison graduate students' opinions on social media as a tool for public engagement. The majority of students had positive views toward social media used for public engagement (*N* = 929). (5 point scale, from 1 = 'Strongly disagree' to 5 = 'Strongly agree.')
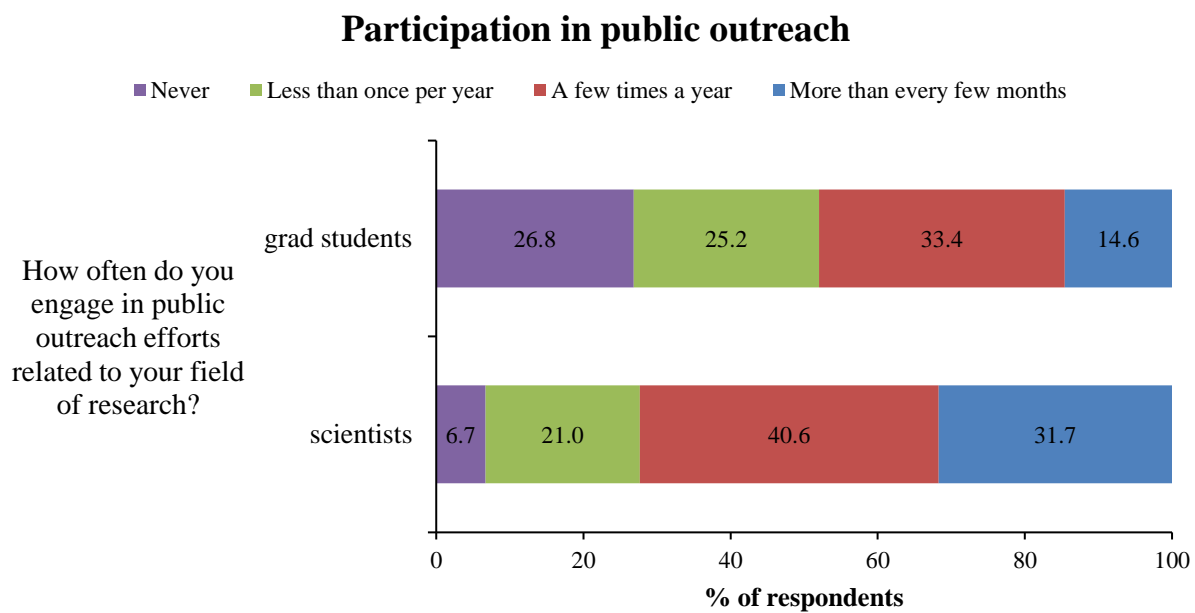
Social media as engagement

Figure 4.

Figure 5. Graduate students' participation in public outreach activities related to their own research. Most students indicated participating in outreach efforts at least once a year ($N = 857$). (5 point scale, where 1 = 'Never,' 2 = 'Less than once per year,' 3 = 'A few times a year,' 4 = 'Every few months to once a month,' and 5 = 'A few times a month.' 'Every few months to once a month' and 'A few times a month' were combined into 'More than every few months.' Missing values excluded.)

Participation in public outreach

■ Never ■ Less than once per year ■ A few times a year ■ More than every few months

grad students: 26.8 | 25.2 | 33.4 | 14.6

scientists: 6.7 | 21.0 | 40.6 | 31.7

How often do you engage in public outreach efforts related to your field of research?

% of respondents

Figure 5.

Figure 6. External factors that may affect graduate student engagement in public science communication. The majority of students indicate they receive support in their science communication efforts ($N = 806$). (5 point scale, from 1 = 'Strongly disagree' to 5 = 'Strongly agree.' Missing values excluded.)

Extrinsic factors affecting public engagement
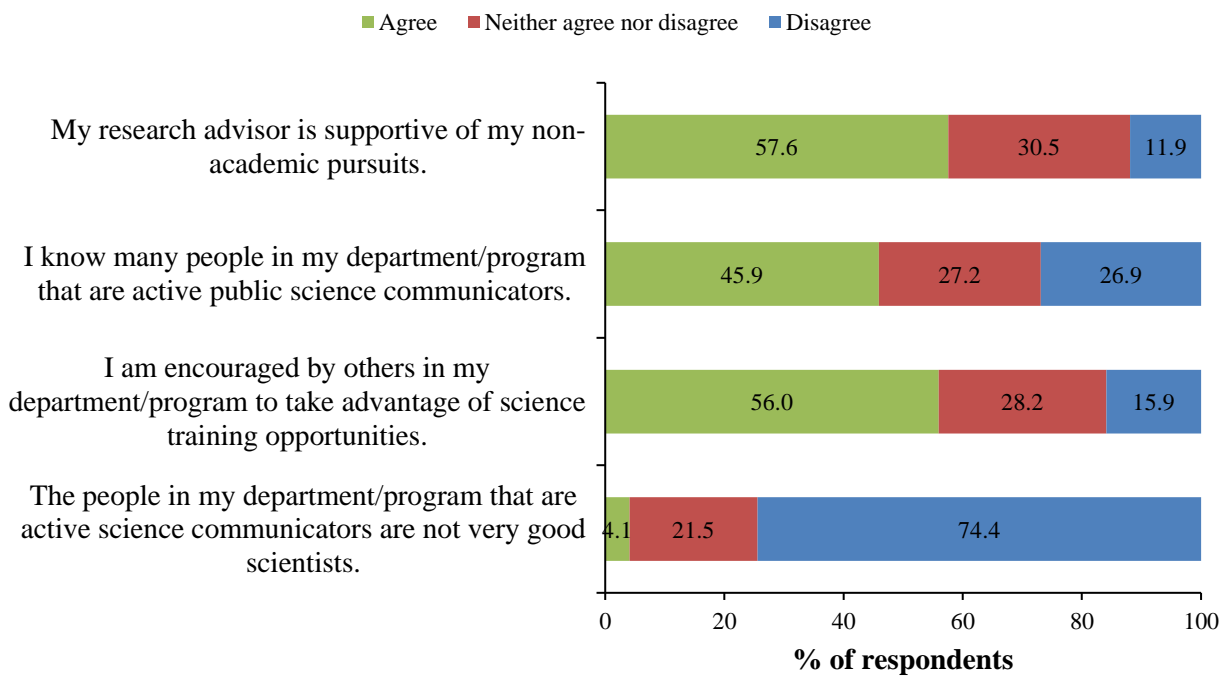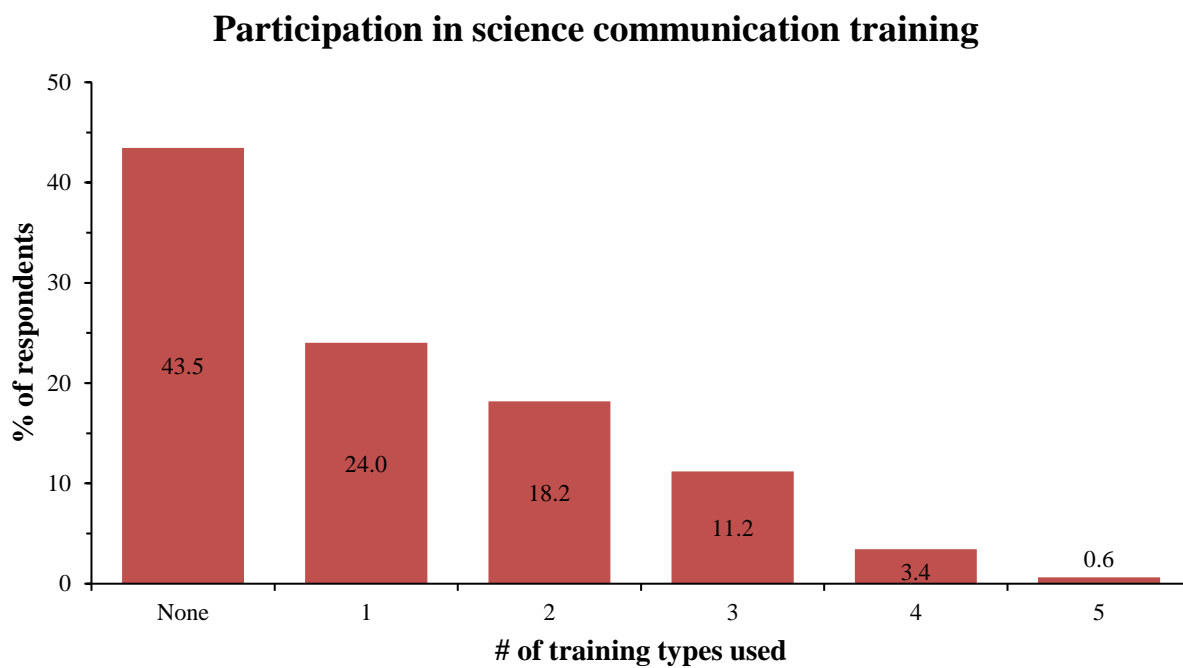
Figure 6.

Figure 7. Intrinsic factors that may affect graduate student engagement in public science communication. Most students feel they are able to contribute to science communication efforts, but are unaware of opportunities to learn about and participate in science communication ($N =$ 744). Answers to the questions 'I am able to contribute to public science communication efforts' and 'I can explain a difficult science concept to a layperson' were combined into 'I am able to contribute to public science communication/communicate science to a layperson' for this report. (5 point scale, from 1 = 'Strongly disagree' to 5 = 'Strongly agree.' Missing values excluded.)

Intrinsic factors affecting public engagement
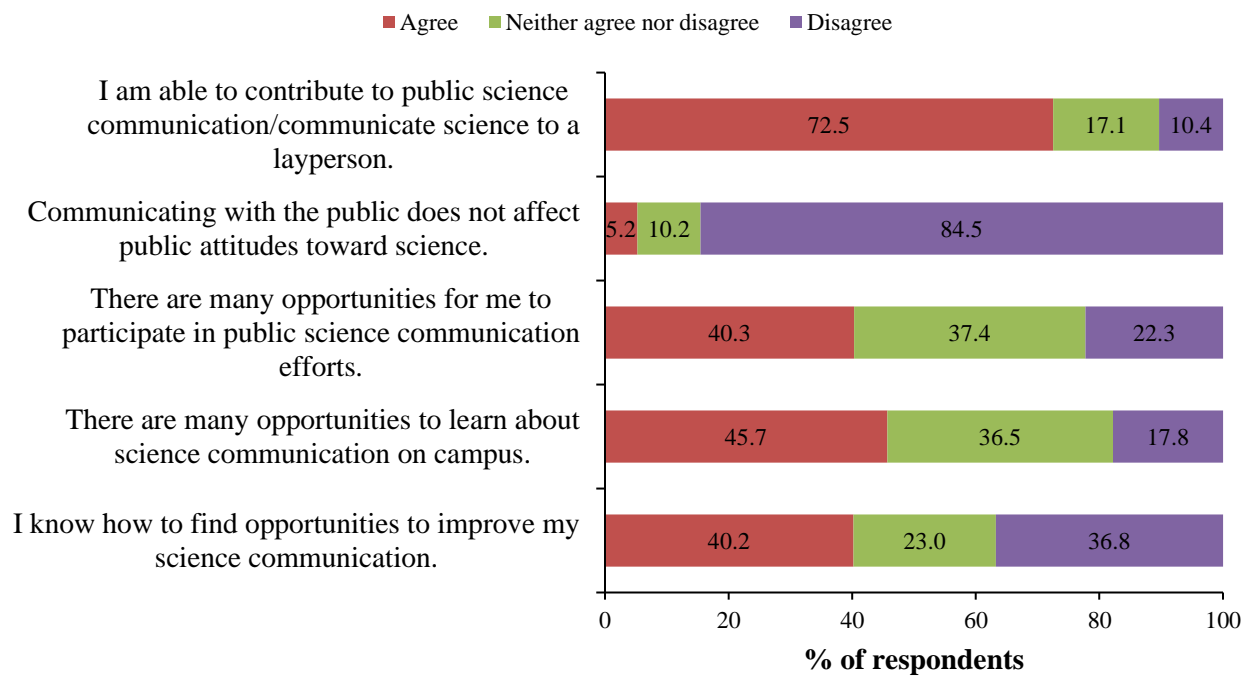
Figure 7.

Figure 8. UW-Madison graduate students' participation in science communication training opportunities. Most students have participated in at least one type of science communication training ($N = 787$).

Figure 8.

Figure 9. Types of science communication training used by graduate students at UW-Madison. Short workshops or seminars were the most frequently used training opportunities ($N = 787$).

Participation in science communication training
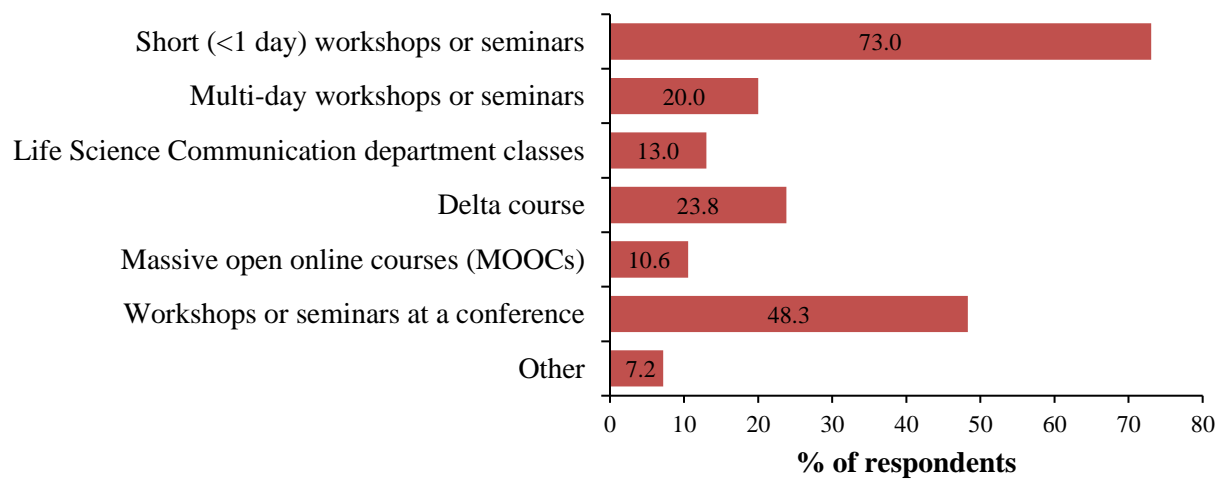
Figure 9.

Figure 10. Perceptions of public engagement in relation to graduate students' career goals. (A) Most students agree that engaging in science communication is relevant to their future career goals (*N* = 786). (5 point scale, from 1 = 'Strongly disagree' to 5 = 'Strongly agree.' Missing values excluded.) (B) The most commonly reported career goal was teaching at a college level (*N* = 746).

A

Public engagement and career goals

■ Agree ■ Neither agree nor disagree ■ Disagree
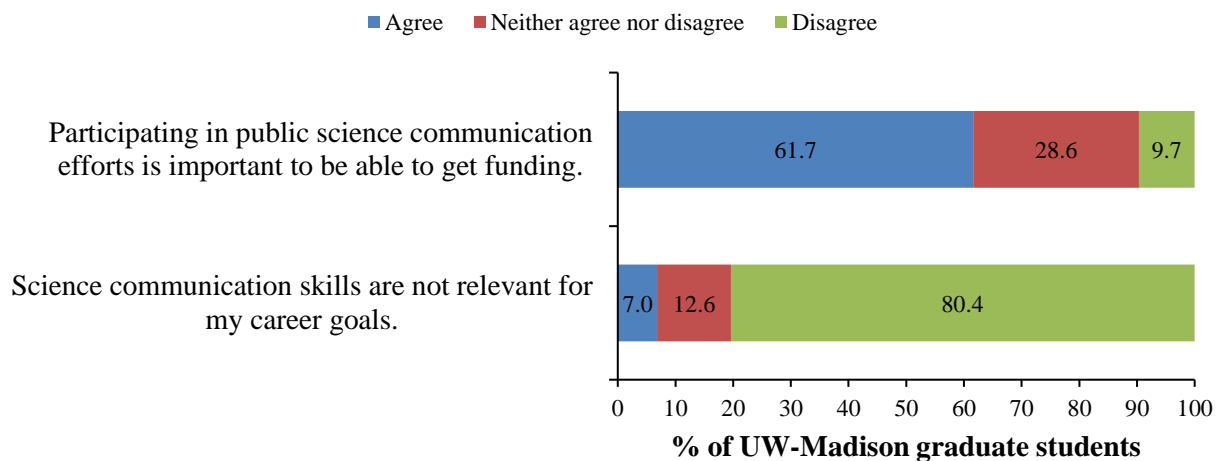
B

UW-Madison graduate student career goals
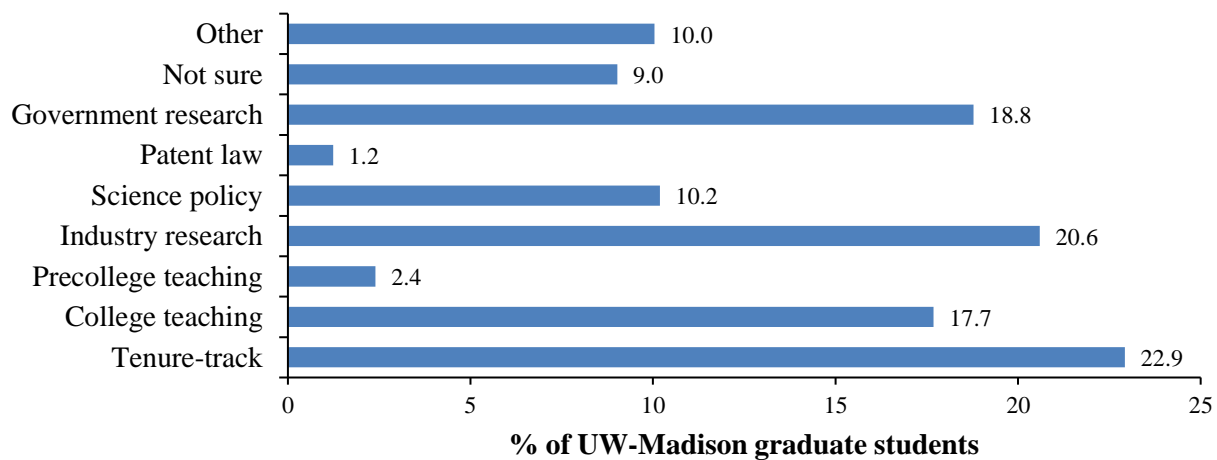
Figure 10.

Figure 11. Graduate students' views of the relationship between science and the public. Most UW-Madison graduate students agree that lay audiences can have valuable perspectives, but disagree that public opinion should be considered more important than scientist opinions on scientific ethics ($N = 744$). (5 point scale, from 1 = 'Strongly disagree' to 5 = 'Strongly agree.' Missing values excluded.)

Graduate student perceptions of lay audiences
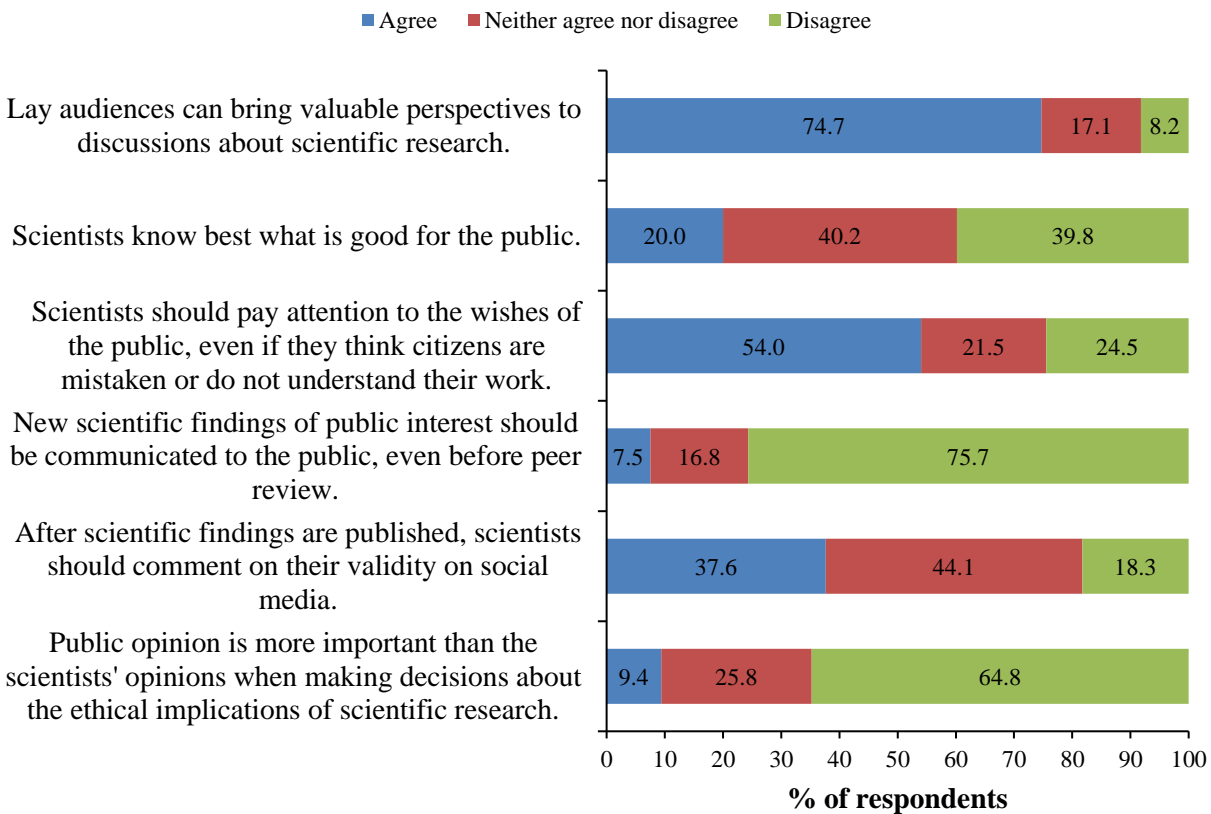
Figure 11.

Appendix—Survey

To begin, here are a few questions about your typical use of media. Please read each item

carefully and select the appropriate option to indicate your response.

1. Do you use social media?

Social media: any online platforms that allow the exchange of user-created content, such as

Facebook, Twitter, ResearchGate, and blogs.

    A. Yes

    B. No

(If "Yes," show 2-4)

2. For each of the following media, please indicate your level of use for **science-related**

purposes. (randomize and record order)

Never	Less than once a month	A few times a month	Once to a few times a week	Every day
1	2	3	4	5

    A. Newspapers and news magazines (online & offline)

    B. Popular science magazines

    C. Public radio (online & offline)

    D. Television (online & offline)

    E. Blogs maintained by other scientists

    F. Other blogs

G. Podcasts

H. Wikipedia/wikis

I. RSS feeds

J. YouTube

K. Restricted online communities, such as ResearchGate or Mendeley

L. General social networks (e.g. Facebook, Twitter)

M. reddit

3. Now thinking specifically about **your own research**, how often do you use social media to do each of the following? (randomize and record order)

Never	Less than once a month	A few times a month	Once to a few times a week	Every day
1	2	3	4	5

A. Post or comment on topics related to your research

B. Participate in discussion about your field of research

C. Write about topics related to your research

D. Share announcements about new studies

E. Engage with peers on post-publication content about your research

4. Now thinking about **research more broadly** (not related to your own field), how often do you use social media to do each of the following? (randomize and record order)

Never	Less than once a month	A few times a month	Once to a few times a week	Every day

1	2	3	4	5

A. Post or comment on science-related content

B. Seek specific information about a scientific issue

C. Follow discussions or debates about science

D. Engage with scientists on post-publication content

E. Share announcements about new studies

F. Use for personal (not research or professional) reasons

5. Here are some statements some scientists have made about using social media in relation to their work. Whether you use social media or not, how much do you disagree or agree with the following? (randomize and record order)

Scientists: all who are in involved in empirical research, including the social sciences.

Lay audiences: individuals who do not have graduate training and expertise in science and research.

Strongly disagree	Disagree	Neither agree nor disagree	Agree	Strongly agree
1	2	3	4	5

A.   There are lay audiences interested in what I have to say on social media.

B.   Using social media enables me to more effectively engage with fellow scientists.

C.   Using social media increases my academic impact, such as my citation rates.

D.   Using social media is too time-consuming.

E.   Social media are good sources for high quality information about science and research.

F.   Using social media negatively impacts my reputation as a scientist.

G.   Scientists should not discuss potentially controversial topics, such as climate change, on social media.

6. Here are a number of statements people have made related to science news and research. Please indicate how much you agree or disagree with each of the following statements. (randomize and record order)

Strongly disagree	Disagree	Neither agree nor disagree	Agree	Strongly agree
1	2	3	4	5

- A. Most of the science news I consume is about my field of research.
- B. The social sciences rely on case studies rather than quantitative evidence.
- C. Social science research mostly yields common sense results.
- D. I consume news about science that is outside my realm of expertise.
- E. My colleagues consume about the same amount of science news as I do.
- F. Social science research is not as rigorous as research in the physical and life sciences.

7. Thinking about your public outreach activities, how often do you....

Public outreach: any scientific communication outside of an academic setting, excluding communication on social media.

Never	Less than once per year	A few times a year	Every few months to once a month	A few times a month
1	2	3	4	5

A. engage in public outreach efforts related to your field of research?

B. engage in public outreach efforts related to science in general?

C. talk to reporters about your research?

8. Continuing to think about your participation in public outreach activities, how much do you agree or disagree with the following statements? (randomize and record order)

Strongly disagree	Disagree	Neither agree nor disagree	Agree	Strongly agree
1	2	3	4	5

A. I am able to contribute to public science communication efforts.

B. I can explain a difficult science concept to a layperson.

C. I enjoy participating in public science communication.

D. Public science communication is stressful.

E. I don't want to participate in public science communication.

F. Participating in public science communication efforts is important to be able to get funding

9. Now thinking about your campus relationships, how much do you agree or disagree with the following statements? (randomize and record order)

Strongly disagree	Disagree	Neither agree nor disagree	Agree	Strongly agree
1	2	3	4	5

A. My research advisor does not want me to participate in activities not related to my research.

B. I am encouraged by my research advisor to take advantage of science communication training opportunities.

C. I am encouraged by others in my department/program to take advantage of science training opportunities.

D. The people in my department/program that are active science communicators are not very good scientists.

E. My research advisor is supportive of my non-academic pursuits.

F. I know many people in my department/program that are active public science communicators.

10. Next, we'd like to ask you about science communication training. Which of the following science communication training opportunities have you participated in?

❑ Short (<1 day) workshops or seminars

❑ Multi-day workshops or seminars

❑ Classes in the Life Science Communication department

❑ Delta courses

❑ Massive open online courses (MOOCs)

❑ Workshops or seminars at a conference

❑ Other (please specify) _____

❑ None

11. How much you agree or disagree with the following statements about training?

Strongly disagree	Disagree	Neither agree nor disagree	Agree	Strongly agree
1	2	3	4	5

    A. There are many opportunities to learn about science communication on campus.

    B. I know how to find opportunities to improve my science communication.

    C. There are many opportunities for me to participate in public science communication efforts.

    D. Science communication skills are not relevant for my career goals.

12. Now thinking about the relationship between scientists and the public, how much do you agree or disagree with the following statements?

Keep in mind that lay audiences are defined as individuals who do not have graduate training and expertise in science and research.

Strongly disagree	Disagree	Neither agree nor disagree	Agree	Strongly agree
1	2	3	4	5

    A. Scientists know best what is good for the public.

    B. Communicating with the public does not affect public attitudes toward science.

    C. Scientists should pay attention to the wishes of the public, even if they think citizens are mistaken or do not understand their work.

    D. Public opinion is more important than the scientists' opinions when making decisions about the ethical implications of scientific research.

E. We depend too much on science and not enough on faith.

F. My scientific work sometimes conflicts with my religious beliefs.

G. Scientists should be actively involved in political debates about controversial issues like climate change.

H. New scientific findings of public interest should be communicated to the public, even before peer review.

I. After scientific findings are published, scientists should comment on their validity on social media.

J. Lay audiences can bring valuable perspectives to discussions about scientific research.

K. It's important to get the next generation excited about science.

Finally, we would like to ask you some questions about yourself.

13. Which of the following best describes your current position?

○ Master's student

○ Doctoral student

○ Postdoc

○ Research scientist

○ Not University affiliated

○ Other _____

14. Are you affiliated with a UW-Madison department in any of the following areas?

❑ Biological sciences (e.g. biochemistry, genetics, entomology)

❑ Physical sciences (e.g. mathematics, physics, chemistry)

❑ Social sciences (e.g. psychology, communication)

❑ Engineering

❑ Not University affiliated

❑ Other (please specify) _____

15. Please write the name of your graduate program, if applicable.

16. What are your future career goals? (select up to 3)

    A. Tenure-track faculty

    B. Teaching (college level)

    C. Teaching (pre-college level)

    D. Industry research

    E. Science policy

    F. Patent law

    G. Government research

    H. Not sure

    I. Other (please specify) _____

17. What is your gender?

❑   Male

❑   Female

❑   Other

❏   Prefer not to answer

18. In what year were you born? (YYYY)

19. Check all of the following categories that describe your race or ethnicity.

❏   American Indian or Alaskan Native

❏   Asian

❏   Black or African American

❏   Hispanic or Latino

❏   Native Hawaiian or Other Pacific Islander

❏   White

❏   Other (please specify)_____

20. The terms "liberal" and "conservative" may mean different things to people, depending on the kind of issue one is considering.

A.   In terms of economic issues, would you say you are...

B.   In terms of social issues, would you say you are…

Prefer not to answer	Very liberal	Liberal	Moderate	Conservative	Very conservative
0	1	2	3	4	5

21. In politics today, do you consider yourself a... (dropdown box)

❑ Republican

❑ Independent

❑ Democrat

❑ Prefer not to answer

22. How much guidance does religion provide in your everyday life?

No guidance at all										Great deal of guidance
0	1	2	3	4	5	6	7	8	9	10

23. What is your present religion, if any?

❑ Agnostic (not sure if there is a God)

❑ Atheist (do not believe in God)

❑ Christian

❑ Hindu

❑ Jewish (Judaism)

❑ Mormon (Church of Jesus Christ of Latter-day Saints/LDS)

❑ Muslim (Islam)

❑ Orthodox (Greek, Russian, or some other orthodox church)

❑ Protestant (such as Baptist, Methodist, Non-denominational, Lutheran, Presbyterian,

Pentecostal, Episcopalian, Reformed, Church of Christ, Jehovah's Witness, etc.)

❏　　　　Roman Catholic (Catholic)

❏　　　　Unitarian (Universalist)

❏　　　　Nothing in particular

❏　　　　Other (please specify)_____

Thank you for taking the time to complete our questionnaire.