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Objective Lens
Double-Blind Peer Review

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Since our inception in 2013, *Fine Focus* has utilized a double-blind peer review system for submitted manuscripts, ensuring that the most objective recommendations are made by members of our Editorial Board. Your submitted manuscripts are redacted of all identifiers (including names, affiliations, and acknowledgements) before they are made available to 2-3 reviewers. This approach is unusual in scientific journals. Etkin and colleagues (2017) have reported that for one well-known academic journal publisher, 95 percent of physical science and health science journals operate single- blind peer review while 72 percent of life sciences journals are single blind. In fact, I am not aware of any journals in the fields of microbiology or science education other than *Fine Focus* which utilize a double-blind review process. Data to substantiate the claim that double-blind minimizes bias in scientific review does vary according to what is specifically being measured, and by what methods. However, the general consensus viewpoint, reflected in several studies indicates that double-blind manuscript review effectively reduces or eliminates nepotism, gender bias, geographic bias, and personal bias against (or in favor of) specific research groups, laboratories, and/or PIs (Thomkins *et al.*, 2017; Okike *et al.*, 2017), and that most scholars prefer it over a single-blind review process (Ware, 2008).

The purpose of this writing is not to explore the topic comprehensively, but rather to inform and justify to our readers as to why the first international journal in undergraduate microbiology research, *Fine Focus*, has opted to use double-blind review over the traditional single – blind process. No system is perfect, but thus far, no more suitable manuscript review system has been adopted that has enjoyed any degree of practicality and persistence above that of single- or double-blind peer-review. The reason that the single-blind approach remains dominant in the sciences (but not in social sciences and humanities journals, where only 15% use single-blind review according to Etkin *et al.*, 2017) is unclear, but change comes very slowly in the global community of scientists and educators, where collaboration and information-driven action can generate a myriad of opinions and arguments with little hope of decisive action and fundamental change in this regard.

Arguments against double-blind peer review in the sciences include the notion that preparation of manuscripts with redacted identifiers (or blinding the papers by editorial staff after authors have submitted them) would be overly burdensome or too complicated. However, as any active author, or a managing or section editor for an academic journal could attest, this process is quite straightforward and certainly not as time-consuming or technically challenging as most of the other actions necessary during the manuscript submission/uploading process. Critics of double-blind review also indicate that reviewers could probably guess the authors identity and/or affiliations based on the content of the research, key words, or the authors' results. This is unlikely, and in any case a reviewer who would go to this length in a petty attempt to guess the authors is not someone who is likely to be an effective reviewer anyway, and probably should not be on the Board. Following my service as a reviewer, and/or member of the Editorial Board for the *CUR Quarterly, Journal of Food Protection, Journal of Dairy Science, Biologia,* and now Managing Editor of *Fine Focus*, I can submit that most reviewers are well-meaning, sincere, and ethical. They want to do a good job, and evaluate the science more than the authors or the place in which the work was done. Double-blind peer review is a good way to ensure that they stay honest and maintain confidence in our system by the submitting authors and editorial staff.

References

1. Etkin, A. , *et al.* 2017. Peer Review: Reform and Renewal in Scientific Publishing DOI: http://dx.doi. org/10.3998/mpub.9944026

2. Tomkins, A., Zhang, M., Heavlin, W.D. 2017. Reviewer bias in single-versus double-blind peer review. PNAS 114(48):12708–12713. doi: 10.1073/pnas.1707323114.

3. Okike, K., Hug, K.T., Kocher, M.S., Leopold, S.S. 2016. Single-blind vs double-blind peer review in the setting of author prestige. JAMA 316(12):1315–1316. doi: 10.1001/jama.2016.11014.

4. Ware, M. 2008. "Peer Review: Benefits, Perceptions and Alternatives." Publishing research consortium summary papers. Web

Biogenesis of Lipoproteins in Gram-Negative Bacteria: 50 Years of Progress

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Abstract

The outer membrane is the defining characteristic of Gram-negative bacteria and is crucial for the maintenance of cellular integrity. Lipoproteins are an essential component of this outer membrane and regulate broad cellular functions ranging from efflux, cellular physiology, antibiotic resistance, and pathogenicity. In the canonical model of lipoprotein biogenesis, lipoprotein precursors are first synthesized in the cytoplasm prior to extensive modifications by the consecutive action of three key enzymes: diacylglyceryl transferase (*Lgt*), lipoprotein signal peptidase A (LspA), and apolipoprotein N-acyltransferase (Lnt). This enzymatic process modifies lipoprotein precursors for subsequent trafficking by the Lol pathway. The function of these three enzymes were originally thought to be essential, however, in some Gram-negative bacteria, namely *Acinetobacter baylyi*, the third enzyme Lnt is dispensable. Here we review the function and significance of *Lgt*, LspA, and Lnt in outer membrane biogenesis and how non-canonical models of lipoprotein processing in *Acinetobacter* spp. can enhance our understanding of lipoprotein modifications and trafficking.

Introduction

The general structure of Gram-negative bacteria such as *Escherichia coli* and *Acinetobacter baylyi* consists of the inner membrane (IM) and outer membrane (OM) separated by an aqueous periplasm containing a thin peptidoglycan layer (1). The outer membrane is the defining characteristic of Gram-negative bacteria and is essential for the maintenance of cellular integrity. The OM is composed of phospholipids and a lipopolysaccharide asymmetric bilayer containing β-barrel OM proteins (OMPs) (2). In order for correct biogenesis of the OM to occur, lipopolysaccharides must be delivered to the OM by the Lpt machinery while the Beta-barrel assembly machinery (BAM) complex assembles OMPs into the OM (3). Proteins destined for the OM are synthesized in the cytoplasm with a signal peptide used for translocation to the IM by the general secretion (Sec) pathway for unfolded proteins or through the twin-arginine translocation (Tat) pathway for fully folded proteins (4–6). Deviations or loss of function in the assembly machinery needed to synthesize the OM results in profound defects causing morphological defects such as antibiotic and temperature sensitivity (7).

Outer membrane lipoproteins have vital functions including aiding in lipopolysaccharide (LPS) insertion in the OM and forming secretion systems (8, 9). LPS is found in the outer leaflet of the OM in most Gram-negative bacteria and is thus essential because it interacts directly with the outside environment of the bacterium and maintains the impermeability of the OM (10–13).

The proteins, LptD and LptE are components of the Lpt machinery which function to transport the essential LPS to the OM (8, 9). LptE and LptD form a complex in which LptE is buried inside LptD and functions as a plug to prevent the passage of molecules through LptD into the bacteria (14– 18). There are also surface-exposed lipoproteins such as Lpp and Pal which function to increase cell wall stability by attaching the OM to the cell wall (19–21). Other surface-exposed lipoproteins are part of stress responses including RcsF which is the sensory component of the Rcs envelope stress response (22, 23). This stress response can be activated by LPS stress and osmotic stress (24–27).

Lipoproteins are also essential in assembly of the OM and regulating the traffic of molecules inside and outside of the Gram-negative cell (28). These OM lipoproteins can release virulence factors to the surrounding environment resulting in infection (29, 30). They also can release toxins or hazardous chemicals for defense (31). Another assembly of OM proteins is the BAM complex which functions to facilitate the formation of beta- barrels in the OM (32). Blocking OM lipoprotein trafficking or inhibiting their function can result in cell death (33). If a lipoprotein is not trafficked, the cell cannot regulate the efflux of molecules across the membrane, depriving it of resources and preventing the cell from expelling hazardous chemicals. Therefore, the lipoprotein sorting pathway is integral to Gram-negative bacteria because lipoproteins in the OM are essential to maintaining the cell envelope.

Synthesis of lipoproteins begins in the cytoplasm. Lipoproteins are synthesized as precursor pre-prolipoproteins in the cytoplasm prior to being trafficked through the ABC transporter localization of lipoproteins (Lol) pathway (34). Once unmodified pre- lipoproteins destined for the OM are translated, they enter the IM through the Sec or Tat pathway (35) (Fig. 1a).

The signal peptide embeds the protein to the IM and allows for recognition for the first step of modification (36). Di-acylglyceryl transferase (*Lgt*) is the first protein in the pathway and transfers di-acylglycerol to the cysteine residue in the lipobox of the prolipoprotein (37). *Lgt* has been thought to be essential in all Gram-negative bacteria because it is the first step of lipoprotein maturation which is required to be recognized and translocated using the Lol system (37, 38). However, insertion mutations have been created in the *lgt* gene of *Acinetobacter baumannii* which have yielded viable cultures (39). The second step of lipoprotein maturation is done by prolipoprotein signal peptidase (LspA) which cleaves the signal peptide leaving the lipobox at the N-terminus. LspA is also essential due to its ability to cleave the anchor peptide from the IM. The heavily hydrophobic region is what allows and maintains the protein in the IM, therefore its release is integral for lipoprotein movement to the OM as well as function and folding. The third and final step in lipoprotein maturation is the addition of one more acyl

group to the cysteine residue by apolipoprotein transacetylase (Lnt) (Fig. 1a). Together, these processes define the canonical lipoprotein modification pathway in Gram-negative bacteria (34).

Classically it was thought that Lnt-mediated tri-acylation was an essential requirement for recognition by the Lol system and subsequent lipoprotein trafficking. However, Lnt was found to be nonessential in some Gram-negative bacteria including *Francisella, Neisseria, and Acinetobacter* (40,41). The loss of Lnt is not without detriment, as the absence of a third acylation interrupts lipoprotein trafficking resulting in visible growth defects such as increased OM permeability in *Acinetobacter* spp. (42). The viability of *Acinetobacter* spp. after *lnt* deletion suggests a non-canonical function of the existing lipoprotein modification and trafficking pathway (Fig. 1b).

If an apo-lipoprotein is successfully tri-acylated and cleaved, to become a mature lipoprotein and reach the OM, it must be translocated there by the Lol system (34). The LolCDE proteins exist in the IM where it receives the tri-acylated lipoproteins, as studied in *E. coli* (43). If destined for the OM the periplasmic chaperone LolA then shuttles the lipoproteins to the OM receptor LolB for inclusion into the OM (38, 44–46). In *Francisella* spp., a fusion protein resembling *E. coli* LolC/LolE exists that shares high homology among functional domains of the transport system (41). This high homology of the fusion protein and subsequent pathway probing identified this protein as LolF, a distinct protein among certain gram- negative bacteria namely *Neisseria, Francisella, and Acinetobacter* (42, 47). LolF was shown to accept di-acylated lipoproteins as compared to the rejection of di-acylated lipoproteins by LolCDE (41).

The LolF arrangement and successful trafficking of di-acylated lipoproteins in Lnt-deficient *A. baylyi* suggests the presence of a non-canonical function of the lipoprotein modification and trafficking pathway (Fig. 1b). In the process of exploring the non-canonical function of this pathway, it is essential to review the known structures, functions, and mechanisms of the canonical pathway. Here we compile research spanning 50 years for the creation of a concise and accessible resource

Figure 1.

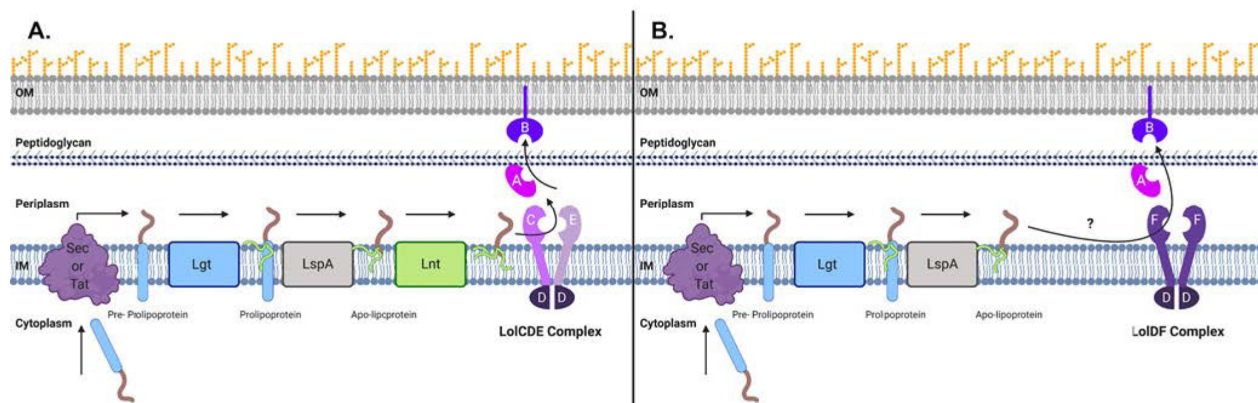

Figure 1. Lipoprotein biogenesis and trafficking in Gram-negative bacteria. (A) In the canonical lipoprotein biogenesis and trafficking pathway, pre-prolipoproteins from the cytoplasm are transported to the periplasm by the Sec or Tat pathway where Lgt adds two acyl chains (green) to the conserved cysteine residue of the lipoprotein precursor. Here, LspA cleaves the signal sequence (blue) and the third and final acyl chain is added to the amino terminus by Lnt. The IM bound LolCDE proteins then receive tri-acylated lipoproteins and can be transported by the periplasmic chaperone LolA to shuttle the lipoprotein to the OM receptor, LolB, for inclusion into the OM. (B) In the non-canonical lipoprotein trafficking pathway of Gram-negative bacteria with LolDF fusion proteins, di-acylated lipoproteins are still capable of transportation to the OM by an unknown mechanism or alternative pathway. Created with Biorender.com.

regarding lipoprotein processing and associated enzymes within Gram-negative bacteria.

Diacylglyceryl Transferase (*Lgt*).

Before lipoproteins are translocated to the OM through the Lol system, they are first modified by *Lgt* in the IM (37). This IM protein di-acylates the prolipoprotein which is the first step to becoming a mature lipoprotein. All lipoproteins that have been identified have N-acyldiacylglyceryl-cysteine as their N-terminal amino acid (48). The *lgt* gene was first discovered in temperature sensitive mutants of *S. typhimurium* in which there was an accumulation of unmodified prolipoproteins at 42°C (49). The prolipoproteins in these temperature sensitive mutants did not have any glyceryl modification. Furthermore, a complementation test with a cloned insert of this deletion restored glyceryl modification activity. This cloned gene was determined to code for diacylglyceryl transferase (*Lgt*). In *E. coli*, the *umpA* gene was determined to code for *Lgt* after demonstrating high levels of similarity to the *lgt* gene in *S. typhimurium* (50).

***Lgt* is generally essential in Gram-negative bacteria when compared to Gram-positive bacteria.** Lipoprotein modification has been studied more extensively in Gram-negative bacteria compared to Gram-positive bacteria. It has been determined that Gram-negative bacteria in which *Lgt* is essential include *E. coli* and *S. typhimurium* (49, 51, 52). However, there are some Gram-negative bacteria such as *A. baumannii* in which insertion mutations are present due to transposons in the *lgt* gene, yet there viable cells persist (39). Furthermore, *Lgt* has been determined to not be essential in Gram-positive organisms such as *Bacillus subtilis* and *Streptococcus pneumoniae* (53, 54). *B. subtilis* and *S. pneumoniae* cultures were still viable after *lgt* deletion (53). However, *lgt* was deemed essential for virulence in *S. pneumoniae* (54). It has been hypothesized that the function of *Lgt* in Gram-positive bacteria may be analogous to substrate-specific sorting enzymes which translocate wall-anchored proteins (55, 56).

***Lgt* consists of 291 amino acids and is comprised of seven transmembrane helices.** The crystal structure of *Lgt* was identified in *E. coli* where it is composed of 291 amino acids (33 kDa) (57). There are seven transmembrane helices which form the core of the protein and there are two phosphatidylglycerol binding sites, R143 and R239 (Fig. 2a). These two binding sites were determined to be critical through the use of complementation tests with *lgt* knockout cells and different mutant variants. There are six beta strands and four short helices. One of the critical catalytic sites, R143, which faces towards the periplasm, directly binds the substrate phosphatidylglycerol for transfer. This site is positively charged which makes it a prime residue for binding the negatively charged phosphatidylglycerol. The other phosphatidylglycerol binding site is E151. The residue R239 was also determined to be essential for diacylglyceryl transfer and functions by forming a hydrogen bond with the C3 of diacylglycerol. These two key residues are the catalytic sites which transfer di-acylglyceryl to the pre-prolipoprotein.

Diacylation of the prolipoprotein begins by transferring a non-acylated glyceryl of phosphatidylglycerol to the sulfhydryl group followed by O-acyltransferase catalyzed acylation of the glyceryl moiety. To determine the mechanism of action for this diacyl modification, Braun's lipoprotein was used in *E. coli* (25, 27, 28). Braun's lipoprotein is a murein lipoprotein which has the same composition as previously identified lipoproteins with a glyceryl cysteine (S-propane-2',3'-diol)-3-thio-2-aminopropanic acid) at the peptide end attached to two fatty acids with another fatty acid bound at the N-terminal (48, 58). To become a mature lipoprotein, the first step of modification is done by *Lgt* which di-acylates the prolipoprotein (37). The mechanism of di-acylating the prolipoprotein was proposed to begin by transferring a non-acylated glyceryl of phosphatidylglycerol to the sulfhydryl group of the cysteine residue at the N-terminal of the prolipoprotein (48, 60). The sn-2 and sn-3 hydroxyls of the glyceryl moiety are then acylated by O-acyltransferase enzymes to form the di-acylglycerylated lipoprotein.

Lipoprotein Signal Peptidase (LspA)

Signal II peptidase encoded by *lspA* in Gram-negative bacteria cleaves the signal peptide from the pre-prolipoprotein at the lipobox residue consensus sequence LAGC (L−3A−2G−1C+1) during the intermediate step of prolipoprotein processing

(61). The cleavage event results in an invariable Cys residue becoming the N-terminal +1 residue, allowing for the prolipoprotein to proceed to the final acylation step by Lnt(62).

LspA is ubiquitous among all known Gram-negative bacteria and homologs have been found in select Gram-positive bacteria. LspA is ubiquitously conserved among all known Gram-negative bacteria and homologs have been found in select Gram-positive bacteria (e.g. *Staphylococcus aureus*) (63). Structurally, among both Gram-positive and Gram-negative eubacteria LspA contains one conserved residue essential for stability, Asp-14, and five conserved residues important for catalytic function; Asn- 99, Asp-102, Asn-126, Ala-128, and Asp- 129 (64) (Fig. 2b). Functionally, in Gram-negative bacteria LspA is considered essential under standard laboratory conditions while in Gram-positive bacteria LspA is considered conditionally essential for virulence (65, 66). There are no known homologs in the domain Eukaryota (63).

LspA consists of 169 amino acids and is comprised of two main domains containing four transmembrane helices. From the crystal structure of LspA isolated from *Pseudomonas aeruginosa*, the 169 amino acid long (18 kDa) inner membrane protein is comprised of two main domains (67). The first domain consists of four transmembrane helices culminating in N and C termini located in the cytoplasm (68) and the second domain consists of a periplasmic domain further separated into two subdomains (67) (Fig. 2b). The larger subdomain is a β-cradle that rests on the membrane extending away from the protein's helical core presenting its polar surface to the periplasm. The smaller and second subdomain contains a periplasmic helix which extends perpendicularly from the β-cradle into the periplasm (67).

Figure 2.

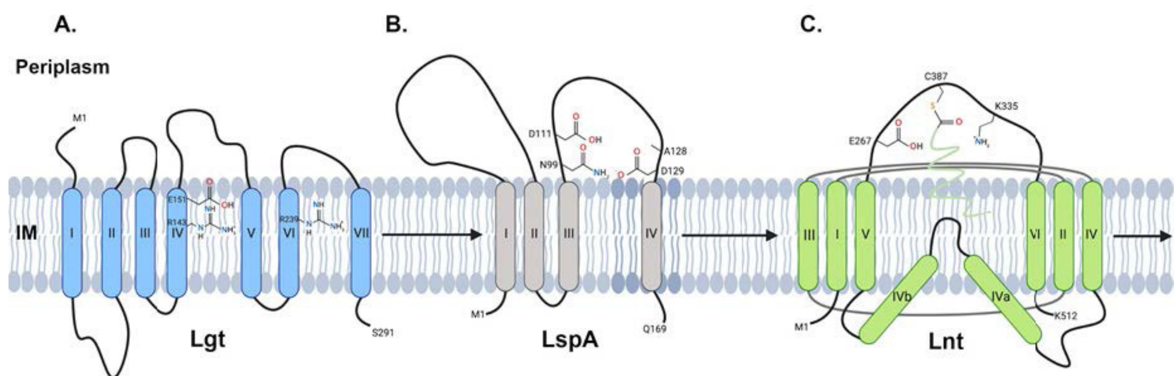

Figure 2. Proposed model of peptide topology for Lgt (blue), LspA (grey), and Lnt (green) in the inner membrane. Roman numerals indicate transmembrane domains. (A) Lgt contains seven transmembrane domains with six beta strands. PG molecules are bound to the Arg143 and Arg239 residues in transmembrane domains IV and VI respectively, with Glu151 in transmembrane IV essential to the acyl transfer. The loop between transmembrane VI and VII is a gate for the entrance of the pre-prolipoprotein. After the acyl transfer, another PG molecule docks on the protein and the process repeats. Adapted from Pailler et al., 2012. (B) LspA in addition to the transmembrane domains, has a β-cradle in the first periplasmic exposed domain that retains the majority of the lipoprotein during proteolytic cleavage. LspA also contains a periplasmic helix in the second periplasmic domain. The signal peptide of the prolipoprotein is wedged between transmembrane II, III, and IV from recognition sites Asp111, Asp129, and Asn99. Adapted from Muñoa et al., 1991 and Tjalsma et al., 1999. (C) Lnt exists as a thioester acyl intermediate with acyl group attached to C387. Lnt has a beta-barrel-like structure with a catalytic cavity. The apolipoprotein enters the cavity laterally, stabilized by the loop between transmembrane domains IVa and IVb. Adapted from Gélis- Jeanvoine et al., 2015. Created with Biorender.com.

LspA shares a mechanism of action similar to that of the aspartic protease family. Based on structural context and the functional domain homology to the family of aspartic proteases, LspA is proposed to form a catalytic dyad at residues Asp-102 and Asp-129 while residues Asn-45, Asn-99, Asp-111, Asn-126, and Ala-128 create the geometry and recognition site for the lipobox of prolipoproteins (64). The proposed mechanism of LspA mediated signal peptide cleavage is as follows. Upon binding to a lipid-modified precursor, the carbonyl carbon of the scissile peptide bond is hydrated creating a tetrahedral intermediate (64). At this point a proton is transferred by means of a lytic water molecule on the initial protonated aspartic acid residue to another aspartic acid residue. The tetrahedral intermediate then donates a proton from one hydroxyl group to the recently charged aspartic acid residue (64). Simultaneously the nitrogen atom of the scissile peptide bond receives a proton from the catalytic aspartic acid residue resulting in the peptide bond cleavage of the signal peptide from the lipobox motif of the prolipoprotein. This proposed mechanism of LspA enzymatic action is further supported by LspA ability to function in the absence of metal ions suggesting LspA does not use classical catalytic mechanisms of metalloproteases (69).

LspA presents novel targets for drug intervention of bacterial infection. LspA is of particular significance to the development of antibiotics in response to the increasing epidemic of antibiotic resistant bacteria (70). Since LspA is broadly conserved and essential in Gram-negative bacteria and some Gram-positive bacteria it presents as a suitable target for broad-spectrum antibiotic development (63). Additionally the correct synthesis of lipoproteins by LspA-mediated enzymatic processes has been implicated in pathogenicity even in bacteria where LspA is not considered essential (e.g. *Mycobacterium tuberculosis*) (71). LspA is also absent in all eukaryotic cells avoiding the possibility of off-target effects on host organisms (63). In comparison to the chronological development of other antibiotics and their respective targets, LspA as a target for antibiotic development is relatively new decreasing the risk associated with "legacy" antibiotics and acquired resistance (70).

In early antibiotic discovery trials, a cyclic peptide antibiotic Globomycin was isolated from *Streptomyces* spp. and administered to a panel of bacteria to determine antibiotic sensitivities (72). In *E. coli* and other Gram-negative bacteria, growth was severely inhibited by this molecule and resulted in the formation of spheroplasts indicating profound membrane assembly defects (72). Further studies in *E. coli* showed Globomycin treatment resulted in the bioaccumulation of di-acylated prolipoproteins in the cytoplasmic membrane and subsequent death of the affected bacterial cell (73). In vitro incubation of LspA enzyme and prolipoprotein substrates in the presence of Globomycin showed an inhibition of LspA enzymatic activity on the cleavage of the signal peptide present on the prolipoprotein (74). Decades later, the mechanism by which Globomycin prevented LspA enzymatic activity was determined by the crystal structure of Globomycin bound to LspA from *P. aeruginosa* (67). Globomycin was found to infiltrate the LspA binding pocket consisting of conserved residues, typically specific for prolipoprotein substrates, and tightly bind both aspartic residues implicated in cleavage as described above (67). The occupation of both active enzymatic residues prevents typical enzymatic function resulting in the observed OM defects and subsequent death of the bacterium. Globomycin is an efficient inhibitor of Gram-negative bacterial growth and affords the benefit of targeting LspA, which has no eukaryotic homolog. Therefore, the development of Globomycin analogs which can more efficiently mimic prolipoproteins and bind LspA is considered a promising avenue of research in the development of new antimicrobials (67).

Another bacterial secondary metabolite derived antibiotic, myxovirescin, was found to inhibit Gram-negative bacteria prolipoprotein processing and subsequent growth in a mechanism similar to that of Globomycin despite having a unique molecular structure (75). Even though operating on convergent mechanisms of LspA inhibition, myxovirescin was shown to be rapidly bactericidal by a magnitude of almost 10-fold compared to Globomycin (75). The advent of another LspA targeted antibiotic with even less incidence of spontaneous resistance compared to Globomycin reinforces the importance of LspA as a viable target for antibiotic discovery (63).

Apolipoprotein N-acyltransferase (Lnt)

The last post-translational modification in the lipoprotein sorting pathway is performed by apolipoprotein N-acyltransferase (Lnt), an IM bound protein. Lnt acquires an acyl group from a glycerophospholipid and transfers to the +1 cysteine, the same residue previously bonded to the now cleaved signal peptide, via an amide bond (52, 76). This final modification acts as a conformational structure for Lol system translocation to the OM (77).

Lnt is conserved among Gram-negative bacteria but has been found in *Mycobacterium* spp. Lnt is extremely well conserved among Gram-negative bacteria but has also been found in *Mycobacterium* (78, 79). While *Lgt* and LspA are universally prominent among both Gram-negative and Gram-positive bacteria, Lnt is not. The purpose of the third acylation by Lnt is thought to be for recognition for translocation to the OM, therefore Lnt does not have a function in Gram-positives. Interestingly, Lnt homologs have been identified from BLASTp in *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* as Ppm1. Ppm1 has also been confirmed to transfer an acetyl group to the N-terminus, similar to the Lnt mechanism (78). The tri-acylated lipoproteins are suspected to contribute to *M. tuberculosis* virulence factors (71).

Glycerophospholipids are the prominent substrates for Lnt. Lnt transfers an acyl group from 3 different glycerophospholipids. Phosphatidylethanolamine (PE), the most abundant phospholipid, constituting around 70% of the cellular lipid content, is the prominent substrate for Lnt, but not essential for Lnt function (80). Phosphatidylglycerol (PG), the second most abundant phospholipid, is a substrate for Lnt as well, but not used as efficiently as PE (81, 82). Interestingly, in mutants lacking PE, PG was used for final acylation without defects. Phosphatidic acids (PA) is one of the least abundant membrane lipids but can be used as a substitute for PG efficiently.

Lnt consists of 512 amino acids and is comprised of six transmembrane segments. Lnt has six transmembrane (TMS) segments with both the carboxy-terminus and amino-terminus exposed towards the cytoplasm (83). A large, 79 amino acid long, cytoplasmic loop (CL-2) is present between TMS-IV and TMS-V and contains two hydrophobic segments (TMS-IVa and TMS-IVb) (83). These hydrophobic segments are not fully integrated into the membrane and are partially exposed to the cytoplasm (Fig. 2c). Similar morphology to a reentrant loop motif, these segments may be used for channeling in substrates, but CL-2 lacks key secondary structure for a reentrant loop (84). It is hypothesized that instead, CL-2 forms a titled fold similar to an intramembrane protease GlpG in *E. coli* (85, 86). Lnt also contains a periplasmic exposed nitrilase domain between TSM-V and TSM-VI (83). It is proposed that CL-2, TSM-IVa, and TSM-IVb are surrounded by the six TMS forming a beta-barrel-like structure (85). Lnt contains a catalytic cavity inside the beta-barrel-like structure, but the lipoprotein substrate enters the structure laterally, a mechanism similar to integral membrane proteins (87), Lnt also exists as a thioester acyl intermediate to allow for higher processivity and acylation for essential OM-bound lipoproteins (52).

The mechanism for acylation transfer is a two-step process comprised of auto-acylation followed by acyl transfer. The mechanism for acylation transfer is most likely a two-step process, auto-acylation followed by acyl transfer (52, 81). Due to the massive amount of lipoproteins transported to the OM and the low abundance of the enzyme, Lnt existing as an acylated intermediate allows for higher processivity of the acyl transfer (52, 81). The active site of Lnt is in the nitrilase domain exposed to the periplasm. For auto-acylation, C387 sulfhydryl group initiates with a nucleophilic attack on the alpha carbonyl of a phospholipid. The resonance from E267 attacks the hydrogen on C387 sulfhydryl group allowing the fatty acid chain to remain on C387, yielding a thioester acyl Lnt (52). For the acyl transfer, the newly exposed nitrogen on the di- acylated lipoprotein then attacks the alpha-carbon of the acyl group on C387. The next step requires C387, K335, and E267 on the nitrilase domain, W237 on the β1/α1 loop, F358 and M362 on β5/ β6 loop, and R139 and P147 on CL-2 (85).

Lnt is essential in *E. coli* containing the LolCDE pathways, but not other Gram-negative bacteria which have LolFD pathways. In *E. coli*, *lnt* is essential. Without the final acyl

modification, OM destined lipoproteins are mislocalized (77). This results in envelope stress and an inability to produce functional channel proteins (73). However, in *Acinetobacter baylyi, Acinetobacter baumannii, Francisella tularensis, and Neisseria gonorrhoeae, lnt* is not essential (88). It is unclear if these Gram-negative bacteria can translocate di-acylated lipoproteins or another enzyme performs the same function. *Francisella* and *Acinetobacter* produce a novel Lol complex, LolDF, as opposed to the LolCDE characterized in *E. coli* and many other Gram-negative bacteria (41) (Fig. 1b). In transcriptomic studies of Lnt deficient *A. baylyi,* LolA is upregulated significantly (88) but it is improbable that this upregulation can solely account for *A. baylyi* survival in Lnt deficient states. Data from the same transcriptome set shows a twenty-fold increase in *hslJ*, gene expression in Lnt deficient *A. baylyi* (88). Additionally, a crystal structures of *E. coli* LolA (89) and putative structure of *E. coli HslJ* (NP_415897.1, EMBL-EBI) are very similar in structure. Due to the highly specific mouth-to-mouth transfer mechanism (90) that LolA and LolB interact with one another to transfer their inner lipoprotein cargo, homology in structure among LolA and *HslJ* is a reasonable cause to investigate HslJ as a potential chaperone suppressing the deleterious effects of Lnt deficiency.

Concluding Remarks

The lipoprotein sorting pathway is more complex than initially thought, the complexity and conditionally essential nature of the genes involved in the processing and transportation pathways provides opportunities to develop additional antimicrobial compounds for both clinical and small molecule pathway-probing applications. Though it was initially assumed that the action of *Lgt*, LspA, and Lnt were needed for lipoprotein biogenesis in all Gram-negative bacteria, this now seems to be an over-simplification. It is certainly true that most Gram-negatives require functional *Lgt*, LspA, and Lnt to be viable (50, 52, 83). However, it is now clear that there are many exceptions to this rule. *A. baylyi* is viable without Lnt and requires no other genetic manipulations to grow (88). In this species, the potentially more promiscuous LolCDE analog LolDF is used and a di-acylated lipoprotein is able to be recognized for transport instead of one that is tri-acylated by a mechanism that is still unknown (Fig. 1b) (88). By exploiting the ability of *A. baylyi* to survive in Lnt-deficient cellular environments we can use *A. baylyi* as a model to explore novel lipoprotein processing and transportation constituents. Uncovering the mechanism of which *A. baylyi* can overcome Lnt deficiency is essential and in doing so, we open new avenues of non-canonical OM biogenesis pathways and chemical interventions.

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Glossary of Terms

β-barrel assembly machine (BAM): Five-protein complex that assembles β-barrel proteins into the outer membrane

General secretion (Sec) pathway: System for exporting unfolded proteins from the cytoplasm into the inner membrane

Twin arginine translocation (Tat) pathway: System for exporting folded proteins from the cytoplasm into the inner membrane

Lipopolysaccharide Transport (Lpt) machinery: Transporter system to shuttle lipopolysaccharides across the periplasm to the outer membrane

Localization of lipoprotein (Lol) pathway: Pathway responsible for trafficking mature lipoproteins from the inner membrane to the outer membrane

Pre-prolipoprotein: Precursor lipoproteins exported from the cytoplasm prior to acylation by *Lgt* Prolipoprotein: Precursor lipoprotein with two acyl chains from the action of *Lgt* and with a still-intact signal peptide

Apo-lipoprotein: Di-acylated precursor lipoprotein with cleaved signal peptide prior to final acylation from Lnt

References

1. Grabowicz M, Silhavy TJ. 2017. Redefining the essential trafficking pathway for outer membrane lipoproteins. *Proc Natl Acad Sci* 114:4769–4774.
2. Okuda S, Sherman DJ, Silhavy TJ, Ruiz N, Kahne D. 2016. Lipopolysaccharide transport and assembly at the outer membrane: the PEZ model. *Nat Rev Microbiol* 14:337–345.
3. Hagan CL, Silhavy TJ, Kahne D. 2011. β-Barrel membrane protein assembly by the Bam complex. *Annu Rev Biochem* 80:189–210.
4. Mori H, Ito K. 2001. The Sec protein-translocation pathway. *Trends Microbiol* 9:494–500.
5. Sargent F, Bogsch EG, Stanley NR, Wexler M, Robinson C, Berks BC, Palmer T. 1998. Overlapping functions of components of a bacterial Sec-independent protein export pathway. *EMBO J* 17:3640–3650.
6. The bacterial twin-arginine translocation pathway. - PubMed - NCBI.
7. Choi U, Lee C-R. 2019. Distinct Roles of Outer Membrane Porins in Antibiotic Resistance and Membrane Integrity in *Escherichia coli*. *Front Microbiol* 10:953.
8. Chng S-S, Ruiz N, Chimalakonda G, Silhavy TJ, Kahne D. 2010. Characterization of the two- protein complex in Escherichia coli responsible for lipopolysaccharide assembly at the outer membrane. *Proc Natl Acad Sci U S A* 107:5363–5368.
9. Wu T, McCandlish AC, Gronenberg LS, Chng S-S, Silhavy TJ, Kahne D. 2006. Identification of a protein complex that assembles lipopolysaccharide in the outer membrane of *Escherichia coli*. *Proc Natl Acad Sci U S A* 103:11754–11759.
10. Sperandeo P, Martorana AM, Polissi A. 2017. The lipopolysaccharide transport (Lpt) machinery: A nonconventional transporter for lipopolysaccharide assembly at the outer membrane of Gram-negative bacteria. J Biol Chem 292:17981–17990. 11. Kamio Y, Nikaido H. 1976. Outer membrane of *Salmonella typhimurium*: accessibility of phospholipid head groups to phospholipase C and cyanogen bromide activated dextran in the external medium. *Biochemistry* 15:2561–2570.
11. Nikaido H. 2003. Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev MMBR* 67:593–656.
12. Nikaido H. 2005. Restoring permeability barrier function to outer membrane. *Chem Biol* 12:507–509.
13. Qiao S, Luo Q, Zhao Y, Zhang XC, Huang Y. 2014. Structural basis for lipopolysaccharide insertion in the bacterial outer membrane. *Nature* 511:108–111.
14. Dong H, Xiang Q, Gu Y, Wang Z, Paterson NG, Stansfeld PJ, He C, Zhang Y, Wang W, Dong C. 2014. Structural basis for outer membrane lipopolysaccharide insertion. *Nature* 511:52–56.
15. Freinkman E, Chng S-S, Kahne D. 2011. The complex that inserts lipopolysaccharide into the bacterial outer membrane forms a two-protein plug-and-barrel. *Proc Natl Acad Sci U S A* 108:2486– 2491.

16. Malojčić G, Andres D, Grabowicz M, George AH, Ruiz N, Silhavy TJ, Kahne D. 2014. LptE binds to and alters the physical state of LPS to catalyze its assembly at the cell surface. *Proc Natl Acad Sci* 111:9467–9472.

17. Grabowicz M, Yeh J, Silhavy TJ. 2013. Dominant Negative lptE Mutation That Supports a Rol for LptE as a Plug in the LptD Barrel. *J Bacteriol* 195:1327–1334.

18. Hirota Y, Suzuki H, Nishimura Y, Yasuda S. 1977. On the process of cellular division in *Escherichia coli*: a mutant of *E. coli* lacking a murein-lipoprotein. *Proc Natl Acad Sci U S A* 74:1417–1420.

19. Suzuki H, Nishimura Y, Yasuda S, Nishimura A, Yamada M, Hirota Y. 1978. Murein-lipoprotein of *Escherichia coli*: A protein involved in the stabilization of bacterial cell envelope. *Mol Gen Genet MGG* 167:1–9.

20. Lazzaroni JC, Portalier R. 1992. The excC gene of *Escherichia coli* K-12 required for cell envelope integrity encodes the peptidoglycan-associated lipoprotein (PAL). *Mol Microbiol* 6:735–742.

21. Majdalani N, Heck M, Stout V, Gottesman S. 2005. Role of RcsF in Signaling to the Rcs Phosphorelay Pathway in *Escherichia coli*. *J Bacteriol* 187:6770–6778.

22. Castanié-Cornet M-P, Cam K, Jacq A. 2006. RcsF Is an Outer Membrane Lipoprotein Involved in the RcsCDB Phosphorelay Signaling Pathway in *Escherichia coli*. *J Bacteriol* 188:4264–4270.

23. Parker CT, Kloser AW, Schnaitman CA, Stein MA, Gottesman S, Gibson BW. 1992. Role of the rfaG and rfaP genes in determining the lipopolysaccharide core structure and cell surface properties of *Escherichia coli* K-12. *J Bacteriol* 174:2525–2538.

24. Farris C, Sanowar S, Bader MW, Pfuetzner R, Miller SI. 2010. Antimicrobial peptides activate the Rcs regulon through the outer membrane lipoprotein RcsF. *J Bacteriol* 192:4894–4903.

25. Ophir T, Gutnick DL. 1994. A role for exopolysaccharides in the protection of microorganisms from desiccation. *Appl Environ Microbiol* 60:740–745.

26. Sledjeski DD, Gottesman S. 1996. Osmotic shock induction of capsule synthesis in *Escherichia coli* K-12. *J Bacteriol* 178:1204–1206.

27. Silhavy TJ, Kahne D, Walker S. 2010. The Bacterial Cell Envelope. *Cold Spring Harb Perspect Biol 2*: a000414.

28. Mei JM, Nourbakhsh F, Ford CW, Holden DW. 1997. Identification of Staphylococcus aureus virulence genes in a murine model of bacteremia using signature-tagged mutagenesis. *Mol Microbiol* 26:399–407.

29. Tidhar A, Flashner Y, Cohen S, Levi Y, Zauberman A, Gur D, Aftalion M, Elhanany E, Zvi A, Shafferman A, Mamroud E. 2009. The NlpD lipoprotein is a novel Yersinia pestis virulence factor essential for the development of plague. *PloS One* 4:e7023.

30. Zhuk I, Jariwala F, Attygalle AB, Wu Y, Libera MR, Sukhishvili SA. 2014. Self-Defensive Layer- by-Layer Films with Bacteria-Triggered Antibiotic Release. *ACS Nano* 8:7733–7745.

31. Kim KH, Aulakh S, Paetzel M. 2012. The bacterial outer membrane β-barrel assembly machinery. *Protein Sci Publ Protein Soc* 21:751–768.

32. Grabowicz M. 2018. Lipoprotein Transport: Greasing the Machines of Outer Membrane Biogenesis: Re-Examining Lipoprotein Transport Mechanisms Among Diverse Gram-Negative Bacteria While Exploring New Discoveries and Questions. *BioEssays News Rev Mol Cell Dev Biol* 40: e1700187.

33. Okuda S, Tokuda H. 2011. Lipoprotein Sorting in Bacteria. *Annu Rev Microbiol* 65:239–259.

34. Narita S-I, Tokuda H. 2017. Bacterial lipoproteins; biogenesis, sorting and quality control. *Biochim Biophys Acta Mol Cell Biol Lipids* 1862:1414–1423.

35. Seydel A, Gounon P, Pugsley AP. 1999. Testing the "+2 rule" for lipoprotein sorting in the *Escherichia coli* cell envelope with a new genetic selection. *Mol Microbiol* 34:810–821.

36. Sankaran K, Wu HC. 1994. Lipid modification of bacterial prolipoprotein. Transfer of diacylglyceryl moiety from phosphatidylglycerol. *J Biol Chem* 269:19701–19706.

37. Matsuyama S, Tajima T, Tokuda H. 1995. A novel periplasmic carrier protein involved in the sorting and transport of *Escherichia coli* lipoproteins destined for the outer membrane. *EMBO J* 14:3365–3372.

38. Gallagher LA, Ramage E, Weiss EJ, Radey M, Hayden HS, Held KG, Huse HK, Zurawski DV, Brittnacher MJ, Manoil C. 2015. Resources for Genetic and Genomic Analysis of Emerging Pathogen Acinetobacter baumannii. *J Bacteriol* 197:2027–2035.

39. Chahales P, Thanassi DG. 2015. A more flexible lipoprotein sorting pathway. *J Bacteriol* 197:1702–1704.

40. LoVullo ED, Wright LF, Isabella V, Huntley JF, Pavelka MS. 2015. Revisiting the Gram-negative lipoprotein paradigm. *J Bacteriol* 197:1705–1715.

41. Gwin CM, Prakash N, Christian Belisario J, Haider L, Rosen ML, Martinez LR, Rigel NW. 2018. The apolipoprotein N-acyl transferase Lnt is dispensable for growth in Acinetobacter species. *Microbiol Read Engl* 164:1547–1556.

42. Yakushi T, Masuda K, Narita S, Matsuyama S, Tokuda H. 2000. A new ABC transporter mediating the detachment of lipid-modified proteins from membranes. *Nat Cell Biol* 2:212–218.

43. Yakushi T, Yokota N, Matsuyama S, Tokuda H. 1998. LolA-dependent release of a lipid-modified protein from the inner membrane of *Escherichia coli* requires nucleoside triphosphate. *J Biol Chem* 273:32576–32581.

44. Matsuyama S i, Yokota N, Tokuda H. 1997. A novel outer membrane lipoprotein, LolB (HemM), involved in the LolA (p20)-dependent localization of lipoproteins to the outer membrane of *Escherichia coli*. *EMBO J* 16:6947–6955.

45. Narita S, Tanaka K, Matsuyama S, Tokuda H. 2002. Disruption of lolCDE, Encoding an ATP- Binding Cassette Transporter, Is Lethal for *Escherichia coli* and Prevents Release of Lipoproteins from the Inner Membrane. *J Bacteriol* 184:1417–22.

46. da Silva RAG, Churchward CP, Karlyshev AV, Eleftheriadou O, Snabaitis AK, Longman MR, Ryan A, Griffin R. 2017. The role of apolipoprotein N-acyl transferase, Lnt, in the lipidation of factor H binding protein of Neisseria meningitidis strain MC58 and its potential as a drug target. *Br J Pharmacol* 174:2247–2260.

47. Hantke K, Braun V. 1973. Covalent binding of lipid to protein. Diglyceride and amide-linked fatty acid at the N-terminal end of the murein-lipoprotein of the *Escherichia coli* outer membrane. *Eur J Biochem* 34:284–296.

48. Gan K, Gupta SD, Sankaran K, Schmid MB, Wu HC. 1993. Isolation and characterization of a temperature-sensitive mutant of Salmonella typhimurium defective in prolipoprotein modification. *J Biol Chem* 268:16544–16550.

49. Gan K, Sankaran K, Williams MG, Aldea M, Rudd KE, Kushner SR, Wu HC. 1995. The umpA gene of *Escherichia coli* encodes phosphatidylglycerol:prolipoprotein diacylglyceryl transferase (*lgt*) and regulates thymidylate synthase levels through translational coupling. *J Bacteriol* 177:1879–1882.

50. Chattopadhyay PK, Lai JS, Wu HC. 1979. Incorporation of phosphatidylglycerol into murein lipoprotein in intact cells of Salmonella typhimurium by phospholipid vesicle fusion. *J Bacteriol* 137:309–312.

51. Buddelmeijer N, Young R. 2010. The Essential *Escherichia coli* Apolipoprotein N-Acyltransferase (Lnt) Exists as an Extracytoplasmic Thioester Acyl-Enzyme Intermediate. *Biochemistry* 49:341–346.

52. Leskelä S, Wahlström E, Kontinen VP, Sarvas M. 1999. Lipid modification of prelipoproteins is dispensable for growth but essential for efficient protein secretion in *Bacillus subtilis*: characterization of the *lgt* gene. *Mol Microbiol* 31:1075–1085.

53. Petit CM, Brown JR, Ingraham K, Bryant AP, Holmes DJ. 2001. Lipid modification of prelipoproteins is dispensable for growth *in vitro* but essential for virulence in *Streptococcus pneumoniae*. *FEMS Microbiol Lett* 200:229–233.

54. Hutchings MI, Palmer T, Harrington DJ, Sutcliffe IC. 2009. Lipoprotein biogenesis in Gram- positive bacteria: knowing when to hold 'em, knowing when to fold 'em. *Trends Microbiol* 17:13–21.

55. Marraffini LA, Dedent AC, Schneewind O. 2006. Sortases and the art of anchoring proteins to the envelopes of Gram-positive bacteria. *Microbiol Mol Biol Rev MMBR* 70:192–221.

56. Mao G, Zhao Y, Kang X, Li Z, Zhang Y, Wang X, Sun F, Sankaran K, Zhang XC. 2016. Crystal structure of *E. coli* lipoprotein diacylglyceryl transferase. *Nat Commun* 7:1–12.

57. Braun V, Rehn K. 1969. Chemical Characterization, Spatial Distribution and Function of a Lipoprotein (Murein-Lipoprotein) of the *E. coli* Cell Wall. *Eur J Biochem* 10:426–438.

58. Hantke K, Braun V. 1973. Covalent Binding of Lipid to Protein. *Eur J Biochem* 34:284–296.

59. Hayashi S, Wu HC. 1990. Lipoproteins in bacteria. J *Bioenerg Biomembr* 22:451–471.

60. Tokunaga M, Loranger JM, Wolfe PB, Wu HC. 1982. Prolipoprotein signal peptidase in *Escherichia coli* is distinct from the M13 procoat protein signal peptidase. *J Biol Chem* 257:9922–9925.

61. Tokunaga M, Tokunaga H, Wu HC. 1982. Post-translational modification and processing of *Escherichia coli* prolipoprotein in vitro. *Proc Natl Acad Sci* 79:2255–2259.

62. Xiao Y, Wall D. 2014. Genetic redundancy, proximity, and functionality of lspA, the target of antibiotic TA, in the *Myxococcus xanthus* producer strain. *J Bacteriol* 196:1174–1183.

63. Tjalsma H, Zanen G, Venema G, Bron S, Dijl JM van. 1999. The Potential Active Site of the Lipoprotein-specific (Type II) Signal Peptidase of *Bacillus subtilis*. *J Biol Chem* 274:28191–28197.

64. Hussain M, Ichihara S, Mizushima S. 1982. Mechanism of signal peptide cleavage in the biosynthesis of the major lipoprotein of the *Escherichia coli* outer membrane. *J Biol Chem* 257:5177– 5182.

65. Tjalsma H, Kontinen VP, Prágai Z, Wu H, Meima R, Venema G, Bron S, Sarvas M, Dijl JM van. 1999. The Role of Lipoprotein Processing by Signal Peptidase II in the Gram-positive Eubacterium *Bacillus subtilis* Signal peptidase ii is required for the efficient secretion of α-amylase, a non-lipoprotein. *J Biol Chem* 274:1698–1707.

66. Vogeley L, Arnaout TE, Bailey J, Stansfeld PJ, Bo land C, Caffrey M. 2016. Structural basis of lipoprotein signal peptidase II action and inhibition by the antibiotic globomycin. *Science* 351:876–880.

67. Muñoa FJ, Miller KW, Beers R, Graham M, Wu HC. 1991. Membrane topology of Escherichia coli prolipoprotein signal peptidase (signal peptidase II). *J Biol Chem* 266:17667–17672.

68. Novak P, Ray PH, Dev IK. 1986. Localization and purification of two enzymes from *Escherichia coli* capable of hydrolyzing a signal peptide. *J Biol Chem* 261:420–427.

69. Nodwell JR. 2007. Novel Links between Antibiotic Resistance and Antibiotic Production. *J Bacteriol* 189:3683–3685.

70. Sander P, Rezwan M, Walker B, Rampini SK, Kroppenstedt RM, Ehlers S, Keller C, Keeble JR, Hagemeier M, Colston MJ, Springer B, Böttger EC. 2004. Lipoprotein processing is required for virulence of *Mycobacterium tuberculosis*. *Mol Microbiol* 52:1543–1552.

71. Inukai M, Nakajima M, Osawa M, Haneishi T, Arai M. 1978. Globomycin, a new peptide antibiotic with spheroplast-forming activity. *J Antibiot (Tokyo)* 31:421–425.

72. Hussain M, Ichihara S, Mizushima S. 1980. Accumulation of glyceride-containing precursor of the outer membrane lipoprotein in the cytoplasmic membrane of *Escherichia coli* treated with globomycin. *J Biol Chem* 255:3707–3712.

73. Tokunaga M, Loranger JM, Wu HC. 1983. Isolation and characterization of an *Escherichia coli* clone overproducing prolipoprotein signal peptidase. *J Biol Chem* 258:12102–12105.

74. Xiao Y, Gerth K, Müller R, Wall D. 2012. Myxobacterium-Produced Antibiotic TA (Myxovirescin) Inhibits Type II Signal Peptidase. *Antimicrob Agents Chemother* 56:2014–2021.

75. Gupta SD, Wu HC. 1991. Identification and subcellular localization of apolipoprotein N-acyltransferase in *Escherichia coli*. *FEMS Microbiol Lett* 62:37–41.

76. Pugsley AP. 1993. The Complete General Secretory Pathway in Gram-Negative Bacteria. *Microbiol Rev* 57:59.

77. Tschumi A, Nai C, Auchli Y, Hunziker P, Gehrig P, Keller P, Grau T, Sander P. 2009. Identification of Apolipoprotein N-Acyltransferase (Lnt) in Mycobacteria. *J Biol Chem* 284:27146–27156.

78. Brülle JK, Tschumi A, Sander P. 2013. Lipoproteins of slow-growing Mycobacteria carry three fatty acids and are N-acylated by Apolipoprotein N-Acyltransferase BCG_2070c. *BMC Microbiol* 13:223.

79. Gupta SD, Dowhan W, Wu HC. 1991. Phosphatidylethanolamine is not essential for the N-acylation of apolipoprotein in *Escherichia coli*. *J Biol Chem* 266:9983–9986.

80. Hillmann F, Argentini M, Buddelmeijer N. 2011. Kinetics and Phospholipid Specificity of Apolipoprotein N-Acyltransferase. *J Biol Chem* 286:27936–27946.

81. Schaechter M. 2001. *Escherichia coli* and Salmonella 2000: the View From Here. *Microbiol Mol Biol Rev* 65:119–130.

82. Robichon C, Vidal-Ingigliardi D, Pugsley AP. 2005. Depletion of Apolipoprotein N-Acyltransferase Causes Mislocalization of Outer Membrane Lipoproteins in *Escherichia coli*. *J Biol Chem* 280:974–983.

83. Yan C, Luo J. 2010. An analysis of reentrant loops. *Protein J* 29:350–354.

84. Gélis-Jeanvoine S, Lory S, Oberto J, Buddelmeijer N. 2015. Residues located on membrane- embedded flexible loops are essential for the second step of the apolipoprotein N-acyltransferase reaction. *Mol Microbiol* 95:692–705.

85. Ben-Shem A, Fass D, Bibi E. 2007. Structural basis for intramembrane proteolysis by rhomboid serine proteases. *Proc Natl Acad Sci U S A* 104:462–466.

86. Lu G, Xu Y, Zhang K, Xiong Y, Li H, Cui L, Wang X, Lou J, Zhai Y, Sun F, Zhang XC. 2017. Crystal structure of *E. coli* apolipoprotein N-acyl transferase. *Nat Commun* 8:1–8.

87. Gwin CM, Prakash N, Christian Belisario J, Haider L, Rosen ML, Martinez LR, Rigel NW. 2018. The apolipoprotein N-acyl transferase Lnt is dispensable for growth in *Acinetobacter* species. *Microbiol Read Engl* 164:1547–1556.

88. Takeda K, Miyatake H, Yokota N, Matsuyama S, Tokuda H, Miki K. 2003. Crystal structures of bacterial lipoprotein localization factors, LolA and LolB. *Embo J* 22:3199–3209.

89. Okuda S, Tokuda H. 2009. Model of mouth-to-mouth transfer of bacterial lipoproteins through inner membrane LolC, periplasmic LolA, and outer membrane LolB. *Proc Natl Acad Sci* 106:5877– 5882.

90. Pailler J, Aucher W, Pires M, Buddelmeijer N. 2012. Phosphatidylglycerol::prolipoprotein diacylglyceryl transferase (*Lgt*) of *Escherichia coli* has seven transmembrane segments, and its essential residues are embedded in the membrane. *J Bacteriol* 194:2142–2151.

Nylon Oxygen Barrier Tubing Reduces Biofouling in Beer Draught Lines

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Abstract

Beer draught lines are frequently contaminated with biofilm-forming microorganisms, which forces retailers to spend considerable time and money cleaning and replacing lines. In light of this financial burden, draught tubing composition was examined for its role in the prevention of biofouling in beer lines. Three types of draught tubing - vinyl, polyethylene, and nylon barrier - were inoculated with a combination of biofilm-forming microorganisms *(Hafnia paralvei, Raoultella planticola, Pediococcus damnosus and Saccharomyces cerevisiae)* and used to simulate a bar environment for sixteen weeks. Following simulation, the degree of biofouling in each draught line was determined by spectrophotometry and microscopy. Absorption values and fluorescence images showed that nylon barrier tubing was superior to the other lines at resisting biofilm maturation.These results suggest that tubing composition plays a significant role in the prevention of biofilm formation in beer draught lines and supports the adoption of nylon barrier tubing as an effective strategy against biofouling in a variety of applications.

Introduction

Biofouling creates a considerable financial burden at various levels of the beer industry. Arguably, the most challenging environment to keep clean is found in beer draught lines where yeast and spoilage organisms readily adhere, altering the taste, aroma, and quality of the beer. Although the deleterious effects of microbial biofilms have been recognized for several decades, relatively little scientific research has been applied to combat these issues, and most methods focus on routine maintenance of beer draught lines. For example, the Brewers Association recommends cleaning draught lines, at a minimum, every two weeks. The cleaning solution should be recirculated through the line for at least 15 minutes at a velocity of two gallons per minute. Additionally, all draught lines should be replaced annually as, despite regular and consistent cleaning, biofilms can still form (1).

Beer spoilage can be caused by a variety of microbial species. Perhaps the most notorious beer contaminants are lactic acid bacteria such as *Lactobacillus brevis*, and *Pediococcus damnosus*. These species are able to survive the harsh conditions of the brewing environment due to the presence of genes for hop resistance and polysaccharide production (2; 25). While the biofilm formation of most lactic acid bacteria is relatively weak, their persistence and prevalence is enhanced by the presence of ubiquitous environmental species that have also been isolated from brewing equipment (14). Many isolates, such as *Stenotrophomonas maltophilia, Pseudomonas putida, and Citrobacteri freundii*, are known to be prolific biofilm producers and may aid lactic acid bacteria as secondary colonizers (11; 24; 28). *P. damnosus* was recently isolated in our lab as a component of a multispecies biofilm in beer draught tubing from a local brewery (Russell *et al*, unpublished). Notably, a human commensal organism, *Halfnia paralvei*, a common soil inhabitant, *Raoultella planticola*, and brewer's yeast, *Saccharomyces cerevisiae* were the other predominant species in this biofilm.

As many retailers and distributers know, standard draught line cleaning procedures are very time-consuming, expensive, and only moderately effective (20). Although proper maintenance can delay biofilm growth and formation, cleaning of beer draught lines needs to be done consistently, as biofilms can form quickly and are more difficult to remove when mature due to the vast and multi-faceted defense mechanisms they exhibit (5). Consequently, alternative treatments have been developed for beer draught lines and other industrial applications that utilize enzymatic digestion (10; 16; 29; 31). These approaches are moderately effective; however, they are often marketed as an additional step to a routine maintenance schedule, adding unnecessary time and expense.

Recently, the medical and industrial fields have shifted their focus to proactive methods of biofilm prevention rather than reactive treatment options. For example, a number of natural and artificial chemicals have been shown to possess anti-biofilm properties that block quorum sensing (9), disperse extracellular polysaccharide (19), inhibit curli biosynthesis (6), or alter membrane permeability (13). A variety of studies also have been published in recent years describing options for making surfaces more resistant to biofouling, including development of novel materials (17; 18), improvement of manufacturing methods (15; 32), and creation of post-production coatings (7; 8; 22). Likewise, beer-draught line manufacturers have begun to experiment with various materials and manufacturing procedures to create lines that are more resistant to biofilm formation and, consequently, require less routine maintenance. One such product, known as Gen-X (Valpar), utilizes both a novel manufacturing method and addition of a nylon barrier layer to stop oxygen permeation and preserve beverage characteristics. Consequently, Gen-X tubing promises to ensure draught quality and hinder microbial growth 2-3 times more effectively than other alternatives (30). While this and other new proprietary compositions show promise at combatting biofouling, no peer-reviewed research has been done to characterize the effectiveness of these lines.

Our project aims to test the qualitative and quantitative effect that various tubing compositions have on biofilm formation in beer draught lines. We hypothesized that nylon barrier tubing would exhibit reduced biofouling compared to other tubing materials due to its potential to reduce oxygen permeation. To test this, three beer draught lines—vinyl, polyethylene, and nylon barrier (Gen-X)—were inoculated with a microbial cocktail consisting of *Hafnia paralvei,*

Raoultella planticola, Pediococcus damnosus, and Saccharomyces cerevisiae liquid cultures. Following inoculation, the lines were connected to a keg-draught system from which beer was regularly dispensed for sixteen weeks to simulate a bar environment. Next, spectrophotometry and microscopy were used to determine the ability of the different draught tubing compositions to resist biofouling. Results from our experiments consistently showed that nylon barrier tubing was significantly better at resisting biofilm growth than traditional vinyl or polyethylene lines. The nylon barrier draught line showed impressive resistance to biofouling after the sixteen-week simulation, supporting the use of nylon oxygen barriers as a preventative measure against beer- spoilage biofilms.

Methods

Microbial Cultures

Four microbial species were obtained and cultured for this study as follows. *Hafnia paralvei* (ATCC 29927) and *Raoultella planticola* (ATCC 33431) were streaked onto nutrient agar plates and incubated at 37°C. *Pediococcus damnosus* (ATCC 29358) was cultured on Lactobacilli MRS plates and incubated at 25°C. *Saccharomyces cerevisiae* (Wyeast 1728) was streaked onto a malt agar plate and incubated at 25°C. Individual 500mL cultures of each species were inoculated with a single colony from each streak plate and incubated at the appropriate temperature until cultures reached late exponential phase (~24 hours). Following incubation, all four cultures were mixed in a 1:1:1:1 ratio at OD600 ~4.8 to create a microbial cocktail for inoculation of our beer draught lines.

Simulated Dispensary System

To simulate a standard dispensary system, three types of beer-draught lines, vinyl (Micromatic 550C), polyethylene (Micromatic 550NE), and nylon barrier (Micromatic 550BF), were attached to a single keg—containing an amber ale from Dakota Territory Brewing company, Aberdeen, SD—and kept at 4ºC. Before being connected to the keg, all lines were cleaned with alkaline liquid beer line cleaner (Micromatic MM-B68), inoculated with a biofilm-forming microbial cocktail (described above), and left to incubate at room temperature for one hour. After inoculation, the culture was drained, and the lines were connected to the same keg using line splitters; the lines were then filled with beer. Once connected, 500 mL of beer was drawn from each line every-other day for sixteen weeks to simulate a bar environment, and kegs were replaced as needed. After the sixteen-week simulation, the lines were disconnected from the keg, wrapped in parafilm, and stored at 4ºC for further processing; some beer was left in the line to prevent the line from drying while in storage.

Quantifying Biofilm Formation

To quantify biofilm formation in each tube, 25 mm long segments were cut at various regions from each line using a completely randomized design. Two segments were cut from each end and two segments were cut from the middle for a total of six samples in each treatment group.The samples were, then, rinsed lightly with deionized (DI) water, and one end was sealed with parafilm. Next, the samples were filled with 0.1% crystal violet and allowed to incubate for 10 mins. After incubation, the crystal violet was removed, and the samples were rinsed with DI water a second time to remove any unbound dye. Next, the tubes were filled with 1 mL of dimethylsulfoxide (DMSO) and incubated for 10 minutes. A stainless-steel spatula was used to gently agitate and break uplarger chunks of biofilm.

The sample was diluted 1:1 with more DMSO, loaded into a 2 mL cuvette, and the absorbance was measured at λ600 on a spectrophotometer. Results were analysed using a one factor ANOVA to assign statistical significance.

Fluorescence Microscopy

One foot of tubing was taken from each type of beer line and three 12.5mm samples were cut from each foot-long segment. The samples were then cut longitudinally into semi-circles and stained using the LIVE/DEAD BacLight bacterial viability kit (Invitrogen), according to the manufacturer's recommendations. The sections were then attached to a slide using tape and observed with fluorescence microscopy. Images

were acquired using a semi-randomized, single blind method on a Leica DM8 confocal microscope with an excitation wavelength of 480 nm and a 63x oil immersion objective.

Results

Quantifying Biofilm Formation

After disassembling our simulated draught system, each draught line was stained with crystal violet to compare total biofilm formation between the three tube types. Visual inspection of each line showed a dramatic difference between the nylon barrier tubing and the other two types. Some biofouling was evident in the nylon barrier tubing, but it was visibly cleaner than both the polyethylene and the vinyl lines (Fig. 1). To quantify this difference, each tube was stained with crystal violet and the absorbance was measured using a spectrophotometer. Matching its visible appearance, the vinyl beer draught line had the highest average absorbance value at 2.771 with a standard deviation of ±0.464 (2.771 ± 0.464, n=3). Biofilm from the polyethylene line measured an absorbance value of 1.601 with a standard deviation of ±0.447 (1.601 ± 0.447, n=3). Notably, the nylon barrier line exhibited significantly lower absorbance than both the vinyl (p= 0.00001) and the polyethylene (p= 0.0004) lines, measuring an average value of 0.253 with a standard deviation of 0.139 (0.253 ± 0.139, n=3) (Fig. 2). It should be noted that crystal violet stain does not differentiate between viable and dead cells in this assay. Therefore, the mean absorbance values reported herein are a measurement of cells that are alive and those that are not.

Fluorescence Microscopy

While the crystal violet staining of the nylon barrier tubing was significantly different from the other two materials, these results gave little insight into the biofilm characteristics and microarchitecture on each tube type. In particular, they did not indicate if bacteria and yeast were prevented from adhering to the nylon barrier tubing altogether, or if microbial cells were just concentrated in small, localized biofilms dispersed along its surface. Therefore, confocal fluorescence microscopy was utilized to obtain three-dimensional images of the biofilms on each tube type. After removing three 25 mm segments from each line, the cells were stained with a fluorescent dye and observed by confocal microscopy to view the characteristics of biofilms in each microenvironment. Image analysis revealed that most of the inner surface on the vinyl line was covered with a thin, but mature biofilm (Fig. 3, left column). By comparison, the polyethylene line exhibited substantially less biofouling, but still had a moderate level

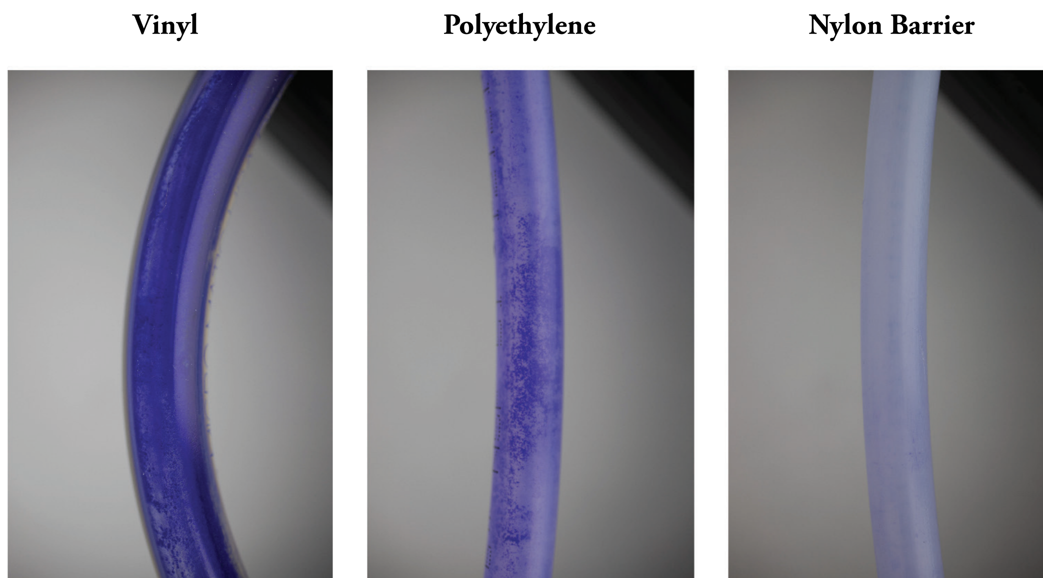

Figure 1. Crystal violet staining of draught lines. Following a sixteen-week simulation, each draught line was drained, rinsed with DI water, and stained with crystal violet. Qualitative images were taken to compare relative biofouling.

of bacteria adhered to its surface (Fig. 3, middle column). Similar to the polyethylene line, the nylon barrier tubing exhibited considerable resistance to biofouling. Only single cells were adhered to its surface, with no evidence of a cohesive biofilm (Fig. 3, right column).

Discussion

The maintenance of beer draught lines is an expensive and time-consuming process that affects thousands of businesses around the globe. Standard line maintenance is essential to ensure taste, aroma, and quality of the product, but it involves cleansing of the line using acidic or alkaline chemicals at a minimum every 2-3 weeks and complete line replacement yearly. Improper cleaning of beer draught lines allows for microorganisms to adhere in the lines while feeding off the nutrients provided by the beer. The adhered microorganisms multiply in the line, and once there are sufficient organisms in the surrounding environment, the organisms begin to produce a polysaccharide matrix around themselves for protection. This biofilm makes the beer-spoiling organisms difficult to eradicate,costing retailers and distributors money and time. For these reasons, the prevention of biofilm formation is of great importance.

In recent years, tubing manufacturers have been developing new chemical compositions that promise increased resistance to microbial biofilms and, therefore, longer intervals between line cleanings.To our knowledge, our results represent the first peer-reviewed study to support the claims that nylon barrier tubing with reduced oxygen permeability does, indeed, resist biofouling. Our findings indicate that the nylon barrier line

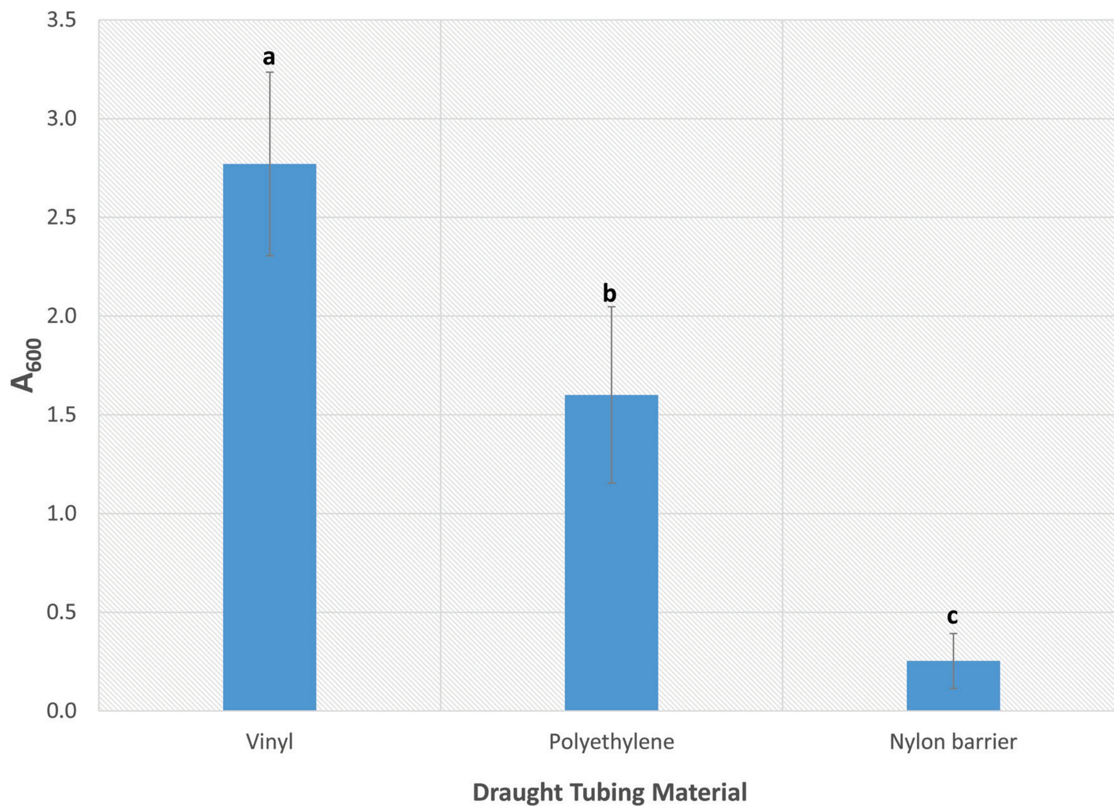

Figure 2. Quantifying biofouling of draught lines. Six 25 mm segments were taken from each draught line and stained with crystal violet. The stained biofilm was solubilized with DMSO, transferred to acuvette, and absorbance was measured at λ600. Errorbarsrepresentstandarddeviation in each group. Significant differences wereobtained between groups indicated with different letters;

*$P<0.01$.

is roughly six times more resistant to biofilm formation than polyethylene tubing and ten times better than standard vinyl tubing (Fig. 2). Fluorescence microscopy revealed that this is due to fewer cells adhering to the surface of the nylon barrier line compared to the others (Fig. 3). While moderate levels of microbial cells still adhere to the surface of both polyethylene and nylon barrier tubing, the microenvironments in these tubes do not promote biofilm maturation. It is likely that the microbial population density required to establish a biofilm has not been reached.

Although the results from this experiment show that the structural and chemical composition of beer line affects biofouling, there are several caveats that deserve consideration. First, it could be assumed that the nylon barrier line helps preserve the quality of the beer longer because it inhibits biofouling. However, the taste and quality of the beer was not evaluated as a part of this study. More research might be needed to substantiate the benefit to consumers. Second, only one style of beer – an amber ale – was used in this study; results may vary with beer styles containing higher alcohol content, greater alpha-acid levels, differing pH, etc. Research suggests that each beer style may provide a unique growth environment, supplying specific micronutrients and antimicrobial compounds (21). For example, hops content is known to have a significant effect on the types of microorganisms that survive in different beer styles (23; 26; 27). Knowing these differences could be useful to optimize cleaning protocols for beer draught lines dedicated to certain beer styles. Third, each line was directly inoculated with a very high concentration (OD600~4.8) of microorganisms and those cells were allowed to establish themselves in the absence of a regular cleaning regimen for sixteen weeks. These conditions were chosen to ensure adequate adherence of primary biofilm colonizers and to accentuate the potential impact of each tubing material. These conditions may not

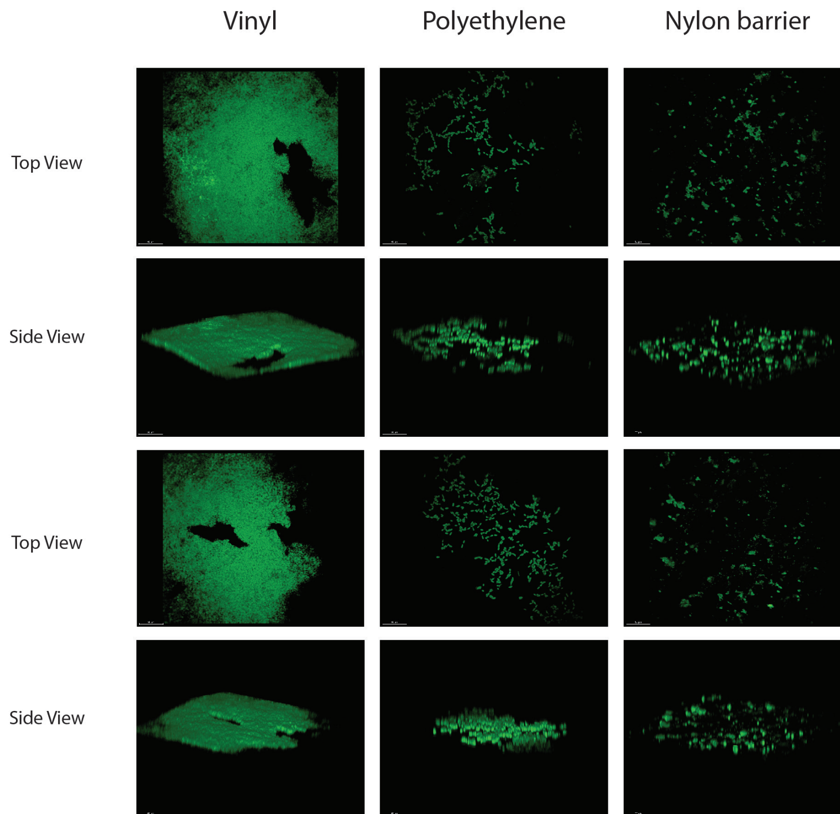

Fig. 3. Confocal imaging of adhered biofilm. Six 25 mm segments were taken from each draughtline, stained with a fluorescent dye, and observed on a confocal microscope with an excitation wavelength of 480 nm and a 63x oil immersion objective.

reflect the typical biological conditions that most beer retailers and distributors encounter. Finally, there are many anaerobic organisms such as *Megasphaera* and *Pectinatus* species that are known to adhere to brewery surfaces and cause beer spoilage (3; 4). Since all four of the test organisms in our study were aerobic or facultative anaerobes, our results cannot predict the potential impact that nylon barrier tubing might have on biofilms formed by strict anaerobes. Given that the benefit of nylon barrier tubing is due to a presumed reduction in oxygen permeability (12), it is likely that lines containing this barrier would show little to no impact on the growth of anaerobic microorganisms.

Despite the caveats described above, our study provides convincing evidence that nylon barrier tubing substantially reduces biofouling when used for beer dispensing. The adoption of such tubing is a low-cost option for retailers to improve the quality of their product and reduce the frequency of line cleaning, resulting in less long-term expense. Moreover, the use of such nylon barriers could have wide-ranging benefits in other applications such as water and soft drink lines, diagnostic equipment, and medical devices.

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Disclosure Statments:

The authors declare no conflict of interest.

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References

1. A1. Association, B. (2014). Brewers Association Draught Beer Quality for Retailers. Retrieved from https://www.brewersassociation.org/educational-publications/draught-beer-quality-for-retailers/

2. Bergsveinson, J., Baecker, N., Pittet, V., & Ziola, B. (2015). Role of Plasmids in Lactobacillus brevis BSO 464 HopTolerance and Beer Spoilage. Appl Environ Microbiol, 81(4), 1234-1241. doi:10.1128/ Aem.02870-14Arnesen, L., P. Stenfors, A. Fagerlund, and P.E. Granum. 2008. From soil to gut: *Bacillus cereus* and its food poisoning toxins. FEMS Microbiol. Rev. 32:579-606.

3. Bittner, M., de Souza, A. C., Brozova, M., Matoulkova, D., Dias, D. R., & Branyik, T. (2016). Adhesion ofanaerobicbeerspoilagebacteriaMegasphaeracerevisiaeandPectinatusfrisingensistostainlesssteel. Lwt-Food Science and Technology, 70, 148-154. doi:10.1016/j.lwt.2016.02.044

4. Bittner, M., Strejc, J., Matoulkova, D., Kolska, Z., Pustelnikova, L., & Branyik, T. (2017). Adhesion of Megasphaeracerevisiaeontosolidsurfacesmimickingmaterialsusedinbreweries.JournaloftheInstitute of Brewing, 123(2), 204-210. doi:10.1002/jib.415

5. Bridier, A., Briandet, R.,Thomas,V.,& Dubois-Brissonnet, F. (2011). Resistanceofbacterialbiofilmsto disinfectants: a review. Biofouling, 27(9), 1017-1032. doi:10.1080/08927014.2011.626899

6. Cegelski, L., Pinkner, J. S., Hammer, N. D., Cusumano, C. K., Hung, C. S., Chorell, E., . . . Hultgren, S. J.(2009). Small-moleculeinhibitorstargetEscherichiacoliamyloidbiogenesisandbiofilmformation. Nat Chem Biol, 5(12), 913-919. doi:10.1038/nchembio.242

7. Dundas, A. A., O. Sanni, J. F. Dubern, G. Dimitrakis, A. L. Hook, D. J. Irvine, P. Williams, and M. R. Alexander. "Validating a Predictive Structure-Property Relationship by Discovery of Novel Polymers Which Reduce Bacterial Biofilm Formation." [In English]. Advanced Materials 31, no. 49 (Dec 2019).

8. R. (2019). Validating a Predictive Structure-Property Relationship by Discovery of Novel Polymers whichReduceBacterialBiofilmFormation.AdvancedMaterials,31(49).doi:ARTN190351310.1002/ adma.201903513

9. Fisher, L., Ostovapour, S., Kelly, P., Whitehead, K. A., Cooke, K., Storgards, E., & Verran, J. (2014). Molybdenumdopedtitaniumdioxidephotocatalyticcoatingsforuseashygienicsurfaces:theeffectof soiling on antimicrobial activity. Biofouling, 30(8), 911-919. doi:10.1080/08927014.2014.939959

10. Hentzer, M., Riedel, K., Rasmussen, T. B., Heydorn, A., Andersen, J. B., Parsek, M. R., . . . Givskov, M. (2002). InhibitionofquorumsensinginPseudomonasaeruginosabiofilmbacteriabyahalogenated furanone compound. Microbiology-Sgm, 148, 87-102. doi:Doi 10.1099/00221287-148-1-87

11. Itoh, Y., Wang, X., Hinnebusch, B. J., Preston, J. F., & Romeo, T. (2005). Depolymerization of beta-1,6- N-acetyl-D-glucosaminedisruptstheintegrityofdiversebacterialbiofilms.JBacteriol,187(1),382-387. doi:10.1128/Jb.187.1.382-387.2005

12. Jucker, B. A., Harms, H., & Zehnder, A. J. (1996). Adhesion of the positively charged bacterium *Stenotrophomonas (Xanthomonas) maltophilia* 70401toglassandTeflon.JBacteriol,178(18),5472- 5479. doi:10.1128/jb.178.18.5472-5479.1996

13. Khanna, Y. P., Day, E. D., Tsai, M. L., & Vaidyanathan, G. (1997). Re-examining the oxygen barrier of nylon6films.1.Roleofmoistureandprocessinginducedvariables.JournalofPlasticFilm&Sheeting, 13(3), 197-211. doi:Doi 10.1177/875608799701300304

14. Kharidia, R., & Liang, J. F. (2011). The Activity of a Small Lytic Peptide PTP-7 on *Staphylococcus aureus* Biofilms. Journal of Microbiology, 49(4), 663-668. doi:10.1007/s12275-011-1013-5

15. Maifreni, M., Frigo, F., Bartolomeoli, I., Buiatti, S., Picon, S., & Marino, M. (2015). Bacterial biofilm asapossiblesourceofcontaminationinthemicrobreweryenvironment.FoodControl,50,809-814. doi:10.1016/j.foodcont.2014.10.032

16. Narayana, P. S. V. V. S., & Srihari, P. S. V. V. (2019). Biofilm Resistant Surfaces and Coatings on Implants: A Review. Materials Today-Proceedings, 18, 4847-4853.

17. Nijland, R., Hall, M. J., & Burgess, J. G. (2010). Dispersal of Biofilms by Secreted, Matrix Degrading, Bacterial DNase. PLoS One, 5(12). doi:ARTN e1566810.1371/journal.pone.0015668

18. Park, H. H., Sun, K., Lee, D., Seong, M., Cha, C., & Jeong, H. E. (2019). Cellulose acetate nanoneedle arraycoveredwithphosphorylcholinemoietyasabiocompatibleandsustainableantifoulingmaterial. Cellulose, 26(16), 8775-8788. doi:10.1007/s10570-019-02681-w

19. Park, H. H., Sun, K., Seong, M., Kang, M., Park, S., Hong, S., . . . Jeong, H. E. (2019). Lipid-Hydrogel- NanostructureHybridsasRobustBiofilm-ResistantPolymericMaterials.AcsMacroLetters,8(1),64-69. doi:10.1021/acsmacrolett.8b00888

20. Park, S. C., Park, Y., & Hahm, K. S. (2011). The Role of Antimicrobial Peptides in Preventing Multidrug-ResistantBacterialInfectionsandBiofilmFormation.InternationalJournalofMolecular Sciences, 12(9), 5971-5992. doi:10.3390/ijms12095971

21. Quain,D.E.(2016).Draughtbeerhygiene:cleaningofdispensetapnozzles.JournaloftheInstituteof Brewing, 122(3), 388-396. doi:10.1002/jib.335

22. Riedl, R., Goderbauer, P., Brandl, A., Jacob, F., & Hutzler, M. (2017). Bavarian Wheat Beer, an Example ofaSpecialMicrobeHabitat-Cultivation,Detection,BiofilmFormation,CharacterizationofSelected Lactic Acid Bacteria Hygiene Indicators and Spoilers. Brewing Science, 70(1-2), 39-50.

23. Sae-ung, P., Wijitamornloet, A., Iwasaki, Y., Thanyasrisung, P., & Hoven, V. P. (2019). Clickable Zwitterionic Copolymer as a Universal Biofilm-Resistant Coating. Macromolecular Materials and Engineering, 304(9). doi:ARTN 190028610.1002/mame.201900286

24. Sami, M., Suzuki, K., Sakamoto, K., Kadokura, H., Kitamoto, K., & Yoda, K. (1998). A plasmid pRH45of*Lactobacillusbrevis*confershopresistance.JournalofGeneralandAppliedMicrobiology, 44(5), 361-363. doi:DOI 10.2323/jgam.44.361

25. Shrove, G. S., Olsen, R. H., & Vogel, T. M. (1991). Development of pure culture biofilms of *P. putida* on solid supports. Biotechnol Bioeng, 37(6), 512-518. doi:10.1002/bit.260370604

26. Snauwaert, I., Stragier, P., De Vuyst, L., & Vandamme, P. (2015). Comparative genome analysis of *Pediococcus damnosus* LMG28219,astrainwell-adaptedtothebeerenvironment.BMCGenomics, 16, 267. doi:10.1186/s12864-015-1438-z

27. Suzuki, K., Iijima, K., Ozaki, K., & Yamashita, H. (2005). Isolation of a hop-sensitive variant of *Lactobacillus lindneri* and identification of genetic markers for beer spoilage ability of lactic acid bacteria. Appl Environ Microbiol, 71(9), 5089-5097. doi:10.1128/Aem.71.9.5089-5097.2005

28. Suzuki, K., Iijima, K., Sakamoto, K., Sami, M., & Yamashita, H. (2006). A review of hop resistance in beer spoilage lactic acid bacteria. Journal of the Institute of Brewing, 112(2), 173-191. doi:DOI 10.1002/j.2050-0416.2006.tb00247.x

29. Thompson, L. J., Gray, V., Lindsay, D., & von Holy, A. (2006). Carbon : nitrogen : phosphorus ratios influence biofilm formation by *Enterobacter cloacae* and *Citrobacter freundii*. Journal of Applied Microbiology, 101(5), 1105-1113. doi:10.1111/j.1365-2672.2006.03003.x

30. Tsiaprazi-Stamou, A., Monfort, I. Y., Romani, A. M., Bakalis, S., & Gkatzionis, K. (2019). The synergisticeffectofenzymaticdetergentsonbiofilmcleaningfromdifferentsurfaces.Biofouling,35(8), 883-899. doi:10.1080 /08927014.2019.1666108

31. Valpar Beverage Tubing. (2000-2019, 2019). Retrieved from https://www.pasp.com.br/valpar- beverage-tubing.html

32. Walker, S. L., Fourgialakis, M., Cerezo, B., & Livens, S. (2007). Removal of microbial biofilms from dispenseequipme nt:Theeffectofenzymaticpre-digestionanddetergenttreatment.Journalofthe Institute of Brewing, 113(1), 61-66. doi:DOI 10.1002/j.2050-0416.2007.tb00257.x

33. Zhou, C., Song, H. Q., Loh, J. L. C., She, J. Q., Deng, L. H., & Liu, B. (2018). Grafting antibiofilm polymer hydrogel film onto catheter by SARA SI-ATRP. Journal of Biomaterials Science-Polymer Edition, 29(17), 2106-2123. doi:10.10 80/09205063.2018.1507268

Real-time Screening of Foods Using Repetitive Element PCR Reveals a DNA Marker Characteristic for Enterotoxigenic *Bacillus* Species

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Abstract

Bacillus cereus is traditionally thought to be the only member of its genus accepted as a pathogen in foods like grains, fruits, vegetables, and milk due to the presence of the nonhemolytic (Nhe) operon. However, many other *Bacillus* spp. may also harbor the Nhe operon and be pathogenic, including not just food-associated gastrointestinal toxicoinfections, but human endophthalmitis as well. Real-time PCR targeted the *nheA* gene in 37 samples obtained from food, soil, and reference cultures by analyzing the standard deviations of melt peaks. Repetitive element PCR was used to compare the banding patterns of each sample against *B. cereus* ATCC 14579 and three *B. thuringiensis* strains to "fingerprint" each isolate. Of the original 43 isolated tested, 37 were Gram-positive rods. The remaining six samples were Gram-positive cocci. Twenty-five of the 37 Gram-positive *Bacillus* spp. were *nheA* positive, while twelve were negative. Many of the *nheA* positive strains were species not previously known to contain Nhe and were capable of causing gastroenteritis in consumers.

Introduction

Bacillus spp. are Gram-positive endospore-forming rods ubiquitous in soil worldwide and are primarily aerobic to facultatively anaerobic saprophytes (3). Over 148 distinct *Bacillus* species have been described. This large number of individual species reflects a high degree of genetic diversity. Taxonomical identification of species within the *Bacillus* genus has changed over time as differentiation methods have improved (17). Currently, *Bacillus* species are divided into two groups - the *B. subtilis* and *B. cereus* divisions. The *B. cereus* group includes *B. cereus, B. mycoides, B. anthracis, B. thuringiensis,* and *B. weihenstephanensis*. These species are also grouped under the name *B. cereus sensu lato* (51). Phylogenetically, *B. cereus* is quite closely related to the entomopathogen *B. thuringiensis* and the human pathogen *B. anthracis*, a fact that has led to vigorous discussion on shared virulence properties, DNA sequence conservation among strains, and prevalence in the environment (19, 39, 48). Along with *B. weihenstephanensis*, these species constitute a single genetic subgroup, a rather arbitrary classification designation that brings into question how a species is even defined in this family of bacteria. For example, it seems clear that at least for many *Bacillaceae* other than *B. cereus*, presence and expression of enterotoxin genes is not uncommon (7, 11, 18, 20); nor is it atypical to identify strains of *B. cereus* lacking detectable enterotoxin genes (5, 48). Moreover, *B. cereus* may harbor virulence genes on plasmids more commonly associated with *B. anthracis* (49). Many pathogenic strains of *Bacillus* spp. are primary isolates from clinical, food, and environmental sources. Naturally, many published studies on the *B. cereus* group mention the difficulty in selecting features for reliable identification of these species. The involvement of species in the *B. cereus* group in foodborne illness, as a leading cause of ocular infections (endophthalmitis), and as an indicator of water quality (4, 25), begs the question of how such a closely related set of species and strains could manifest itself so differently in varied environments. Accordingly, a reassessment of identification strategies is in order as even more reports appear in the literature of *Bacillus* spp. involvement in novel ecological niches.

The search for a reliable DNA-based typing approach for *Bacillaceae* has explored several technologies in recent

Table 1.

Lesaffre Yeast	Smuckers French Vanilla
Seasoning	**Snacks**
Basil	Beef Taco from Taco Bell
Oregano	Food Club Quick Oats
Mustard	Ann's House Healthy Energy Blend Nuts
Nutmeg	Kraft Jet-Puffed Marshmallows
Paprika	Dannon Yogurt
Milk	**Coffee Creamer**
Parmalat 2% Reduced Fat Milk	Coffee Mate
Mix N' Drink Skim Milk	Wholesome Farms
Great Value Evaporated Milk	Great Value
Myenberg Vitamin D Goat Milk	Glenview Farms
Carnation Evaporated Milk	Flavor Right Half and Half
Prairie Farms	

Table 1: Foods screened for the presence of *Bacillus* spp. by food type as described in Materials and Methods.

years, including repetitive element PCR (rep-PCR) (34), next generation sequencing (NGS) of whole genomes to identify polymorphic regions (12), and multilocus sequence typing (MLST) (6, 21, 22), which relies on the PCR-based amplification of 400- 600bp internal fragments of housekeeping genes. However, these conserved gene targets are frequently not adequate to effectively resolve *Bacillus* species or strains for identification. Clearly, a repertoire of both phenotypic and novel genotypic-based methods must be utilized for the ever-increasing number of strains appearing in the literature. This trend reflects a growing interest in this group of bacteria (4). The objective and hypothesis of this research is that one may develop a genotypic screening method to reliably detect enterotoxigenic *Bacillus* spp. from contaminated food without the need for culture-based methods. Use of DNA typing/fingerprinting compared to positive control enterotoxigenic (Nhe-producing) *Bacillus*, we show the potential in rep-PCR as a rapid and high-throughput screening tool for a variety of contaminated foods.

MATERIALS AND METHODS

Bacillus spp. isolation from soil and food.

Using a previously described method (46), soil was collected (at 4-inch depths) from multiple locations around the Ball State University campus in Muncie, IN. A total of 41 varieties of flavoring/ powder, seasonings, milk, coffee creamers, cheese, snacks, spreads, and drink additives were obtained at local retailers in order to isolate *Bacillus* spp. from these naturally contaminated foods (Table 1). Each sample (5g) was added to 100 mL of brain heart infusion broth (BHIB; BD Diagnostic Systems, Franklin Lakes, NJ). After mixing, the solution was incubated at 32°C while shaking at 160 RPM for 72h. Samples were heat-treated at 80°C for 30 min on a hot plate using a water jacketed vessel and constant shaking, after which the suspension (1 mL) was pipetted onto quadruplicate tryptic soy agar (TSA; Alpha Biosciences, Baltimore, MD) plates and incubated overnight at 37°C. Streak plates were performed from initial growth and incubated at 37°C overnight to obtain pure cultures, confirmed by Gram and endospore staining. Reference strains (Table 2) were obtained from Presque Isle Cultures (Erie, PA USA) and Dr. James Mitchell (Ball State University, Muncie, IN) and subcultured

Table 2.

Strain	Identifying Code	Source
B. subtilis globigii	6201	Presque Isle Cultures
B. stearothermophilus	627	Presque Isle Cultures
Geobacillus stearothermophilus	627A	Presque Isle Cultures
B. sphaericus	633	Presque Isle Cultures
B. megaterium	616	Presque Isle Cultures
B. macerans	626	Presque Isle Cultures
B. pumulis	6222	Presque Isle Cultures
B. brevis	630	Presque Isle Cultures
B. polymyxa	625	Presque Isle Cultures
B. coagulans	6221	Presque Isle Cultures
B. circulans	628	Presque Isle Cultures
B. subtilis	620	Presque Isle Cultures
B. laterosporos	629	Presque Isle Cultures
B. cereus	14579	ATCC
B. thuringiensis kurstaki HD1	33679	Dr. James Mitchell
B. thuringiensis japanensis B23	T23 001	Dr. James Mitchell
B. thuringiensis israelensis	T14 001	Dr. James Mitchell

Table 2: *Bacillus* reference strains used in this study.

onto TSA slants. All cultures were refrigerated at 4°C until DNA extraction and real-time PCR analysis.

DNA Extraction.

Each isolate, including reference strains, was separately cultured in tryptic soy broth (TSB, Weber Scientific, Hamilton, NJ USA) grown aerobically by shaking for 24h as previously described (31). Bacteria were pelleted by centrifugation at 9,000 x g for 3 min at 4°C and the pellets were resuspended in 300µl TE buffer (Amresco, Solon, OH) containing 30 µl of 10% SDS (Promega, Madison, WI), and 20 µl of 20 mg/mL Proteinase K (Ambion, Austin, TX). Following a 37°C 30 min. incubation, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) solution (Amresco) was added, vortexed, and centrifuged for 10 min. at 10,000 x g and 4°C. The aqueous phase was carefully transferred into clean microcentrifuge tubes and mixed with 0.1 volume of cold 3M sodium acetate (Fisher Scientific, Pittsburgh, PA) and one volume of cold 95% isopropanol (Greenfield Ethanol Co., Brookfield, CT). The microcentrifuge tubes were inverted to mix and centrifuged for 30 min at 16,000 x g at 4°C. The supernatant was discarded, and the dried DNA pellets were quantified spectrophotometrically to assess yield and purity.

Uniplex PCR.

All DNA samples were normalized to a concentration of 1 µg/µL in sterile water. All reactions were performed in triplicate. Real-time PCR was used initially to target the *nheA* gene (32). Primer sequences used for this and other experiments are shown in Table 3.

PCR was performed as previously described (18) with minor modifications. The annealing temperature was changed from 55°C to 52°C to better support annealing of the *nheAF* primer to template DNA, and a melting curve was used to resolve and validate amplicon identity. Each PCR reaction consisted of 1X iQ Sybr Green Supermix (Bio Rad, Hercules, CA), 100 pmol *nheAF* and *nheAR* primers (Integrated DNA Technologies, Coralville, IA), and 0.5 µg of template DNA. Nuclease-free water (Promega) was added for a final volume of 25 µL in 0.2 mL PCR tubes (Corbett Research, Concord, NSW). A positive control was included in each set of reactions, consisting of template DNA from *B. cereus* ATCC 14579, (American Type Culture Collection, Manassas, VA), previously shown to harbor the *nheABC* operon (41).

PCR reactions were performed in a Rotor Gene RG-3000 thermocycler (Corbett Research) using an initial 94°C 120s denaturation step followed by 35 cycles of 94°C for 20s, 52°C for 60s, and 72°C for 60s. A final 72°C 6 min. extension step preceded melting curve analysis (40°C to 95°C in 0.7°C per second increments). Amplicon melting peaks were plotted using Rotor Gene 6 software and melt peak data were exported into Microsoft Excel for analysis. Only melt peaks within 1 standard deviation of the average melt peak of positive control *B. cereus* ATCC 14579 were considered as positive for the presence of *nheA*.

Rep-PCR.

DNA templates from all strains analyzed (Table 1) were subjected to repetitive element- PCR (rep-PCR) using a Diversilab kit (Bacterial Barcodes, Athens, GA) specific for fingerprinting *Bacillus* spp. DNA of each sample previously isolated for real-time PCR was re-standardized to 50 ng/µl. Primers for repetitive elements within *Bacillaceae* were included in the Diversilab kit and are shown in Table 3 (24).

All reactions were completed in triplicate and consisted of 18 µl rep-PCR MM1 buffer, 2.5 µl of GeneAmp® 10X PCR Buffer, 2.0 µl of primer mix, and 0.5 µl of Taq DNA Polymerase (5 PRIME, Gaithersburg, MD) and 100 ng of template DNA. Positive kit controls were included with each set of replicates, as were no template controls (NTC). All reactions were performed using a Rotor Gene instrument and consisted of an initial 94°C 2 min. denaturation followed by 35 cycles of 94°C for 30s, 55°C for 30s, and 70°C for 90s. Following a final extension step for 3 min at 70°C, a subset of reactions was subjected to melting curve analysis as described earlier, while others were analyzed using agarose gel electrophoresis. For the latter samples, 5µl of each PCR product was loaded into a 1.5% (w/v) agarose gel (BioExpress, Kaysville, UT) containing 0.625 µg/µl ethidium bromide

(Invitrogen, Carlsbad, CA) (43) and the gel electrophoresed for 1.5h at 70V (constant). The gel was visualized on a Gel Doc XR (Bio-Rad, Hercules, CA) using UV light. The resulting banding patterns were recorded in Microsoft Excel as a virtual gel (Table 4). Banding patterns of *nheA* positive and *nheA* negative were compared against the *B. cereus* reference strain and three *B. thuringiensis* strains, representing additional members of the B. cereus genetic subgroup. Sample bands identical to each reference strain (*B. cereus, B. thuringiensis* var. *kurstaki*, *B. thuringiensis* var. *japanensis,* and *B. thuringiensis* var. *israelensis*) were divided by the number of total bands in each reference strain. The resulting number was multiplied by 100 to determine the percent each sample was identical to *B. cereus, B. thuringiensis* var. *kurstaki, B. thuringiensis* var. *japanensis,* and *B. thuringiensis* var. *israelensis*.

Sequencing of diagnostic rep-PCR product.

The 1,230bp diagnostic band (11) earlier found to be unique to enterotoxigenic *Bacillus* spp. was identified in real-time melting curve plots of *B. cereus* ATCC 14579 and other strains.

RESULTS

Samples

Excluding reference microbes purchased from Presque Isle Cultures, a total of 45 food and soil samples were screened for the presence of *Bacillus* spp. Of these, 21 isolates (48.9%) were found to contain no detectable *Bacillaceae*. Twenty isolates (44.4%) were Gram-positive, spore-forming rods after heat-treatment and subsequent streak-plating on TSA. These included: basil seasoning, nutmeg seasoning, Tazo tea powder, a beef taco from Taco Bell, Lesaffre Yeast, Prairie Farms Whole Milk, Food Club Quick Oats, Ann's House Healthy Energy Blend Nuts, Peter Pan Peanut Butter, Great Value Peanut Butter, Dannon Yogurt, Chevre Fresh Goat Cheese, Saputo Stella Gorgonzola Cheese, Black Creek Extra Sharp Cheddar Cheese, Pilgrim's Choice Blue Stilton Cheese, Cooper Science Building Soil, Lucina Building Soil, Christy Woods Soil, and Ball Gymnasium Soil. An additional six isolates, three of which were isolated from Jiffy Corn Muffin Mix, were Gram-positive cocci. Aside from Jiffy Corn Muffin Mix, Gram-positive cocci were isolated from mustard seasoning and Mix n' Drink Powdered Skim Milk. These Gram-positive cocci accounted for a total of 6.7% of the entire sample pool.

Seventeen *Bacillus* spp. reference strains were purchased from Presque Isle Cultures for subsequent real-time PCR analysis. Overall, a total of 37 samples were either pure-type cultures or Gram-positive rods that were subsequently subjected to DNA extraction in preparation for real-time and rep-PCR.

Real-time PCR

B. cereus ATCC 14579 was used as a positive control to test for the presence of *nheA* and had an average melt peak of

Table 3.

nheA	Primer Sequence 5' → 3'	Position	Accession #	Ref	T_m	G/C
F	TACGCTAAGGAGGGGCA	344 – 360 →	Y19005	9	55.5°C	58.80%
R	GTTTTTATTGCTTCATCGGCT	843 – 823 ←	-	-	51.8°C	38.10%
Direction	**Primer Sequence 5'→ 3'**	**Primer Name**	-	**Ref**	**T_m**	**G/C**
F	ICG ICT TAT CIG GCC TAC	REP 2-I	-	15	57.5°C	50%
R	III ICG ICG ICA TCI GGC	REP 1R-I	-	15	68.3°C	50%

Table 3: Primer sequences, melting temperatures, and guanine and cytosine content for the *nheA* gene used in uniplex PCR and repetitive element (rep-PCR) palindromic sequences in *Bacillus* spp.

81.96°C over three runs. As shown in Table 5, fourteen test samples (37.84% = green highlighted) were consistently within 1 standard deviation (SD) of the positive control over three separate real- time PCR runs (SD determined using SigmaStat for Windows). Standard deviations of samples positive all of three replications for *nheA* are included in Table 6, part B. These samples included reference strains *B. macerans, B. brevis, B. cereus, B. thuringiensis var. kurstaki, B thuringiensis var. japanensis,* and *B. thuringiensis var. israelensis*. Food samples consistently within one SD of the positive control originated from Prairie Farms Whole Milk, Ball Gymnasium soil, Lucina Hall soil, Cooper Science soil, Christy Woods soil, basil seasoning powder, "Clean" Peter Pan Peanut Butter, and Great Value Peanut Butter. Five samples (13.51% = yellow highlighted had two melt peaks within 1 SD of the positive control strain. These included the pure strain *B. laterosporos* and food samples from a beef soft taco from Taco Bell, nutmeg powder, Chevre Fresh Goat Cheese, and Saputo Stella Gorgonzola Cheese.

Table 4.

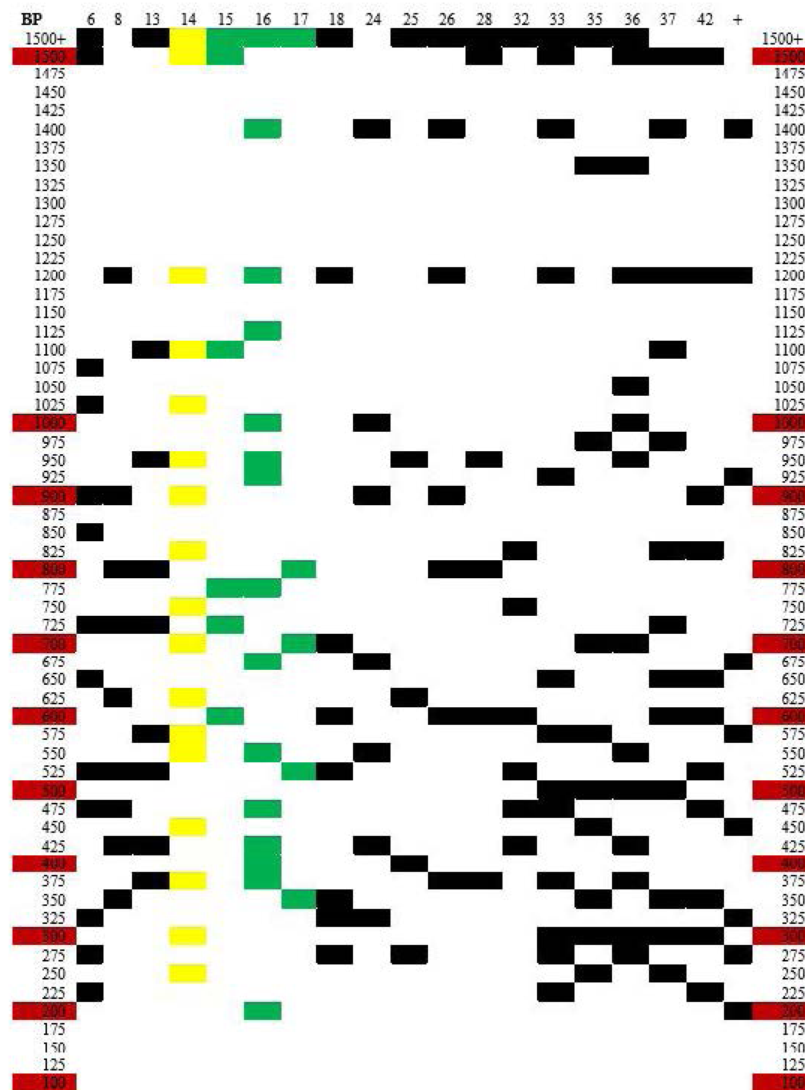

Table 4: Banding patterns of all *nheA* positive samples, excluding samples 9, 10, 22, 27, 31, 39, and 43. Red cells represent the length in base pairs of each DNA ladder band. Yellow cells represent the column of positive control *B. cereus* ATCC 14579. Green cells represent banding of *B. thuringiensis var. kurstaki* (#15), *B. thuringiensis var. japanensis* (#16), and *B. thuringiensis var. israelensis* (#17).

Table 5.

#	Sample	#	Sample
1	*B. subtilis globigii*	23	Jiffy Cornbread Cream Colony
2	*B. stearothermophilus*	24	Ball Gym Soil
3	*Geobacillus stearothermophilus*	25	Lucina Building Soil
4	*B. spaericus*	26	Basil Seasoning Powder
5	*B. megaterium*	27	Taco Bell Soft Beef Taco
6	*B. macerans*	28	Cooper Science Soil
7	*B. pumulis*	29	Mustard Seasoning Powder
8	*B. brevis*	30	Jiffy Cornbread Translucent Colony
9	*B. polymyxa*	31	Nutmeg Powder Seasoning
10	*B. coagulans*	32	Christy Woods Soil
11	*B. circulans*	33	Chevre Fresh Goat Cheese
12	*B. subtilis*	34	Black Creek Sharp Cheddar Cheese
13	*B. laterosporos*	35	Saputo Stella Gorgonzola Cheese
14	*B. cereus*	36	Pilgrim's Choice Blue Stilton Cheese
15	*B. thuringiensis kurstaki*	37	"Clean" Peter Pan Peanut Butter
16	*B. thuringiensis japanensis*	38	Tazo Tea Powder
17	*B. thuringiensis israelensis*	39	Ann's House Energy Blend Nuts
18	Prairie Farms Whole Milk	40	Dannon Yogurt
19	Jiffy Cornbread Yellow Colony	41	Lesaffre Yeast Isolate
20	Mix N' Drink Powdered Milk	42	Great Value Peanut Butter
21	Nesquick	43	*Salmonella*-Contaminated Peter Pan Peanut Butter
22	Food Club Quick Oats		

Color	Legend
(green)	= 3 of 3 melt peaks within 1°C of *B. cereus* control (82°C) melt peak
(yellow)	= 2 of 3 melt peaks within 1°C of *B. cereus* control (82°C) melt peak
(orange)	= 1 of 3 melt peaks within 1°C of *B. cereus* control (82°C) melt peak
(red)	= 0 of 3 melt peaks within 1°C of *B. cereus* control (82°C) melt peak
(blue)	= Not Gram-positive rods, but isolated after heat-treatment

Table 5: Samples were positive or negative for the presence of *nheA* 3/3, 2/3, 1/3, or 0/3 times. Green samples indicate positive detection of *nheA* in real-time PCR over three separate runs. Yellow samples indicate *nheA* positive samples in two of three real-time PCR runs. Orange samples indicate *nheA* positive samples in one of three real-time PCR runs. Red samples indicate negative *nheA* detection in real-time PCR.

Six samples (16.22% = orange highlighted) had one melt peak within 1 SD of the positive control train. These included pure strains *B. polymyxa* and *B. coagulans*. In addition, food samples with only 1 of 3 melt peaks consistent with *B. cereus* positive control were from Food Club Quick Oats, Pilgrim's Choice Blue Stilton Cheese, Ann's House Energy Blend Nuts, and a jar of Peter Pan Peanut Butter involved in a food recall that may have contained *Salmonella*.

Twelve of the 37 samples (32.43% = red highlighted) had either no melt peaks or melt peaks greater or less than one SD of the positive control strain. These included: pure strains *B. subtilis globigii, B. stearothermophilus, Geobacillus stearothermophilus, B. spaericus, B. megaterium, G. pumulis, B. circulans,* and *B. subtilis*. Foods negative for nheA included Black Creek Extra Sharp Cheddar, Tazo Tea powder, Dannon Yogurt, and Lesaffre Yeast.

As shown in Table 6, part A, there were 4 of 16 food samples that tested positive for *nheA* for three melt peaks. Additionally, 4 food samples displayed two positive melt peaks, while 4 more displayed one melt peak. Four food samples displayed zero melt peaks. All soil samples were positive for *nheA* with three melt peaks. Six reference strains displayed three positive melt peaks for *nheA*, while only one strain had two melt peaks consistent with the positive control. Two strains had one melt peak in line with the positive control, while 8 strains were completely negative for the presence of *nheA*.

After real-time PCR, SD were calculated for sample melt peaks to compare against *B. cereus* ATCC 14579. Samples 6, 8, 14, 15, 16, 17, 18, 24, 25, 26, 28, 32, 37, and 42 resulted in standard deviations < 1 when compared against the 82°C average positive control melt peak. Any sample with a SD < 1 indicated a positive detection for the *nheA* gene. Data are shown in Table 6, part B.

Table 6.

A.

# *nheA* +	Total Food	% Food	Total Soil	% Soil	Total Ref.	% Ref.
3/3	4	25	4	100	6	35.29
2/3	4	25	0	0	1	5.88
1/3	4	25	0	0	2	11.76
0/3	4	25	0	0	8	47.06
	16	**100**	**4**	**100**	**17**	**100**

B.

Sample	**6**	**8**	**14**	**15**	**16**	**17**	**18**
S.D.	0.27	0.29	0.18	0.22	0.23	0.12	0.62
Sample	**24**	**25**	**26**	**28**	**32**	**37**	**42**
S.D.	0.38	0.27	0.32	0.34	0.29	0.27	0.30

Table 6: A: Number of samples with *nheA* positive melt peaks three, two, one, and zero times in divisions of food, soil, and reference strains. **B:** Samples with corresponding SDs < 1 when compared against the positive control strain *B. cereus* ATCC 14579 during real-time PCR.

Rep-PCR

Repetitive element PCR was utilized on *B. cereus* ATCC 14579, which was labeled as sample 14 for real-time and rep-PCR. This strain was subsequently used as the standard against which all other *nheA* positive samples in rep-PCR were compared. Sample 14 displayed 16 bands within the range of the DNA ladder, as shown in Table 4. These bands corresponded to lengths of 1500 bp, 1200 bp, 1100 bp, 1025 bp, 950 bp, 900 bp, 825 bp, 750 bp, 700 bp, 625 bp, 575 bp, 550 bp, 450 bp, 375 bp, 300 bp, and 250 bp. Table 7 includes all banding patterns for all *nheA* positive samples. All other banding patterns were compared against *B. cereus* reference strain (#14) and three *B. thuringiensis* spp. reference strains (#15-17). All samples were compared against *B. cereus, B. thuringiensis var. kurstaki, B. thuringiensis var. japonensis,* and *B. thuringiensis var. israelensis*. The resulting percent identities of the banding patterns to each reference strain of each sample are recorded in Table 7.

B. cereus (sample #14)

Samples 17 and 25 were 6% identical to the banding pattern of sample 14. Samples 6, 15, 18, 24, and 32 were 13% identical to sample 14. Samples 8, 13, 26, and 28 were 19% identical to sample 14 banding. Samples 16 and 42 were 25% identical to sample 14, while samples 33 and 35 were 31% identical. Samples 36 and 37 were 44% identical to sample 14. No samples were more than 44% identical to sample 14.

Nine *nheA* negative samples were analyzed using rep-PCR, and include samples 1-5, 7, 9, 11, and 12 (data not shown). Samples 2, 3, 5, and 7 were 6% identical to the banding

pattern of sample 14. Samples 1, 4, and 12 were 19% identical to sample 14. Sample 11 was 44% identical to the banding pattern from sample 14.

B. thuringiensis var. kurstaki (sample #15)

When compared against *B. thuringiensis var. kurstaki*, samples 17, 24, 25, and 35 shared no identical banding. Samples 8, 16, 18, 26, 32, 33, and 36 were 20% identical to B. thuringiensis var. kurstaki. Samples 6, 13, 14, 28, and 42 were 40% identical, while sample 37 was 80% identical to *B. thuringiensis var. kurstaki*.

B. thuringiensis var. japanensis (sample #16)

Samples 6, 17, and 35 shared no identical banding with *B. thuringiensis var. japanensis*, while samples 15 and 18 were 8% identical. Samples 28 and 32 were 17% identical to *B. thuringiensis var. japanensis*, but samples 8, 13, 26, and 37 were 25% identical. Samples 14 and 42 were 33% identical to *B. thuringiensis var. japanensis*. Samples 24 and 33 were 42% identical to *B. thuringiensis var. japanensis* banding, while sample 36 was 50% identical.

Table 7.

Compared against #14																		
Sample	6	8	13	14	15	16	17	18	24	25	26	28	32	33	35	36	37	42
Band 1500+	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0
Exact	2	3	3	16	2	4	1	2	2	1	3	3	2	5	5	7	7	4
Bands in Range	12	8	8	16	5	12	4	7	6	4	6	5	6	12	10	13	14	11
Total Bands	13	9	9	17	6	13	5	8	7	5	7	6	7	13	11	14	15	12
% Identical to Sample #14	13	19	19	100	13	25	6	13	13	6	19	19	13	31	31	44	44	25
Compared against #15																		
Sample	6	8	13	14	15	16	17	18	24	25	26	28	32	33	35	36	37	42
Band 1500+	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0
Exact	2	1	2	2	5	1	0	1	0	0	1	2	1	1	0	1	4	2
Bands in Range	12	8	8	16	5	12	4	7	6	4	6	5	6	12	10	13	14	11
Total Bands	13	9	9	17	6	13	5	8	7	5	7	6	7	13	11	14	15	12
% Identical to Sample #15	40	20	40	40	100	20	0	20	0	0	20	40	20	20	0	20	80	40
Compared against #16																		
Sample	6	8	13	14	15	16	17	18	24	25	26	28	32	33	35	36	37	42
Band 1500+	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0
Exact	0	3	3	4	1	12	0	1	5	1	3	2	2	5	0	6	3	4
Bands in Range	12	8	8	16	5	12	4	7	6	4	6	5	6	12	10	13	14	11
Total Bands	13	9	9	17	6	13	5	8	7	5	7	6	7	13	11	14	15	12
% Identical to Sample #16	0	25	25	33	8	100	0	8	42	8	25	17	17	42	0	50	25	33
Compared against #17																		
Sample	6	8	13	14	15	16	17	18	24	25	26	28	32	33	35	36	37	42
Band 1500+	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0
Exact	1	3	2	1	0	0	4	3	0	0	1	1	1	1	2	1	1	2
Bands in Range	12	8	8	16	5	12	4	7	6	4	6	5	6	12	10	13	14	11
Total Bands	13	9	9	17	6	13	5	8	7	5	7	6	7	13	11	14	15	12
% Identical to Sample #17	25	75	50	25	0	0	100	75	0	0	25	25	25	25	50	25	25	50
(red)	= Between 40% and 99% identical to yellow reference strain																	
(green)	= Between 30% and 39% identical to yellow reference strain																	
(blue)	= Between 20% and 29% identical to yellow reference strain																	
(yellow)	= Sample all others were compared against																	

Table 7: Comparison of all rep-PCR banding patterns with *B. cereus* (sample #14), *B. thuringiensis var. kurstaki* (sample #15), *B. thuringiensis var. japanensis* (sample #16), and *B. thuringiensis var. israelensis* (sample #17). Yellow cells denote the specific sample all other banding patterns were compared against. Blue cells represent banding patterns 20% to 29% identical to each yellow reference strain. Green cells represent banding patterns 30% to 39% identical to each yellow reference strain. Red cells represent banding patterns identical banding that was 40% and above to each yellow reference strain. No samples were more than 44% identical to *B. cereus*. Sample 37 was 80% identical to *B. thuringiensis var. kurstaki*. Sample 36 was 50% identical to *B. thuringiensis var. japonensis*. Samples 13, 35, and 42 were 50% identical to *B. thuringiensis var. israelensis*, while samples 8 and 18 were 75% identical.

***B. thuringiensis var. israelensis* (sample #17)**

When compared against *B. thuringiensis var. israelensis*, samples 15, 16, 24, and 25 were 0% identical. Samples 6, 14, 26, 28, 32, 33, 36, and 37 were 25% identical to *B. thuringiensis var. israelensis*. Samples 13, 35, and 42 were 50% identical to *B. thuringiensis var. israelensis*, while samples 8 and 18 were 75% identical.

As shown in Table 5, samples 9, 10, 22, 39, and 43 had one *nheA* positive amplicon during real-time PCR. Samples 27 and 31 had two *nheA* positive amplicons late in analysis after initially appearing to only contain one positive melt peak. Consequently, these samples were not subjected to rep-PCR, as the real-time results were inconsistent. Rep-PCR efforts were instead directed at samples that had either three melt peaks or two melt peaks early in analysis within 1 SD of the positive control.

DISCUSSION

The debate over the ideal method for identification of *Bacillus* isolates has raged for over 50 years (42). Recent public awareness of potential bioterrorism using the anthrax toxin produced by *B. anthracis* has led government agencies to fund multiple studies aimed at rapidly differentiating *B. anthracis* from other closely related *Bacillus* species, such as *B. cereus* and *B. thuringiensis*. *B. anthracis* produces the anthrax toxin encoded by two plasmid-based operons, pXO1 and pXO2 (3, 17, 42). The anthrax toxin primarily kills herbivore mammals but can also kill humans (42, 48). Not to be underestimated, *B. cereus* can cause severe food poisoning through its production of emetic and diarrheal toxins (3, 19). While heavily used as an insecticidal agent in crops with its Cry crystalline toxins, *B. thuringiensis* has also recently been demonstrated to cause food poisoning symptoms in humans similar to *B. cereus* (3, 42). Ironically, species like *Bacillus coagulans*, which was found to contain *nheA* at least once in this study, are readily used as probiotics in human health (30). It should be noted that the *nhe* genes have been among the most common reference virulence genes targeted in PCR-based assays performed in foods, including dairy foods (37). NheA/*nheA* has thus been widely accepted as an indicator of virulence potential in *Bacillus* spp. *senso lato*.

These strains were originally differentiated into species at a time when biologists did not possess the molecular tools to delve deeper than biochemical tests and phenotypical observations (3, 17, 42). While this strategy worked well for other genera, 16S rRNA analysis of differences among *B. cereus, B. thuringiensis, and B. anthracis* have shown these species to have a nucleotide sequence difference of < 1% (48). Thus, the emerging "holy grail" of *Bacillus* research would be to accurately differentiate these species. Recent advances in molecular biology have allowed scientists to scrutinize the genetic properties of these three "species" (42). After exhaustive studies using DNA-DNA hybridization, 16S and 23S rRNA comparative analyses, multilocus sequence typing (MLST), fluorescent amplified fragment length polymorphism analysis, rep-PCR, and small nucleotide polymorphism (SNP) analyses, scientists have been unable to reliably differentiate these three *Bacillus* species.

While many methods have been pursued, most results have suggested that *B. cereus, B. thuringiensis,* and *B. anthracis* should be considered the same species due to highly conserved nucleoidal genetic sequences (3, 17, 39, 43, 48). Due to the easily identifiable symptoms of *B. anthracis* and *B. cereus*, there is recent concern among biologists that the *"B. anthracis"* species may in fact be an oversampled subset of *B. cereus* (42). Other scientists speculate that *B. anthracis* may have only recently evolved to the point to be considered distinct from *B. cereus* (20). Either way, a separate study confirmed that enough of a difference exists between the genome of *B. anthracis* when compared against *B. cereus* or *B. thuringiensis* to consider *B. anthracis* as identifiable using pulsed-field gel electrophoresis (50).

Of the 45 total food and soil samples in this study, 20 *Bacillus* isolates were obtained (44.4%). Twenty-one samples (48.9%) were not found to contain *Bacillus* isolates. Three isolates were plated from Jiffy Corn Muffin Mix along with two other samples for a total of 6.7% after heat-treatment but were Gram-positive cocci. Because this research examined *Bacillus* spp., any non-Gram-positive rod specimens were not analyzed further.

Bacillus spp. are ubiquitous in nature and form endospores that readily transfer to foods (3, 16, 17). Initially for the *Bacillus* isolation approach, nutrient rich BHIB incubation overnight at 32°C did not allow for endospore formation. Endospores optimally form when the bacteria are stressed and require 1 to 2 days for full development (3, 36). While most samples had already been screened for *Bacillus* presence, the remaining few were instead shaken for three days at the same conditions to allow sufficient time for endospore formation. Consequently, endospores were better isolated after this change. It is likely that *Bacillus* spp. endospores were present in many samples that lacked detectable *Bacillus* isolates initially, like Nestle Nesquik, given their general ubiquity (17). These samples were then subjected to real-time PCR analysis.

There are three *nhe* genes that are encoded on the *nheABC* operon (3) and have been shown to remain conserved as a cluster during genetic recombination (19). It can reasonably be assumed that the presence of the most proximal subunit of *nhe* indicates the presence of the other two genes. In the literature, all genes encoding the Nhe and Hbl enterotoxins have been readily located downstream in both *B. cereus* and *B. thuringiensis* (35). Of 616 *Bacillus* isolates tested, none were found to harbor only a single or two of the genes for each operon.

Over three separate real-time PCR runs, all 4 soil samples had three melt peaks within 1 SD of *nheA* positive *B. cereus* ATCC 14579. Thus, they were also positive for the presence of the *nheA* gene. By extension, these strains were also positive for the presence of the *nheABC* operon and could be considered pathogenic. Samples with three melt peaks consistent with the *B. cereus* positive control also resulted in standard deviations much less than 1, as shown in Table 6B. These melt peaks were extremely similar to each other and to the positive control, meaning that the amplified product was, in fact, *nheA*.

Of 16 total food isolates, four displayed three *nheA* positive melt peaks, while four displayed two *nheA* positive melt peaks. Additionally, 4 food isolates displayed only one *nheA* positive melt peak, while four were found to contain no identifiable *nheA* genes. Over three real-time PCR runs, samples with three melt peaks within 1 SD of the *nheA* positive control strain were also considered positive for the presence of *nheA*. Samples with two of three melt peaks within 1 SD of the positive control strain were also considered to be positive for the presence of *nheA*, even with an erroneous third melt peak. While real-time PCR is an accurate assay for gene detection, it is still sensitive to pipette error as well as PCR inhibitors (29). Thus, it is likely that user error prevented a third melt peak within 1 SD of the positive control.

Samples with one of three melt peaks within 1 SD of the positive control were treated as potentially positive for the presence of *nheA*. However, further research of these strains needs to be performed for a definitive answer. One positive melt peak was not determined to be strong enough evidence to ignore two negative results.

After real-time PCR analysis, reference cultures *B. macerans, B. brevis, B. cereus, B. thuringiensis var. kurstaki, B. thuringiensis var. japanensis,* and *B. thuringiensis var. israelensis* also displayed three melt peaks within 1 SD of *nheA* positive *B. cereus* over three runs. Additionally, *B. laterosporos* displayed two of three total melt peaks consistent with the positive control strain, and by extension contained the *nheABC* operon. The *B. thuringiensis* and *B. cereus* sample results were expected and confirm earlier work indicating that both are pathogenic (33, 39, 48). While the reference strains *B. circulans* and *B. megaterium* were not positive for the presence of *nheA* in this study, they were found to harbor each Hbl gene in a separate study (44). It is very possible these strains contained a polymorphic version of *nheA*.

To the best of our knowledge these samples, minus *B. cereus* and *B. thuringiensis*, are novel findings that are not usually associated with food pathogenicity (3, 16, 17, 18). However, it is an unsurprising find that *Bacillus* isolates harboring the *nheA* gene were identified in food, at least in *B. thuringiensis* and *B. cereus*. *B. cereus* and *B. thuringiensis* are arguably the same species (3) and have been demonstrated to be pathogenic in food (18). There is a general consensus among biologists that most, if not all, *Bacillus* isolates undergo horizontal gene transfer (17).

One study determined that of the *B. cereus* and *B. thuringiensis* isolates obtained from rice, 84.3% and 100% of them produced the *Nhe* enterotoxin, respectively (1). Sixty-one percent and 100% of these same isolates produced the Hbl enterotoxin, respectively. A separate study found that of 136 *B. cereus* isolates obtained from milk, over half were toxic against HeLa cells (10). Additionally, 73.2% were toxic against HEL cells. A third study noted that of emetic strains identified, 77.5% of *B. cereus* strains also produced *Nhe* (27). Yet another study found that the *nheABC* operon was present in every *B. thuringiensis* strain tested (35). The presence of the *nheABC* operon does not necessarily indicate a virulent strain, but has a very high likelihood of expressing these genes in a host environment or in food under permissive conditions (3, 41). Thus, future work to determine the pathogenicity of *nheA* positive samples would include the use of a Tecra VIA immunoassay kit to detect enterotoxin proteins (3, 17, 18, 27). Without this step, the virulence of *nheA* positive samples cannot be definitively determined.

These data suggest that at least 8 of the 16 isolates from food were positive for the presence of the *nheABC* operon. An additional four food isolates may also be enterotoxigenic, meaning that there is a 75% chance of any food isolate consumed being potentially enterotoxigenic. Additionally, three reference strains were identified that have not been previously known to harbor enterotoxigenic genes. A large degree of genetic variation exists in nhe sequences among *Bacillus* spp. (18), giving rise to false negative results in PCR-based detection assays. Strains negative for *nheA* in real-time PCR have been found to produce the enterotoxin *Nhe* as determined using a Tecra VIA kit. It is very possible that some of the *nheA* negative strains from real-time PCR may still be enterotoxigenic due to polymorphism (15).

After real-time PCR analysis, it was necessary to determine how similar the unidentified *Bacillus* food and soil isolates were to the reference strains *B. cereus, B. thuringiensis var. kurstaki, B. thuringiensis var. japanensis,* and *B. thuringiensis var. israelensis* using rep- PCR. If banding patterns of the unidentified isolates were very similar to rep-PCR banding patterns of reference strains, then this research would not have identified new strains harboring enterotoxigenic genes.

Within *Bacillus*, most virulence factors are encoded on plasmids (42), which have been demonstrated to readily transfer between differing species (3, 19). Indeed, a recent study indicated that the virulence genes associated with *B. cereus* infection undergo frequent rearrangement both within the bacterial nucleoid and between species (26). Thus, a better method than traditional biochemical tests to detect pathogenic *Bacillus* strains is to screen for virulence operons present in plasmids or in nucleoidal DNA (28, 42). *Bacillus* genomes that have been sequenced display a high level of genetic synteny in their gene order. Two genes that encode for bacterial ribosomes, 16S and 23S rDNA, contain genetic sequences that are < 1% different when compared between *B. cereus, B. thuringiensis,* and *B. anthracis* (48). A dissimilarity of 3% between 16S or 23S rDNA sequences is the minimal "cut off " between two strains to be considered as distinct species. Additionally, the *gyrB* gene sequence shared among these species is very homologous (38). Because these genes are shared among different species within the *Bacillus* genus, they cannot be used to differentiate species (42, 48). However, 16S and 23S rRNA can be used to differentiate between different strains of *B. anthracis* (13). Ultimately, the many attempts at differentiating *B. cereus, B. thuringiensis,* and *B. anthracis* have led to complete genomic sequencing of 16 strains of these three species (42). This large data pool has allowed *Bacillus* to serve as a good model for genetic conservation and to allow thorough study of virulence gene transfer. Additionally, the abundance of sequencing information on *Bacillus* genomes has allowed scientists to statistically differentiate sequencing error from actual polymorphisms.

Interestingly, there are a number of mechanisms that facilitate the movement of genes between different members of the *Bacillus* genus. One such mechanism is through the natural action of bacteriophage. After lysing its host cell, the bacteriophage will insert its genes into *Bacillus* genomes. While normally either lytic or lysogenic, it is possible for prophage to undergo random mutation, which renders it unable to enter the lysogenic cycle. In this way, genes from one species of bacteria can be transferred to *Bacillus spp*. As previously mentioned, *Bacillus* operons may be on conjugative plasmids. Additionally, *Bacillus* spp. are naturally competent, allowing these microbes to naturally take up random DNA in their vicinity (19).

The virulence genes for *Nhe* are present in more strains of *Bacillus* than is currently accepted within the scientific community. This research identified several "species" of *Bacillus* that were not previously known to harbor the *Nhe* enterotoxigenic operon. Given that a debate is currently underway about the very identity of *B. cereus* and other strains, it is improper for food safety experts to screen food products only for *B. cereus*. Phenotypic-based classification techniques have failed to accurately differentiate *Bacillus* species. Additionally, no molecular-based approach can accurately differentiate *Bacillus* (42). The bottom line is the determination of species within *Bacillus* does not even matter when concerned with food safety. Molecular techniques should instead screen for virulence determinants in microbes instead of identifying said microbes (47). Since endospore formation enables *Bacillus* spp. to be ubiquitous in the environment and on food, all foods should be examined in this way (3, 16, 36). This is the only true way to determine whether food products are safe for human consumption.

References

1. Ankolekar, C., Rahmati, T., and Labbe, R.G. 2009. Detection of toxigenic *Bacillus cereus* and *Bacillus thuringiensis* spores in U.S. rice. *International Journal of Food Microbiology.* 128: 460-66.

2. Antwerpen, M., Ximmermann, P., Bewley, K., Frangoulidis, D., and Meyer, H. 2008. Real-time PCR system targeting a chromosomal marker specific for *Bacillus anthracis*. *Molecular and Cellular Probes.* 22: 313-15.

3. Arnesen, L., P. Stenfors, A. Fagerlund, and P.E. Granum. 2008. From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiol. Rev.* 32:579-606.

4. Bottone, E.J. 2010. *Bacillus cereus* as a volatile human pathogen. *Clin. Microbiol. Rev.* 23:382-398.

5. Brousseau, R., A. Saint-Onge., G. Prefontaine, L. Masson, and J. Cabana, 1993. Arbitrary polymerase chain reaction, a powerful method to identify *Bacillus thuringiensis* serovars and strains. *Appl. Environ. Microbiol.* 59:114-19.

6. Cardazzo, B., Negrisolo, E., Carraro, L. Alberghini, L., Patarnello, T., and Ciaccone, V. 2008. Multiple- locus sequence typing and analysis of toxin genes in *Bacillus cereus* food-borne isolates. *Appl. Environ. Microbiol.* 74:850-860.

7. Chen, M.L. and H.Y. Tsen. 2002. Discrimination of *Bacillus cereus* and *Bacillus thuringiensis* with 16s rRNA and *gyrB* gene-based PCR primers and sequencing of their annealing sites. *J. Appl. Microbiol.* 92: 912-19.

8. Cherif, A., Brusetti, L., Borin, S., Rizzi, A., Boudabous, A., Khyami-Horani, H., and Daffonchio, D. 2003. Genetic relationship in the '*Bacillus cereus* group' by rep-PCR fingerprinting and sequencing of a *Bacillus anthracis*-specific rep-PCR fragment. *J. Appl. Microbiol.* 94:1108-19.

9. Cherif, A., Ettoumi, B., Raddadi, N., Daffonchio, D., and Boudabous, A. 2007. Genomic diversity and relationship of *Bacillus thuringiensis* and *Bacillus cereus* by multi-REP-PCR fingerprinting. *Can. J. Microbiol.* 53: 343-50.

10. Christiansson, A., Naidu, A.S., Nilsson, I., Wadstrom, T., and Pettersson, H.E. 1989. Toxin production by *Bacillus cereus* dairy isolates in milk at low temperatures. *Applied and Environmental Microbiology.* 55: 2595-2600.

11. Cooper, R.M. and J.L. McKillip. 2006. Enterotoxigenic *Bacillus* spp. DNA fingerprint revealed in naturally contaminated nonfat dry milk powder using rep-PCR. *J. Basic Microbiol.* 46:358-64.

12. Cummings, C.A., Bormann Chung, C.A., Fang, R., Barker, M., Brzoska, P.M., Williamson, P., Beaudry, J.A., Matthews, M., Schupp, J.M., Wagner, D.M., Furtado, M.R., Keim, P., and Budowle, B. 2009. Whole-genome typing of *Bacillus anthracis* isolates by next-generation sequencing accurately and rapidly identifies strain-specific diagnostic polymorphisms. *Forensic Sci. Intl.* 2:300-301.

13. Daffonchio, D., Raddadi, N., Merabishvili, M., Cherif, A., Carmagnola, L., Brusetti, L., Rizzi, A., Chanishvili, N., Visca, P., Sharp, R., and Borin, S. 2006. Strategy for identification of *Bacillus cereus* and *Bacillus thuringiensis* strains closely related to *Bacillus anthracis*. *Applied and Environmental Microbiology.* 72: 1295-1301.

14. Didelot, X., Barker, M., Falush, D., and Priest, F.G. 2009. Evolution of pathogenicity in the *Bacillus cereus* group. *Systematic and Applied Microbiology.* 32: 81-90.

15. Ehling-Schulz, M., Guinebretiere, M.H., Monthan, A., Berge, O., Fricker, M. and Svensson, B. 2006. Toxin gene profiling of enterotoxic and emetic *Bacillus cereus*. *FEMS Microbiol Lett.* 260: 232-40.

16. Giffel, M.C. and R.R. Beumer. 1999. *Bacillus cereus*: a review. *The Journal of Food Technology in Africa.* 4: 7-13.

17. Griffiths, M.W. 2010. Pathogens and toxins in foods: challenges and interventions. *ASM Press, Washington, DC.* pp. 1-19.

18. Hansen, B.M. and N.B. Hendriksen. 2001. Detection of enterotoxic *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. *Appl. Environ. Microbiol.* 67:185-89.

19. Helgason, E., Okstad, O.A., Caugant, D.A., Johansen, H.A., Fouet, A., Mock, M., Hegna, I., and Kolsto, A.B. 2000. *Bacillus anthracis, Bacillus cereus,* and *Bacillus thuringiensis* – one species on the basis of genetic evidence. *Appl. Environ. Microbiol.* 66:2627-2630.

20. Henerson, I., Duggleby, C.J., and Turnbull, P.C.B. 1994. Differentiation of *Bacillus anthracis* from other *Bacillus cereus* group bacteria with the PCR. *Int. J. Syst. Bacteriol.* 44:99-105.

21. Hoffmaster, A.R., Novak, R.T., Marston, C.K., Gee, J.E., Helsel, L., Pruckler, J.M., and Wilkins, P.P. 2008. Genetic diversity of clinical isolates of *Bacillus cereus* using multilocus sequence typing. *BMC Microbiol.* 8:191. doi: 10.1186/1471-2180/8/191.

22. Hong, H.A., To, E., Fakhry, S., Baccigalupi, L., Ricca, E., and Cutting, S.M. 2009. Defining the natural habitat of *Bacillus* spore formers. *Res. Microbiol.* 160:375-379.

23. Jackson, P.J., Hill, K.K., Laker, M.T., Ticknor, M.T., and Keim, P. 1999. Genetic comparison of *Bacillus anthracis* and its close relatives using amplified fragment length polymorphism and polymerase chain reaction analysis. *Journal of Applied Microbiology.* 87: 263-9.

24. Jersek, B., Gilot, P., Gubina, M., Klun, N., Mehle, J., Tcherneva, E., Rijpens, N., and Herman, L. 1999. Typing of Listeria monocytongenes strains by repetitive element sequence-based PCR. *Journal of Clinical Microbiology.* 37: 103-9.

25. Jolley, K.A., Chan, M.S., and Maiden, M.C.J. 2004. mlstdbNet – distributed multi-locus sequence typing (MLST) databases. *BMC Bioinformatics* 5:86-93.

26. Kim, Y.R. and Batt, C.A. 2008. Riboprint and virulence gene patterns for *Bacillus cereus* and related species. *J. Microbiol. Biotechnol.* 18: 1146-55.

27. Kim, J.B., Kim, J.M., Kim, S.Y., Kim, J.H., Park, Y,B., Choi, N.J., and Oh, D.H. 2010. Comparison of enterotoxin production and phenotypic characteristics between emetic and enterotoxic *Bacillus cereus*. *Journal of Food Protection.* 73:1219-24.

28. Klee, S.R., Nattermann, H., Becker, S., Urban-Schriefer, M., Franz, T., Jacob, D., and Appel, B. 2006. Evaluation of different methods to discriminate *Bacillus anthracis* from other bacteria of the *Bacillus cereus* group. *Journal of Applied Microbiology.* 100: 673-81.

29. Lauri, A. and P.O. Mariana. 2009. Potentials and limitations of molecular diagnostic methods in food safety. *Genes Nutr.* 4: 1-12.

30. Maity, T.K. and A.K. Misra. 2009. Probiotics and human health: synoptic review. *African Journal of Food Agriculture Nutrition and Development.* 9.

31. Manzano, M, Cocolin, L., Carlo Cantoni, and Comi, G. 2003. *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus mycoides* differentiation using a PCR-RE technique. *International Journal of Food Microbiology.* 81: 249-54.

32. Marchuk, D., Drumm, B., Saulino, A., and Collins, F.S. 1990. Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. *Nucleic Acids Research.* 19: 1154.

33. McKillip, J.L. 2000. Prevalence and expression of enterotoxins in *Bacillus cereus* and other *Bacillus* spp., a literature review. *Antonie Van Leeuwenhoek.* 77: 393-9.

34. McKillip, J.L. and M.A. Drake. 2005. Genetic-based methods for detection of bacterial pathogens. Pp. 187-1-187-12. In (Y.H. Hui, Ed.) *Handbook of Food Science and Technology, vol. 4.* CRC Press, Boca Raton, FL.

35. Ngamwongsatit, P., Buasri, W., Pianariyanon, P., Pulsrikarn, C., Ohba, M., Assavanig, A., and Panbangred, W. 2008. Broad distribution of enterotoxin genes (HBLCDA, NHEABC, cytK, and entFM) among *Bacillus thuringiensis* and *Bacillus cereus* as shown by novel primers. *International Journal of Food Microbiology.* 121: 352-56.

36. Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J., and Setlow, P. 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiology and Molecular Biology Reviews.* 64: 548-72.

37. Owusu-Kwarteng, J., Wuni, A., Akabanda, F., Tano-Debrah, K., and Jespersen, L. 2017. Prevalence, virulence factor genes and antibiotic resistance of *Bacillus cereus sensu lato* isolated from dairy farms and traditional dairy products. *BMC Microbiol.* 17:65. DOI 10.1186/s12866-017-0975-9

38. Park, S.H., Kim, H.J., Kim, J.H., Kim, T.W., and Kim, H.Y. 2007. Detection and identification of *Bacillus cereus* group bacteria using multiplex PCR. *Journal of Microbiology and Biotechnology.* 17: 1177-82.

39. Peruca, A.P.S., G.T. Vilas-Boas, and O.M.N. Arantes. 2008. Genetic relationships between sympatric populations of *Bacillus cereus* and *Bacillus thuringiensis*, as revealed by rep-PCR genomic fingerprinting. *Mem Inst Oswaldo Cruz.* 103: 497-500.

40. Phelps, R.J. and McKillip, J.L. 2002. Enterotoxin production in natural isolates of Bacillaceae outside the *Bacillus cereus* group. *Applied and Environmental Microbiology.* 68: 3147-51.

41. Rahmati, T. and Labbe, R. 2008. Levels and toxigenicity of *Bacillus cereus* and Clostridium perfringens from retail seafood. *Journal of Food Protection.* 71: 1178-85.

42. Rasko, D.A., Altherr, M.R., Han, C.S., and Ravel, J. 2005. Genomics of the *Bacillus cereus* group of organisms. *FEMS Microbiology Reviews.* 29: 303-29.

43. Reyes-Ramirez, A. and Ibarra, J.E. 2005. Fingerprinting of *Bacillus thuringiensis* type strains and isolates by using *Bacillus cereus* group-specific repetitive extragenic palindromic sequence-based PCR analysis. *Applied and Environmental Microbiology.* 71: 1346-55.

44. Rowan, N.J., Caldow, G., Gemmell, C.G., and Hunter, I.S. 2003. Production of diarrheal enterotoxins and other potential virulence factors by veterinary isolates of *Bacillus* species associated with nongastrointestinal infections. *Applied and Environmental Microbiology.* 69: 2372-76.

45. Schoeni, J.L. and Amy C. Lee Wong. 2004. *Bacillus cereus* food poisoning and its toxins. *Journal of Food Protection.* 68: 636-48.

46. Travers, R., S., Martin, P.A.W., and Reichelderfer, C.F. 1987. Selective process for efficient isolation of soil *Bacillus* spp. *Appl. Environ. Microbiol.* 53:1263-66.

47. Vilas-Boas, G., Sanchis, V., Lereclus, D., Lemos, M.V.F., and Bourguet, D. 2002. Genetic differentiation between sympatric populations of *Bacillus cereus* and *Bacillus thuringiensis*. *Applied and Environmental Microbiology.* 68: 1414-24.

48. Vilas-Boas, G.T., A.P.S. Peruca, and O.M.N. Arantes. 2007. Biology and taxonomy of *Bacillus cereus, Bacillus anthracis*, and *Bacillus thuringiensis*. *Can. J. Microbiol.* 53:673-87.

49. Wilson, M.K., Vergis, J.M., Alem, F., Palmer, J.R., Keane-Myers, A.M., Brahmbhatt, T.N., Ventura, C.L., and O'Brien, A.D. 2011. *Bacillus cereus* G9241 makes anthrax toxin and capsule like highly virulent B. anthracis Ames but behaves like attenuated toxigenic nonencapsulated B. anthracis sterne in rabbits and mice. *Infec. Immun.* 79:301203019.

50. Zhong, Y.S., Yoshida, T.M., and Marrone, B.M. 2007. Differentiation of *Bacillus anthracis, Bacillus cereus* and *Bacillus thuringiensis* by using pulsed-field gel electrophoresis. *Applied and Environmental Biology.* 73: 3446-49.

51. Zwick, M.E., J. Sandeep, X. Didelot, P.E. Chen, K.A. Bishop-Lilly, A.C. Stewart, K. Willner, S. Lentz,

52. N. Nolan, M.K. Thomason, S. Sozhammannan, A.J. Mateczun, L. Du, and T.D. Read. 2012. Genomic characterization of the *Bacillus cereus sensu lato* species: backdrop to the evolution of *Bacillus anthracis*. *Genome Res.* doi: 10.1101/gr.134437.111.

Anti-proliferation of Melanoma Cells and Immune Stimulation by the Cyanobacterial Indole-alkaloid Scytonemin

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Abstract

Under the stress of ultraviolet radiation some cyanobacteria synthesize scytonemin, a protective pigment against DNA photodamage. In addition to photoprotection, scytonemin has been shown to have an anti-proliferative effect on various types of malignant cells. In this study the effect of scytonemin on melanoma and spleen cells was assessed both in vitro using tissue cultures and in vivo in mice models. Melanoma and spleen cells were exposed to 0.08 to 10 μM of scytonemin, and cell proliferation was measured using tritiated thymidine uptake. The data suggest that scytonemin acts as an inhibitor for melanoma cells in a concentration-dependent manner while enhancing the proliferation of spleen cells, suggesting that it can potentially augment the immune response. Furthermore, mice injected with melanoma cells and scytonemin produced fewer tumors than mice that did not receive scytonemin, although the data were not significant. This study adds to the growing body of research that scytonemin may be beneficial as a future anticancer agent to prevent tumor cell growth.

Introduction

According to the Centers for Disease Control, skin cancer is the most common type of cancer in the world, with 85,868 people in the United States diagnosed with melanomas of the skin in 2017 (3). Consequently, the demand for a product that is both effective in killing tumor cells and safe for an individual to take is as great as it has ever been. Since ultraviolet radiation (UVR) plays a major role in skin cancer, potential treatments could explore photoprotective effects of various compounds against solar UVR. Long- wavelength (UVA) in the range of 320-400 nm, plays a role in long-term skin damage contributing to aging skin due to its deep penetration of the epidermis and dermis. UVA is known to damage keratinocytes, which are found in the basal layer of the epidermis where most skin cancers occur (8). Short-wavelength UVB in the range of 280- 320 nm is the major contributor in sunburns and contributes to skin cancer alongside UVA by directly damaging DNA and proteins (17). All living cells, including bacteria, can be harmed by UVR (8). As photosynthetic bacteria regularly exposed to UVR, cyanobacteria have developed several mechanisms to defend themselves against its harmful effects. These include physical migration away from UVR (1), synthesis of UV-shock proteins (4), up-regulation of antioxidant defenses (14), and down-regulation of UVR- sensitive proteins (7). Of particular interest is the ability of some cyanobacteria to synthesize scytonemin, a photoprotective sheath pigment that protects primarily against UVA radiation (5). Scytonemin is a lipophilic, yellow-brown, indole-alkaloid pigment (Fig. 1) (10) that efficiently absorbs UVA in vivo at 370 nm (6). It is produced by certain species of cyanobacteria where it is induced upon exposure to UVA, and is then deposited into the sheath surrounding the cells (5).

In addition to its photoprotective properties, scytonemin demonstrates anti- inflammatory and anti-proliferative qualities. Tissue hyperplasia is a hallmark feature of hyperproliferative and inflammatory pathologies, such as rheumatoid arthritis, psoriasis, asthma, and cancer (15). Scytonemin has been shown to exhibit anti-inflammatory properties that could potentially treat these diseases (11, 15, 16). For example, the topical application of scytonemin on mouse ear edema reduced swelling compared to mice receiving no treatment (16). Furthermore, several studies argue that scytonemin inhibits cell proliferation through mechanisms of cell cycle arrest (15, 16, 18, 19). Scytonemin has also been shown to interrupt hyperproliferation of renal cancer cells (18) and slow the proliferation of multiple myeloma cells (19). Possibly the most thorough study on the anti-proliferative properties of scytonemin was that it could hinder actively proliferating cells, including malignant Jurkat T cells, rheumatoid synovial fibroblasts implicated in arthritis, human lung fibroblasts, and human umbilical vein endothelial cells. This study also demonstrated that scytonemin was not cytotoxic towards non-proliferative human monocytes (15). These results convey the possibility for scytonemin to halt malignant cell growth without harming other body cells, which is a quality particularly sought after in cancer treatment research.

Figure 1.

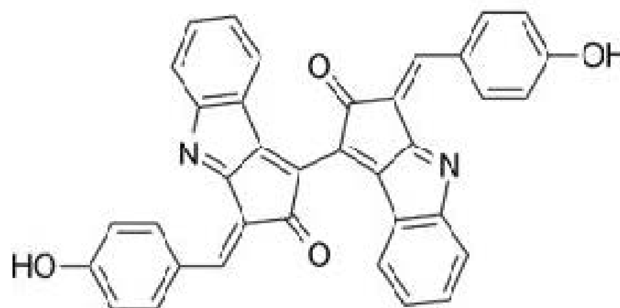

Figure 1. Structure of scytonemin (Proteau et. al. 1993).

Since the literature suggests that the UV- protective agent scytonemin is implicated in cell cycle arrest, this study seeks to determine whether scytonemin will have anti-proliferative effects against melanoma cell growth in animals. Therefore, the objectives of this study were to examine both the *in vitro* melanoma and spleen cell proliferation in the presence of different concentrations of scytonemin, as well as the *in vivo* effect of scytonemin on tumor growth in mice. If scytonemin inhibits melanoma cell proliferation without equally inhibiting spleen cell proliferation, then it has potential as a future therapy for malignant cells and should be further explored as an anti-tumor therapy. However, if scytonemin affects melanoma and spleen cells alike, then it could potentially harm tissue and might not result in the production of an effective therapy. While the findings of other studies already indicate that scytonemin slows malignant proliferation (15, 16, 18, 19), this study takes a slightly different angle by comparing the effects of scytonemin on cancerous cell proliferation (i.e., melanomas) to healthy non-cancerous cell growth (i.e., spleen cells) from other tissues in the same organism. Spleen cells were chosen over skin cells, for example, as the healthy non-cancerous control cells because they are more likely to interact with a therapeutic agent that enters the bloodstream. Furthermore, the spleen plays a critical role in activating immune cells in response to bloodborne antigens. Therefore, the hypothesis of this study is that in the presence of scytonemin, melanoma growth will decrease in tissues and animals while spleen cell proliferation will increase.

Methods

Melanoma and Spleen Cell Assays

B16-F1 melanoma cells were purchased from the American Type Culture Collection (ATCC© CRL-6323) (2). Spleen cells were extracted from male C57BL/6 mice obtained from Charles River Labs that were 4-6 months old at the time of extraction. For each assay, a final concentration of 5 x 104 melanoma or 1 x 106 spleen cells were placed in each well of a 96-well plate and assayed in triplicate for each condition. Samples treated with scytonemin received 10 µL HPLC-purified scytonemin (a gift from Benjamin Philmus, Oregon State University) diluted in DMSO with final concentrations of 10 to 0.08 µM. To better determine the effect of scytonemin on spleen cell growth, the mitogen concanavalin A (Con A), which stimulates T lymphocyte proliferation, was added at a concentration of 0.1 µg per well in a volume of 200 ul of RPMI-1640 media plus 10% FBS, to determine whether scytonemin inhibited rapidly proliferating spleen cells (12). Spleen cell plates were incubated for 48 hours, then 3H-thymidine was added followed by a second incubation for 24 hours, all of which took place at 37 °C under 10% CO2. A cell harvester was then used to transfer the cells onto filters, which were washed approximately ten times using phosphate buffered saline (PBS). Filters were then placed into counting tubes with EcoLume™ Scintillation Cocktail (MP Biomedicals, Solon, Ohio) to count the incorporated H3-thymidine using a Beckman scintillation counter. All assays were done in triplicate and controls received no scytonemin or DMSO treatment. Previous experiments in our lab have demonstrated that DMSO does not have an inhibitory effect on melanoma cell proliferation (unpublished data). Cell counts were averaged and compared to control groups to determine the percent proliferation relative to the control groups. All statistical tests were done using ANOVA and TukeyHSD post-hoc comparisons in R Studio v1.2.1335 (13).

In Vivo Assays

For in vivo scytonemin experiments against melanoma tumor cells, 18 male C57/BL6 mice were obtained from Charles River Labs that were approximately 14 weeks old at the time of experimentation. Each mouse was weighed before the initial intraperitoneal (IP) injection of scytonemin diluted in DMSO to a final concentration of 3.5 µM g-1. Scytonemin was then administered at 3.5 µM g-1 daily for two weeks through IP injections into nine treated mice, and the other nine mice received the same volume of sterile saline that had an equal concentration of DMSO as the scytonemin-treated animals. After two weeks a single tail vein injection of melanoma cells was performed. Melanoma cells were diluted to 5 x 106 melanoma cells ml-1 in sterile saline, and 0.1 ml of this preparation was injected into the tail vein of all 18 mice. IP injections of scytonemin were continued for an additional week and the mice were monitored for any health changes

for two additional weeks while the tumors grew. After two weeks of no injections eight mice in each group survived and were weighed once more and sacrified. When injected into the tail vein of syngeneic mice, melanoma cells migrate to the lungs and produce dark colonies. Upon sacrifice the lungs were teased apart and the melanoma tumor cell colonies were counted on and within the lung tissue and the counts were compared against the control mice that did not receive scytonemin using an ANOVA with TukeyHSD post-hoc analysis. All animal experiments were performed according to IACUC Protocol #1111000244.

Results

Melanoma and Spleen Cell Assays

The melanoma cell assays showed that in the presence of 10 µM scytonemin, the percent inhibition compared to the control was highest at 87.60% ± 1.86 while in the presence of 2 µM scytonemin, the inhibition decreased to 52.03% ± 1.76 (Fig. 2). The percent inhibition compared to the control was significant only at 10 µM and 2 µM scytonemin ($p < 0.0001$). The percent inhibition generally increased as the concentration of scytonemin increased, indicating a positive relationship among these variables.

For the spleen cell assays, in all treatments except the 10 µM scytonemin dilution without Con A (65.28% ± 5.34), the percentage proliferation relative to the control was over 100% (Fig. 3). For the spleen cells receiving the mitogen Con A, the percentage proliferation relative to the control was 211.14% ± 53.24 for the most dilute concentration of scytonemin, 0.08 µM, peaking at 387.00% ± 79.34 with 2 µM scytonemin. The percent stimulation compared to the control was significant only at 2 µM scytonemin, with $p = 0.0012$ regardless of whether Con A was used. The results of the assays with and without Con A display the same general trends, suggesting that the presence of the mitogen was not a confounding variable. Scytonemin did not demonstrate any inhibition of these spleen tissue cultures while the 2 µM and 10 µM levels showed significant inhibition of melanoma cell growth.

In Vivo Assays

After treatment, there was no significant difference in the weights of untreated mice (28.44 ± 0.49 g) and those treated with scytonemin (29.63 ± 0.48 g). There were also no significant differences in the number of melanoma tumors in untreated mice (205.5 ± 51.29) versus those treated with scytonemin (142.50 ± 30.65).

Discussion

Mechanistically, scytonemin inhibits the hyperproliferation of cells by targeting multiple enzymes implicated primarily in cell cycle regulation. For instance, one study examined the effect on several kinases, including PLK1, CDK1/cyclin B, checkpoint kinase 1, protein kinase A, protein kinase C, Myt1 kinase, and Tie2 kinase (16). Of these enzymes, it was determined that scytonemin acted as an inhibitor for PLK1, CDK1/cyclin B, checkpoint kinase 1, protein kinase C, and Myt1 kinase, but did not inhibit protein kinase A or Tie2 kinase (16). These results suggest that scytonemin binds nonspecifically to a variety of enzymes to prevent phosphorylation steps critical to cell cycle progression. Other experiments explored the relationship between scytonemin and PLK1, finding that scytonemin acts as a mixed inhibitor for PLK1 and functions in a concentration-dependent, time-independent manner to induce cell cycle arrest (15, 16, 18). By looking at molecular signals in the cell, the specific mechanism of action of scytonemin appears to be G2 to M phase cell cycle arrest (15, 19).

In light of these prior findings, the results of this study support the anti-proliferative potential of scytonemin as an inhibitor of important cell cycle enzymes implicated in cancer growth. Scytonemin at 2 µM and 10 µM significantly inhibited melanoma cell proliferation by greater than 50% compared to controls in a somewhat concentration-dependent manner. This is consistent with other studies that explore the effect of scytonemin on malignant cells (15, 18, 19).

In addition, the results of the spleen cell assay demonstrates that 2 µM scytonemin significantly stimulated proliferation

Figure 2.

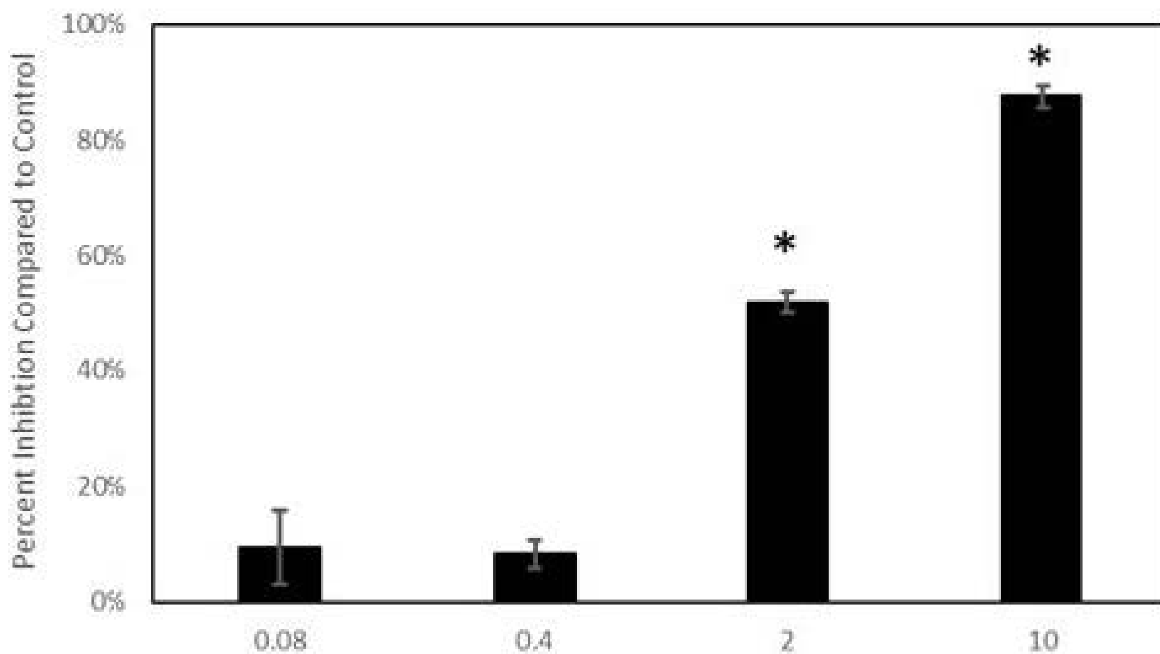

Figure 2. Percent inhibition of melanoma cells compared to the untreated control for various concentrations of scytonemin. Error bars represent the standard error of nine replicates. Significant inhibition compared to the control is marked with an asterisk, for both the 2 µM and 10 µM samples, $p < 0.0001$.

compared to the controls, which was an unexpected outcome. Since scytonemin typically inhibits actively proliferating cell types (15), there was some concern that it would inhibit spleen and melanoma cell growth alike. However, this was not the case, and was especially true for spleen cells receiving Con A. In these cells scytonemin appears to have enhanced proliferation, ranging from 164.79% to 387.00% relative to the control group. However, unlike the melanoma cell assays, the spleen cells were not affected by scytonemin in a concentration-dependent manner through 10 µM of scytonemin. This result could be due to high standard errors or data resolution, which does not show the trend between 0.4 to 2 µM of scytonemin. Nonetheless, the data suggests that scytonemin inhibits melanoma cells without hindering spleen cell proliferation. Given that scytonemin inhibits melanoma cell proliferation and potentially increases spleen cell proliferation, it is a promising therapeutic agent for melanoma treatment because it can slow cell growth while

Figure 4.

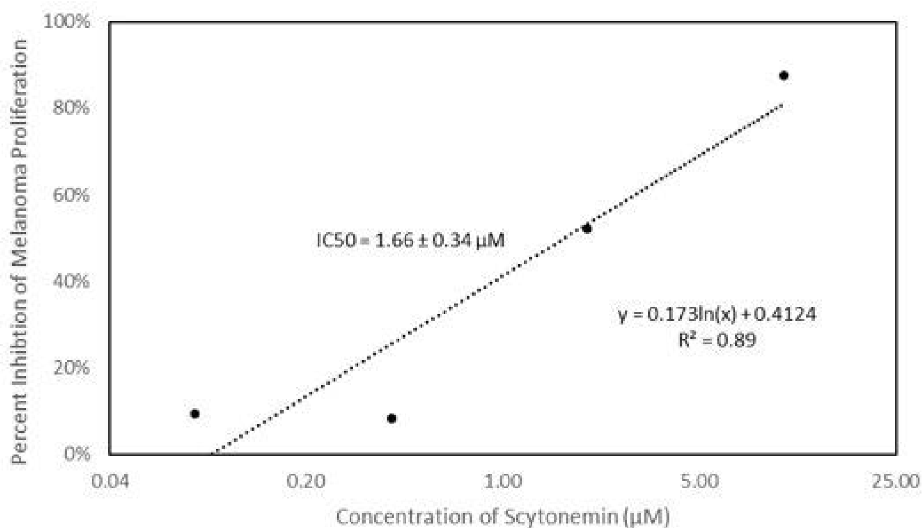

Figure 3.

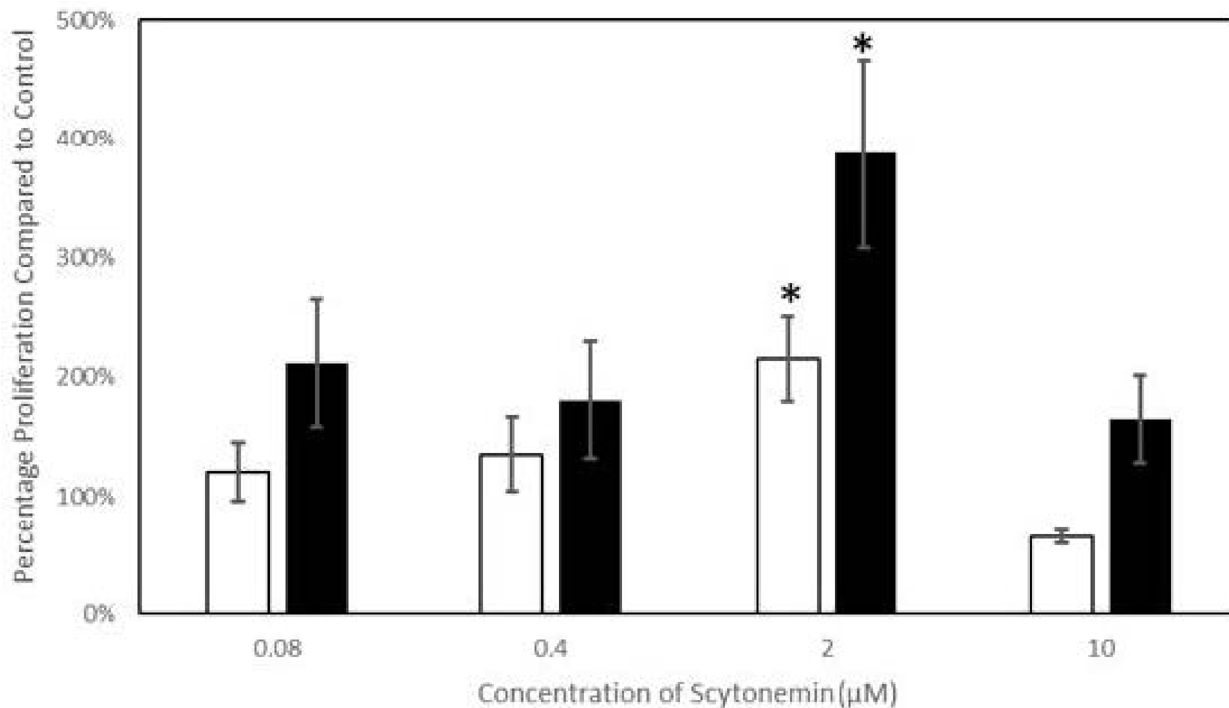

Figure 3. Percent proliferation of spleen cells compared to the control for various concentrations of scytonemin in the absence (white bars) and presence (black bars) of Con A. Error bars represent the standard error of nine replicates. Significant inhibition compared to the control is marked with an asterisk, at 2 µM scytonemin, $p = 0.0012$ for cells with and without Con A. Biorender.com.

simultaneously augmenting the immune system. The in vivo experiments on mice evaluating the number of tumors formed with and without the presence of scytonemin favored the inhibitory effects of scytonemin. Even though there were no significant differences in the number of melanoma tumors in untreated mice versus those treated with scytonemin, the averages themselves indicate that with additional studies there may be some inhibitory effect of scytonemin on melanoma tumor cells. These studies could benefit by a dose-dependent analysis. The concentration of scytonemin used was based on a study using Jurkat T cells (15) and it may have not been ideal to use the same concentration in a study on melanoma tumor development.

To better compare the inhibitory effects of scytonemin with the previous body of knowledge, the inhibitory concentration at 50% inhibition (IC50) was determined. This value represents the concentration of scytonemin at which cell proliferation is stunted by 50% relative to the control. In a regression analysis of scytonemin concentration versus percent inhibition of melanoma proliferation, a positive correlation ($R2 = 0.89$) was identified (Fig. 4). Using this regression plot, the IC50 for scytonemin on melanoma cells was determined to be approximately 1.66 ± 0.34 µM. In a similar study measuring the effects of scytonemin on the cancerous Jurkat T cell line, the IC50 was determined to be 2.5 ± 0.6 µM (15). These relatively close values could help inform future research studies exploring the benefits of scytonemin as an anti-proliferative therapeutic.

The results of this study support that scytonemin inhibits melanoma cell proliferation and enhances the immune response via spleen cell proliferation. As a result, future research should examine the role of scytonemin on in vivo tumor growth beyond our studies to determine whether scytonemin functionally inhibits malignant growth in body systems without cytotoxicity to healthy cells. Further, in vivo systems can be used to examine the response of the immune system to scytonemin, to potentially affirm enhanced spleen cell proliferation and examine other cytokines and immune

cells. Research could also focus on experimental cancer therapies using scytonemin to prevent tumors from growing and metastasizing. Since a growing body of literature suggests that scytonemin acts as an inhibitor for PLK1 (15, 16, 18, 19), the role of PLK1 in melanoma and spleen cell proliferation should be further studied. In addition, since dimethoxyscytonemin binds to PLK1 with high affinity (9), it would be interesting to study how this scytonemin derivative affects proliferation in melanoma and spleen cell assays compared to purified scytonemin. Overall this study, along with prior research, provides evidence that the therapeutic potential of scytonemin should be further explored.

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References

1. Bebout BM, Garcia-Pichel F. 1995. UV-B-induced vertical migrations of cyanobacteria in a microbial mat. *Appl Environ Microbiol* 61:4215-4222.

2. Briles EB, Kornfeld S. 1978. Isolation and metastatic properties of detachment variants of B16 melanoma cells. *J Natl Cancer Inst* 60:1217-1222.

3. CDC. 2020. Skin Cancer. https://www.cdc.gov/cancer/skin/statistics/index.htm. Accessed

4. Ehling-Schulz M, Bilger W, Scherer S. 1997. UV-B-induced synthesis of photoprotective pigments and extracellular polysaccharides in the terrestrial cyanobacterium *Nostoc commune*. *J Bacteriol* 179:1940-1945.

5. Garcia-Pichel F, Castenholz RW. 1991. Characterization and biological implications of scytonemin, a cyanobacterial sheath pigment. *J Phycol* 27:395-409.

6. Garcia-Pichel F, Sherry ND, Castenholz RW. 1992. Evidence for an ultraviolet sunscreen role of the extracellular pigment scytonemin in the terrestrial cyanobacterium *Chlorogloeopsis* sp. *Photochem Photobiol* 56:17-23.

7. Huang L, McCluskey MP, Ni H, LaRossa RA. 2002. Global gene expression profiles of the cyanobacterium *Synechocystis* sp. strain PCC 6803 in response to irradiation with UV-B and white light. *J Bacteriol* 184:6845-6858.

8. Jagger J. 1985. Solar-UV actions on living cells. Praeger, New York.

9. Pathak J, Mondal S, Ahmed H, Rajneesh, Singh SP, RP S. 2019. In silico study on interaction between human polo-like kinase 1 and cyanobacterial sheath pigment scytonemin by molecular docking approach. *Biointerface Res Appl Chem* 9:4374-4378.

10. Proteau PJ, Gerwick WH, Garcia-Pichel F, Castenholz RW. 1993. The structure of scytonemin, an ultraviolet sunscreen pigment from the sheaths of cyanobacteria. *Experientia* 49:825- 829.

11. Rastogi R, Sonani R, Madamwar D. 2015. Cyanobacterial sunscreen scytonemin: Role in photoprotection and biomedical research. *Appl Biochem Biotechnol* 176:1551-1563.

12. Reeke GN, Jr., Becker JW, Cunningham BA, Wang JL, Yahara I, Edelman GM. 1975. Structure and function of concanavalin A. *Adv Exp Med Biol* 55:13-33.

13. RStudio. 2018. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA. http:// www.rstudio.com/.

14. Shibata H, Baba K, Ochiai H. 1991. Near-UV induces shock proteins in *Anacystis nidulans* R-2, possible role of active oxygen. *Plant Cell Physiol* 32:771-776.

15. Stevenson CS, Capper EA, Roshak AK, Marquez B, Eichman C, Jackson JR, Mattern M, Gerwick WH, Jacobs RS, Marshall LA. 2002. The identification and characterization of the marine natural product scytonemin as a novel antiproliferative pharmacophore. *J Pharmacol Exp Ther* 303:858-866.

16. Stevenson CS, Capper EA, Roshak AK, Marquez B, Grace K, Gerwick WH, Jacobs RS, Marshall LA. 2002. Scytonemin, a marine natural product inhibitor of kinases key in hyperproliferative inflammatory diseases. *Inflammation Res* 51:112-114.

17. Urbach F. 1997. Ultraviolet radiation and skin cancer of humans. *J Photochem Photobiol B: Biology* 40:3-7.

18. Zhang G, Zhang Z, Liu Z. 2013. Polo-like kinase 1 is overexpressed in renal cancer and participates in the proliferation and invasion of renal cancer cells. *Tumor Biol* 34:1887-1894.

19. Zhang G, Zhang Z, Liu Z. 2013. Scytonemin inhibits cell proliferation and arrests cell cycle through downregulating Plk1 activity in multiple myeloma cells. *Tumor Biol* 34:2241-2247.

Isolation and identification of dermatophytes from collegiate runners

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Abstract

Competitive runners experience various risk factors that render them more susceptible to superficial cutaneous fungal infections, including the use of occlusive footwear, shared locker rooms, submission of feet to constant maceration, trauma, sweating, and having depressed immune function. The goal of this work was to assess the prevalence of athlete's foot fungi in cross country runners at St. John Fisher College. Toe webs of 16 collegiate runners were sampled and volunteers surveyed about their shoe habits, foot hygiene, and average miles run per week. Lack of tinea pedis-causing fungi in asymptomatic cross- country runners shifted the study to investigate the identities of fungi morphologically similar to athlete's foot and look for correlations with volunteers' running habits and hygiene. Thirty-five distinct fungal cultures were isolated and compared to a known *Trichophyton rubrum* strain both microscopically and macroscopically. Four samples were preliminarily identified as tinea pedis-causing fungi and sequenced to confirm molecular identification. Fungal DNA was isolated, purified, and PCR amplified using primers for the internal transcribed spacer region, D1/D2 region of the 28S subunit, and *ß*-Tubulin gene. Three of the four isolates were identified as *Fusarium equiseti*, a soil-borne plant pathogen with rare human pathogenicity reported. The fourth isolate was *Beauveria bassiana*, a common soil-borne pathogen that can infect immunocompromised individuals. Correct dermatophytic identification and understanding of the interplay between species is important to provide correct treatment, prevent spread among athletes and within facilities, and determine how opportunistic pathogens might play a role in people with immune suppressed function, which includes runners.

Introduction

Athletes, particularly long-distance runners, are at high risk for tinea pedis infection as they train in occlusive footwear, use shared locker rooms, frequently endure minor foot trauma, and have periods of decreased immune function (7,18,20,22,23,29). Tinea pedis, or athlete's foot, is a common superficial fungal infection caused by several dermatophytes, most commonly *Trichophyton, Microsporum, and Epidermophyton* species. With the increase in superficial fungal infections over the past 30 years, correct identification of tinea pedis-causing fungi is critical for epidemiological purposes, recognizing potential infection sources, and providing proper treatment, such as topical and/ or oral antifungals (1,5,9,29). Many symptoms can mimic the symptoms of tinea pedis, which further indicates the need for proper identification, as incorrect treatment can lead to secondary fungal and bacterial infections (9,11). Infection by non- dermatophytic molds can also result in tinea pedis. Little is known about the impact of multiple fungal species on morbidity (3). Sports-related infections can be a major cause of disease in individuals, as well as the whole team, due to close contact and sharing of equipment and facilities (20). Dermatophyte infections are difficult to cure completely and frequently recur at the initial site of infection (7,29).

Clinical identification in practice is typically based on symptoms rather than morphological or molecular identification. Further morphological identification involves assessing growth rate, colony pigmentation, hyphal structure, size and shape of conidia, and examining stained foot scrapings for hyphae or pseudo hyphae (1,3,10).

These methods are imprecise and can lead to incorrect identification of other fungi inhabiting the foot, as the foot hosts a plethora of other organisms. Gram positive bacteria, such as *Corynebacterium minutissimum*, and Candida species can cause scaling and maceration that mimic tinea pedis symptoms (10), while other soil-borne species such as *Microsporum gypseum*, can live on skin and cause acute infections (30). Accurate identification of asymptomatic tinea pedis and other potential opportunistic pathogens needs to be coupled to fungal morphology with a DNA sequence-based approach.

Molecular targets such as ribosomal DNA (rDNA), beta tubulin, and mitochondrial DNA (mDNA) have been successful in identifying dermatophytes to a species level (1). Many fungi can be identified by comparing the internal transcribed spacer (ITS) region of ribosomal DNA; though closely related sister taxa, significant sequence variation, and imprecise typification of species dictate the use of additional conserved sequence regions (26). The ITS region is the most commonly used target for fungal sequencing due to sensitive detection by PCR. Multiple copies of the ribosomal gene are present in all organisms and provide an optimal target for developing specific PCR primers that discriminate among closely related species (31). Additional targets, such as the D1/D2 region of the 28S subunit and the *ß*-tubulin gene, provide a more robust molecular picture for dermatophyte identification (11).

The goal of this research was to investigate the prevalence of tinea pedis-causing fungi on asymptomatic cross-country runners at St. John Fisher College and look for correlations with their running habits and hygiene. It was predicted that approximately a quarter of the runners would be carriers of athlete's foot-causing fungi based on a previous survey of asymptomatic marathon runners (14). Morphological and molecular techniques were used to identify potential tinea pedis isolates to determine prevalence of specific dermatophytes among runners. Results of this work provide basic information on soil-borne fungi that are morphologically similar to tinea pedis but not typically associated with human disease complexes. Further understanding of the prevalence and interactions of these fungi with dermatophytic pathogens can help develop recommendations to reduce spread among teammates and within facilities and improve proper diagnosis and treatment.

Table 1: Participant survey of running habits and hygiene.

Question	Response	%
Gender	Female	19 %
	Male	81 %
On average how many miles a week do you run?	46.3 miles	
Do you wear shoes inside of locker rooms at all times?	Yes	75 %
	No	25 %
Do you wear shoes inside of shared showering facilities at all times (ex. dorm room showers)?	Yes	19 %
	No	81 %
Do you wear your training shoes outside of practice and competition?	Yes	56 %
	No	44 %
How often do you wash your running shoes?	Rarely	56 %
	Never	44 %
If you wash your running shoes, do you wash it in hot, warm or cold water?	Hot	11 %
	Warm	56 %
	Cold	33 %
Do you ever wear your running shoes without socks?	Yes	12.5 %
	No	87.5 %
Do you usually scrub with soap and water your feet in the shower?	Yes	87.5 %
	No	12.5 %

Materials and Methods

In the fall of 2014, male and female cross- country runners at St. John Fisher College were invited to participate in this study. A document describing the purpose, methods, and risks were explained, presented to, and signed by each participant. The voluntary nature of participation and procedures for ensuring confidentiality of participants and their samples were ensured. The study was approved by St. John Fisher College Institutional Review Board (IRB) on October 2nd, 2014 (IRB File no: 3370 - 091814 - 07). Each participant completed an eight-question foot condition survey addressing their individual amounts of running, footwear habits, and foot hygiene.

Sample Collection, Culture Identification, and Maintenance

The stratum corneum of the third and fourth web spaces of both feet of 16 volunteers was sampled using a sterile swab (6). Samples were isolated on BBL Sabouraud dextrose agar (SBA) amended with 0.2 mg/ml chloramphenicol and incubated at 27°C for 6 days, checked daily for visible growth, then subcultured until pure (22). For spore visualization, samples were grown on Potato Dextrose Agar (PDA) at room temperature for 7 days until a visible ring pattern emerged.

DNA Extraction, Sequencing and Analysis

Samples for DNA extraction were isolated from pure fungal colonies and grown in Sabouraud dextrose broth for 7 days at room temperature using a Cel-GroTissue Culture Rotator (ThermoFisher Scientific, Waltham, MA). Fungal DNA was isolated using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA). The internal transcribed spacer region (ITS), ribosomal large subunit D1/D2, and β-tubulin genes were amplified via polymerase chain reaction (PCR) using the following primers: ITS1/ITS4 (31), ITS5/ITS4 (31), NL-1/NL-4 (13,25), Bt2a/T1 (9,17) using a BioRad T100 Thermal-Cycler. Reactions for PCR amplifications (50 µl) consisted of: 35 to 50 ng of template DNA, primers (0.2 µM each), 1x AmpliTaq

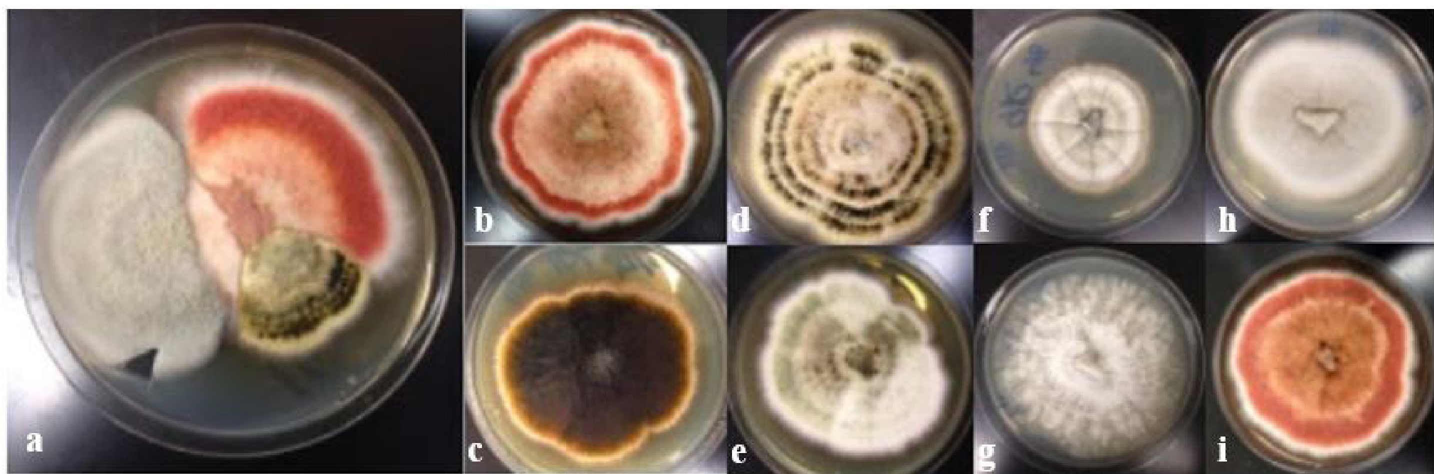

Figure 1: Macroscopic diversity of dermatophyte fungi isolated from collegiate runners. Picture (a) shows an original sample, pictures (b-i) are a representation of morphological diversity of isolated subcultures.

Gold Master Mix containing 1.25 U AmpliTaq Gold DNA Polymerase, 0.2 mM dNTPs, 2.5 mM MgCl2, and GeneAmp PCR Gold Buffer (Applied Biosystems, Waltham, MA). Cycle parameters for ITS primer combinations were an initial denaturation at 94℃ for 5 min, followed by 35 cycles consisting of denaturation for 1 min, annealing at 56℃ for 1 min, extension at 72℃ for 1 min, and a final extension at 72℃ for 10 min (17,18). Cycle parameters for NL-1/NL-4 Bt2a/T1 primer sets were an initial denaturation at 95℃ for 5 min, followed by 35 cycles consisting of denaturation for 45 sec, annealing at 52℃ for 90 sec, extension at 72℃ for 90 sec, and a final extension at 72℃ for 10 min (17,18). PCR products were visualized on a 0.8% agarose gel, purified using the E.Z.N.A. cycle pure kit (Omega bio-tek, Norcross, GA), and submitted for Sanger DNA sequencing (ACGT, Inc. Wheeling, IL). Sequences were compared with sequence entries in GenBank database using the Basic Local Alignment Search Tool for nucleotides (BLASTN) (20). Using the calculated percent identity score, specimens were assigned to a genus and species with a minimum average of > 93% homology across the primer sets used.

Results

Sixteen collegiate cross-country runners, 3 female and 13 male, participated in this study. (Table 1). Runners sampled in this study ran between 35-60 miles per week and none had active tinea pedis infections. Survey results did not yield any consistent patterns in frequency and temperature of washing running shoes, whether running shoes were worn in the locker room or used for outside of training, or if shower shoes were worn in the locker room. All but two volunteers always wore socks with their athletic footwear. Foot washing habits varied among participants; most runners washed their feet daily with soap and water while a few did this periodically or not at all.

Sampling both feet of each volunteer resulted in 35 distinct fungal cultures (from a total of 24 of the samples) that varied in size, shape, and color (Fig. 1). Four isolates (3R, 4R, 11R, 13L) were tentatively identified as tinea pedis-causing fungi based on their macroscopic and microscopic morphology when grown on SBA. Isolates 3R, 4R, and 13L were initially classified as *Trichophyton rubrum* as they exhibited chains of round macroconidia of varying shape and size and fluffy to cottony white colonies. Isolate 11R appeared similar to *Trichophyton mentagrophytes* as it grew numerous small and circular spores and exhibited small, star- shaped, white colonies (Table 2).

The four isolates morphologically similar to tinea pedis-causing fungi came from male runners who ran between 40-50 miles per week, none wore their shoes without socks, and all used soap and water to scrub their feet. From the 3 runners where *F. equiseti* was isolated - none wore shower shoes; all wore their training shoes for other activities. Shoe washing habits varied as one runner didn't wash their running shoes, one washed with cold water, and the third washed with

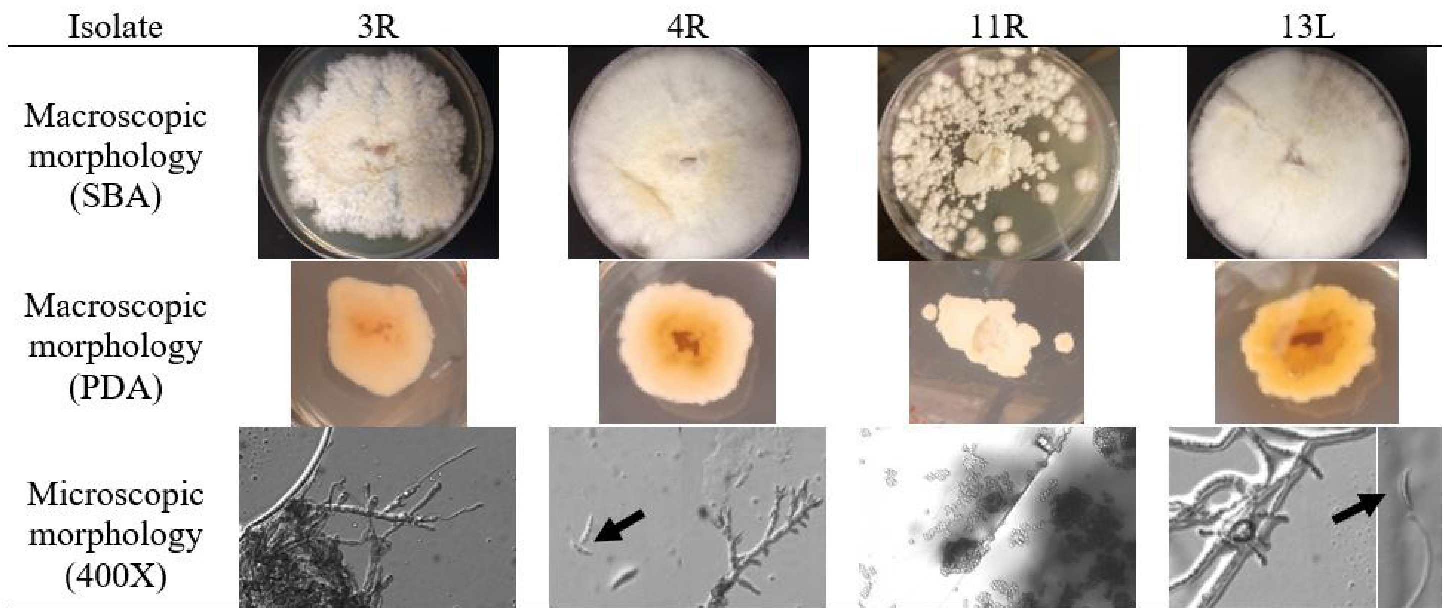

Table 2: Macroscopic and microscopic morphology of tentative tinea pedis-causing fungal isolates. Arrows highlight macroconidia.

warm water. Only one of the three wore shoes in the locker room. The runner with *B. bassiana* wore shoes in the locker room, didn't wear their running shoes outside of training, and infrequently washed their shoes in hot water.

When isolates were grown on PDA, colonies of 3R, 4R, and 13L exhibited a pink/ orange pigmentation on the underside of the colony while 11R still appeared white, with one large colony. Isolates 3R, 4R, and 13L also produced sickle-shaped, septate macroconidia with aerial conidiogenous cells. Macroconidia, typical of Fusarium species, are highlighted with arrows in microscopic morphology of isolates 4R and 13L in Table 2. Colony 11R grew globus, hyaline conidia on aerial hyphae.

PCR products for all samples using primer sets ITS-1/ITS-4, NL-1/NL-4, and T1/Bt2b were in the 500-700 bp range, as expected. The ITS-5/ITS-4 primer set did not amplify products under the conditions tested. Top sequencing results showed 92.89-99.83% identity to the unknown isolates (Table 3). Isolates 4R and 13L were identified as *Fusarium equiseti* and 11R as *Beauveria bassiana* as the sequencing produced the same species for each of the six primers tested. Isolate 3R is likely *Fusarium equiseti* as it was the top result for four of the primers, while the D1/ D2 region of isolate 3R came back as the closely related species *Fusarium incarnatum*

Discussion

A range of fungi were isolated from the feet of collegiate cross-country runners, four of which initially resembled tinea pedis species on SBA and chosen for further identification. This preliminary morphological identification is the most common method of identification of tinea pedis in clinical settings as it is accurate (particularly when an individual is symptomatic) and inexpensive (1,3). DNA sequencing can be costly but has the advantages of increased sensitivity and reproducibility, minimal sample handling, and speed, compared to culture-based diagnosis (1,26). Asymptomatic individuals, variation in fungal morphology, and inconsistent morphological results have created a need for more straightforward and reliable identification methods.

Runner hygiene was variable across the across the sixteen volunteers. Inconsistent habits of shoe, sock, and foot hygiene can influence the spread and survival of fungi, yet there is no standard protocol for hygiene in collegiate runners (8). Consideration of community spread of dermatophytes has led to recommendations of not wearing running shoes in the locker room, implementation of additional sanitation measures for shoes and socks, and increasing washing temperature for clothing and shoes to 60˚C in order to kill fungal spores (8).

	ITS				D1/D2				β-Tubulin			
Isolate	ITS-1	%	ITS-4	%	NL-1	%	NL-4	%	T1	%	Bt2b	%
3R	*Fusarium equiseti*	97.6	*Fusarium equiseti*	98.8	*Fusarium incarnatum*	99.1	*Fusarium incarnatum*	99.6	*Fusarium equiseti*	98.5	*Fusarium equiseti*	98.3
4R	*Fusarium equiseti*	93.5	*Fusarium equiseti*	97.7	*Fusarium equiseti*	99.6	*Fusarium equiseti*	99.5	*Fusarium equiseti*	98.6	*Fusarium equiseti*	98.3
11R	*Beauveria bassiana*	98.9	*Beauveria bassiana*	98.2	*Beauveria bassiana*	99.0	*Beauveria bassiana*	98.1	*Beauveria bassiana*	98.1	*Beauveria bassiana*	97.6
13L	*Fusarium equiseti*	92.9	*Fusarium equiseti*	99.1	*Fusarium equiseti*	99.7	*Fusarium equiseti*	99.8	*Fusarium equiseti*	99.0	*Fusarium equiseti*	98.6

Table 3: Sequencing results and percent identity for ITS, D1/D2, and β-Tubulin regions for dermatophytic isolates 3R, 4R,11R and 13L.

Four isolates appeared to be tinea pedis- causing species based on their morphology on SBA media, commonly used to culture dermatophytic fungi (22). Isolation of these fungi from 25% of runners surveyed was consistent with Lacroix *et al.*'s findings where 27% of European marathon runners were asymptomatic carriers of *Trichophyton* spp. (14). Using a more general growth medium (PDA), cultures demonstrated inconsistent morphologies to those of athlete's foot-causing fungi. Isolates 3R, 4R, and 13L resembled *Trichophyton* when grown on SBA; however, once placed on PDA, they produced curved, septate macroconidia characteristic of *Fusarium* species. Isolate 11R grew as a fluffy white colony on SBA similar to athlete's foot fungi, though microscopic morphology was consistent with *Beauveria bassiana* on both growth media. SBA media did not hinder spore formation for the species as it did for Fusarium species complex isolates. DNA sequence analysis indicated that none of the isolates were typical species that cause athlete's foot.

It was not surprising that no fungal DNA was amplified using the ITS5/ITS4 primers as it was previously documented that some fungal isolates only work with ITS5/ITS4 or ITS1/ITS4 (18). All primer set identities agreed except for isolate 3R. This isolate was identified as *Fusarium equiseti* (*β*-Tubulin and ITS) and *Fusarium incarnatum* (D1/D2). Inconsistencies about species identification and recognition exist in the Fusarium incarnatum-equiseti complex due to significant genetic variability and need for further research on differences among species (2,26,30). The translation elongation factor 1-alpha and partial RNA polymerase second largest subunit genes have distinguished over 20 species within the *Fusarium incarnatum-equiseti* species complex; use of both primer sets could identify isolate 3R to species level (2,29). BLAST scores in the low 90% range can be explained by limited fungal data in databases and sequencing Phred scores between 20 to 40 (99.0-99.99% accuracy).

Asymptomatic infections of tinea pedis, referred to as occult tinea pedis, are common among athletes and complicate the spread and diagnosis of tinea pedis (10). While not previously isolated from runners, both *B. bassiana* and members of the *Fusarium incarnatum-equiseti* species complex are opportunistic human pathogens that can result in superficial dermal lesions to deep tissue, systemic infections (28,29). Long distance runners have depressed immune function and are especially prone to traumatic and environmental dermatoses due to repeated physical stress on their feet and exposure to soil. *B. bassiana* and *Fusarium* species are ubiquitous in soil and track and field athletes may come in contact while running through soil during practice and outdoor track meets, which then gets tracked into locker rooms. Limited washing of running sneakers can contribute to repeated exposure to soil fungi directly or through shared surfaces.

Molecular identification tools, such as sequencing of conserved DNA regions, can improve the consistency and accuracy of identification of dermatophytic fungi (10). This study demonstrated morphological identification could eliminate 75% of the isolates cultured from runners' feet from being tinea pedis-causing fungi. Four isolates resembled *Trichophyton* when grown on SBA media, but exhibited atypical morphology when PDA media was used. DNA sequencing was required to determine no isolates were tinea pedis-causing fungi. The presence of opportunistic human pathogens in areas of common tinea pedis infection highlights the need to identify microbes inhabiting asymptomatic and healthy runners. More diagnostic techniques are being developed for dermatophyte identification, such as Matrix-assisted laser desorption/ionization- time of flight mass spectrometry, and these tools will continue to improve fungal identification (26). Greater understanding of dermatophytic fungal interactions and their role in disease development can further inform methods of prevention, identification, and treatment.

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Citations

1. Ahmadi, B., Mirhendi, H., Shidfar, M.R., Nouripour-Sisaakht, S., Jalaziland, N., Geramishoar, M., & Shokoohi, G.R. 2014. A comparative study on morphological versus molecular identification of dermatophyte isolates. Journal de Mycologie Medicale. 25:29-35.

2. Avila, C.F., Moreira, G.M., Nicolli, C.P., Gomes, L.B., Abreu, L.M., Pfenning L.H., Haidukowski, M., Moretti, A., Logrieco, A., & Del Ponte, E. M. 2019. Fusarium incarnatum-equiseti species complex associated with Brazilian rice: Phylogeny, morphology and toxigenic potential. International Journal of Food Microbiology 306:1-8.

3. Canavan, T.N., & Elewski, B.E. 2015. Identifying Signs of Tinea Pedis: A Key to Understanding Clinical Variables. Journal of Drugs in Dermatology 14:42-47.

4. de-Hoog, G.S., Guamo, J., Gene, J., & Figueras, M.J. 2000. Atlas of Clinical Fungi 2nd Edition. The Netherlands: Central Bureau voor Schimmelcultures Utrecht.

5. Diongue, K., Brechard, L., Diallo, M.A., Seck, M.C., Ndiaye, M., Badiane, A.D., Ranque, S., & Ndiaye, D. 2019. A comparative Study versus ITS-Based Molecular Identification of Dermatophytes Isolated in Dakar, Senegal. International Journal of Microbiology 2019:1-6.

6. El-Said, A.H.M. 2001. Mycological and Physiological Studies on Fungi, Isolated from Skin Diseases. Pakistan Journal of Biological Sciences 4:1432-1436.

7. Field, L.A., & Adams, B.B. 2008. Tinea pedis in athletes. International Journal of Dermatology 47:485-492.

8. Gupta, A.K., &Versteeg, S.G. 2019. The Role of Shoe and Sock Sanitization in the Management of Superficial Fungal Infections of the Feet. Journal of the American Podiatric Medical Association 109:141-149.

9. Glass, N.L., & Donaldson, G.C. 1995. Development of Primer Sets Designed for Use with the PCR To Amplify Conserved Genes from Filamentous Ascomycetes. Applied and Environmental Microbiology 61:1323-1330.

10. Ilkit, M., & Durdu, M. 2015. Tinea pedis: The etiology and global epidemiology of a common fungal infection. Critical Reviews in Microbiology 41:374-388.

11. Jang, J.H., Lee, J.H., Ki, C.S., & Lee, N.Y. 2012. Identification of Clinical Mold Isolates by Sequence Analysis of the Internal Transcribed Spacer Region, Ribosomal Large-Subunit D1/D2, and β-Tubulin. Annals of Laboratory Medicine 32:126-132.

12. Kovitwanichkanout, T., Chong, A. H. 2020. Superficial fungal infections. Australian Journal of General Practice 48: 706-711.

13. Kurztman, C.P., & Robnett. C.J. 1997. Identification of Clinically Important Ascomycetous Yeasts Based on Nucleotide Divergence in the 5' End of the Large-Subunit (26S) Ribosomal DNA Gene. Journal of Clinical Microbiology. 35:1216-1223.

14. Lacroix, C., Baspeyras, M., de La Salmoniere, P., Benderdouche, M., Couprie, B., Accoeberry, I., Weill, F.X., Derouin, F., & de Chauvint, M.F. 2002. Tinea pedis in European marathon runners. Journal of European Academy of Dermatology and Venereology 16:139-142.

15. Nakasone, K.K., Peterson, S.W., Jong, S. 2004. Preservation and Distribution of Fungal Cultures. In M. Foster, G. Bills(Eds.), Biodiversity of Fungi (pp. 37-47). Academic Press.

16. Nakasone, K.K., Peterson, S.W., Jong, S. 2004. Preservation and Distribution of Fungal Cultures. In M. Foster, G. Bills (Eds.), Biodiversity of Fungi (pp. 37-47). Academic Press.

17. Nweze, E.I, Mukherjee, P.K., & Ghannoum, M.A. 2009. Agar-Based Disk Diffusion Assay for Susceptibility Testing of Dermatophytes. Journal of Clinical Microbiology 48:3750-3752.

18. O'Brien, H.E., Miadlikowska, J., Lutzoni, F. 2009. Assessing reproductive isolation in highly diverse communities of the lichen-forming fungal genus Peltigera. Evolution 63: 2076-2086.

19. Okhura, M., Abawi, G.S., Smart, C.D., & Hodge, K.T. 2009. Diversity and aggressiveness of *Rhizoctonia solani* and *Rhizoctonia*-like fungi on vegetables in New York. Plant Disease 93:615-624.

20. Pleacher, M.D., & Dexter, W.W. 2007. Cutaneoud Fungal and Viral Infections in Athletes. Clinic in Sports Medicine 26:397-411.

21. Pruitt, K., Brown, G., Tatusova, T., & Maglott, D. 2013. The Reference Sequence (RefSeq) Database. In: McEntyre, J., Ostell, J. (Eds.), The NCBI Handbook. (pp. 307-328). National Center for Biotechnology Information.

22. Purim, K.S.M., & Leite, N. 2014.Sports related dermatoses among road runners in Southern Brazil. Anais Brasileiros de Dermatologia 89: 457-592.

23. Robert, R., & Pihet, M. 2008. Conventional Methods for Diagnosis of Dermatophytes. Mycopathologia 166:295-306.

24. Sabadin, C.S., Benvegnu, A.A., da Fontoura, M.M.C., Saggin, L.MF., Tomimori, J., & Tischman, O. 2011. Onychomycosis and Tinea Pedis in Athletes from the State of Rio Grande Do Sul (Brazil): A Cross-Sectional Study. Mycopathologia 171:183-189.

25. Sasagawa, Y. 2019. Internal environment of footwear is a risk factor for tinea pedis. The Journal of Dermatology 46:940-946.

26. Sting, R., Eisenberg, T., & Hrubenja, M. 2019. Rapid and reasonable molecular identification of bacteria and fungi in microbiological diagnostics using rapid real time PCR and Sanger sequencing. Journal of Microbiological Methods 159:148-156.

27. Suh, S-O, Grosso, K. M., Carrison, M. 2018. Multilocus phylogeny of the *Trichophyton mentagrophytes* species complex and the application of matrix-assisted laser desorption/ionization- time-flight (MALDI-TOF) mass spectrometry for the rapid identification of dermatophytes. Mycologia 110:118-130.

28. Taschdjian, C. 1955. Fountain Pen Ink As an aid in Mycologic Technic. Journal of Investigative Dermatology 24:77-80.

29. Tucker, D.L., Beresford, C.H., Sigler, L., & Rogers, K. 2004. Disseminated *Beauveria bassiana* Infection in a Patient with Acute Lymphoblastic Leukemia. Journal of Clinical Microbiology 42:5412-5414.

30. Wang, M.M., Chen, Q., Diao, Y.Z., Duan, W.J., & Cai, L. 2019. *Fusarium incarnatum-equiseti* complex from China. Persoonia. 43:70-89

31. White, T.C., Findley, K., et all. 2014. Fungi on the Skin: Dermatophytes and Malassezia. Cold Spring Harbor Perspectives in Medicine.

32. White, T.J., Bruns, T.D., & Leach, L.D. 1990. PCR Protocols: A Guide to Methods and Applications. In M.A. Innis, D.H. Gefland & J.J. Sninsky (Eds.), Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics (pp. 315-322). Academic Press.

Undergraduate Student Perspective - Undergraduate research student interview

Airhenvbahihea Edionwe

Received a B.S. from Ball State University 2021 in Pre-Med Studies

Interviewed by Sammie Campaniello

Received a B.S. from Ball State University 2021 in Science Education

Tell me a little about your research, and the broad goal.

Title: Bacteriophage control of Salmonella enterica in artificially contaminated 1%, pasteurized milk

The genus *Salmonella* is made up of over 2,500 serovars between two species: *Salmonella* enterica and *Salmonella* bongori. *Salmonella* spp. are the number one causative agent of food-borne illnesses in the United States. Consuming food that is contaminated with *Salmonella* can cause a condition known as Salmonellosis. Salmonellosis, also known as food poisoning, occurs when *Salmonella* spp. enter the intestinal tract and invade the epithelial cells of our small and large intestines. It is characterized by vomiting, diarrhea, fever, and abdominal cramps. Usually this infection will only last up to 48 hours but in the immunocompromised community, this can be life threatening. If a person's immune system is not able to fight off the *Salmonella* infection, then the bacteria will enter the bloodstream and cause a systemic infection which can be fatal. An infection of the intestinal tract cannot be treated with antibiotics. Treatment with antibiotics can leave the patient vulnerable to more infections of the GI tract. However, if antibiotics were a treatment option, they would be ineffective against a *Salmonella* infection. *Salmonella* spp. are resistant to multiple antibiotics leaving only a few that can be used to treat these infections. Alternatives to antibiotics are needed to treat infections where the bacteria are resistant to all antibiotics. Bacteriophages are known as the "viruses of bacteria". Bacteriophage, also known as phage, insert their genetic material into their hosts, hijacking their DNA synthesis machinery in order to produce more phage inside the host cell. There are two forms of bacteriophage, lytic and lysogenic. Both phages hijack their hosts in the same way, but how long it takes to kill the host is where these two differ. Lytic phage get their name by causing the host cell to lyse after producing more phage. Lysogenic phage will incorporate their genome into the hosts and not cause lysis until a switch is made. There is no set time for how long a lysogenic phage will keep the host cell alive but once the switch is made, the host cell will produce more phage eventually causing itself to lyse. Bacteriophages have been considered an alternative to antibiotics because of this lytic ability to kill their hosts. The goal of this project is to isolate and characterize bacteriophage

from a raw milk environment to use as a biological control of *Salmonella* enterica contamination of food products.

We expect to see that the bacteriophage are able to control *Salmonella* contamination at a low density but as the density increases, the phage becomes less effective. We also expect that the bacteriophage will have a higher affinity to the S1 (unknown *Salmonella* species we isolated from the farm) bacteria than it does to the lab grown *Salmonella* enterica .

Can you give a brief overview of some of your daily activities in the lab?

Each day I go to to the lab and subculture set of S1 and S. enterica with TSB tubes. If we are out of material we need, I usually make sets of TSA plates and TSB tubes. Each day's task depends on what we have going on in the lab at a particular time.

How has working in the lab helped to better prepare you for your future?

From just in the lab this semester I have gained some confidence on what it takes to be a Microbiology student and work in a Microbiology lab. Also, working in the lab I feel has gotten me ready for my MS program and what it takes to be a scientist and also with having the experience in the lab will increase my chances of getting into medical school.

What are some limitations that you seem to be facing in this pandemic regarding your research?

Some limitations we faced during this pandemic are noting having in materials and phages we need for experiment not getting delivered time due to the pandemic, also due to the pandemic the amount of time we are allowed in lab is limited in other to accommodate all students doing research to help reduce the numbers of students allowed in the lab at a given time.

Do you foresee these limitations changing anytime soon or do you think research has forever been changed due to COVID-19?

I think the limitations we are currently facing could change. We have a vaccine for this virus and the pandemic is over. With that being said I do not think research is forever changed due to COVID-19 because I believe a good scientist/researchers should be able to accommodate changes and be able to work around these unplanned changes but change is the only constant time in life.

What is some advice that you would give a new undergraduate researcher that would have helped you be better prepared for your research experience?

- Do not wait till your last year of your undergraduate degree to start research.
- Failure is part of being a researcher
- Don't be afraid to ask professors to work in their labs from your first of your undergraduate career.
- Take initiative and work hard.
- Don't be afraid to ask questions if unsure.

Do you have any further comments about Fine Focus or undergraduate research in general?

I would encourage any science/biology major student to sign up for a research immersive learning class because it will help prepare them for plans after their undergraduate degrees such as graduate school, phd program. Also it will also look good on your resume and most importantly makes you a better scientist.

Undergraduate Student Perspective

Irene Yi

Linguistics Undergrad Student

UC Berkley

I recently saw a humorous post online, poking fun at society's expectations of younger generations to have life all figured out. It said, "It just hit me that five years ago, I was in high school and they were asking 'Where do you see yourself in five years?' and I want you all to know [that] I was very wrong." I relate to this deeply, but in the best way possible and completely unironically. Five years ago, I could have never imagined all the wonderful paths this life would take me down. Thanks to my incredible family, friends, and department, I have been presented with and supported in endless opportunities that have helped me grow as a person, student, and linguist.

Before I write more about what I've learned from undergraduate research, I'll begin with a very brief introduction of myself. I am, in fact, not a microbiology student--I'm a linguistics undergraduate student at UC Berkeley, finishing up my last semester of college. My passions lie in the linguistics subfields of sociolinguistics, historical linguistics, and Indigenous language revitalization; specifically, I am fascinated by the way that language is used to construct and express identity, on both individual and community levels. I believe that linguistics, as a field, has so much potential for research that directly helps and empowers individuals and communities in their linguistic (and general) identities. In my time at Berkeley, I've had the privilege of working with incredible people on a number of fascinating and important research projects, and I hope to continue working in linguistics academia for the rest of my life. After graduation, I'll be moving across the country to work as a post-baccalaureate researcher in the Linguistics Department at Yale, with a focus on historical linguistics and language revitalization. I am wholly and absolutely in love with the research I do, and the very existence of linguistics is what makes me get out of bed every morning with a smile on my face.

Coming to college, I knew I wanted to study linguistics in some capacity, though I had limited understanding of the wide variety of linguistics research, including methodologies, subfields, and interdisciplinary possibilities. Fortunately, the linguistics department at Berkeley not only has a strong representation of all the subfields of linguistics, they also greatly support undergraduate students' opportunities to do all different types of research. The constant and consistent encouragement to make original contributions to the general linguistics research literature helped fuel my classmates and me to explore the vast expanse of research methods and ultimately find our favorite ones.

While my work isn't in microbiology, I think research contains many universal experiences and teaches us many of the same lessons. For any undergraduate students who are thinking about doing research, my biggest piece of advice is simply this: do it!! Research is a beautiful, rewarding, and sometimes grueling process, and it is so, so worth it. More specifically,

though, here are some things that I have learned throughout the years:

1) Be patient with yourself and your research process. Research is a long, consistent process; it's not something you can cram at the last minute. Not only would it be extremely stressful to try and get everything done in one day (or even one week… or one month), it is realistically impossible. Because there are so many factors outside of one's control during a research project, it's also futile (and unsustainable) to try and speed the whole process up by working yourself to exhaustion every day. There's no "if I just work double the amount and time that I do every day, I can halve the timeline of the process." Often, you actually need months to get approval, collect data, analyze data, and put it all together. Plus, when spread out over months, you give yourself time to make mistakes, space to try new solutions and approaches, and--most importantly--freedom to take a day off and rest. I'm currently in the process of writing my senior thesis, and I always have to remind myself to be patient with the process. It took a few months' time just to write a proposal to the Institutional Review Board (IRB), edit and improve the proposal based on feedback, and ultimately get approval from the IRB to start collecting data. Then, it took months of daily work to collect, transcribe, clean, and analyze the data. Even though it took a long time, that doesn't mean that I wasn't working diligently at it during the entire process. All this is to say that a research project is inherently going to take time, and rather than trying to shorten the timeline by trying to "work harder" (because chances are, you are already working incredibly hard and maybe even at the limits of your capacity), you might as enjoy the process of watching your research develop and grow.

2) It's okay if you realize that the research you're doing--whether it be the methodology, data, or general topic--isn't right for you long term. It's great to explore different types of research, but you don't have to feel guilty about finding out that a certain methodology isn't what you want to do. In linguistics, there are endless ways of conducting research, gathering data, and analyzing it (depending on your subfield, research question, etc.)--and these methods vary quite a bit. Exploring different research methods is for the purpose of finding what works for you. For example, I've done a handful of research projects where I realized quickly that certain ways of gathering data and analyzing it wasn't anything I would want to do in the future. After the conclusion of said projects, I stopped doing those specific types of research. Rather than looking at these experiences negatively as "wasted time," I saw them as experiences that helped me learn about myself as a researcher, and it helped guide me to develop my ideas for future projects. Knowing what research methods I liked and didn't like allowed me to choose projects whose processes I knew would be interesting and enjoyable the whole way through. Namely, when I was choosing my topic for my senior thesis, I knew that this project would be all-consuming, and I would be in this process for a whole year. I decided to pick a topic and a few methodologies that I would never get tired of (after having experiences that I learned from on research projects where I would get tired of a certain method or not enjoy the methods to begin with), and it made all the difference.

3) There is no shame in creating your own opportunities for research by yourself! If I could star, bold, and underline one important piece of advice, it would be this one. Even without institutional funding or structure, there is still so much independent research that you have the capacity to do! In my freshman year of college, before I discovered the official program(s) for undergraduate research opportunities within the linguistics department, I became interested in the topic of gender in language and did my own digging into it. Eventually, my own little pet project turned into a three-year long personal research passion, opportunities to present my work at research conferences and be involved in related (and more official) research, and the foundation of the future research I want to do. The premise is this: in grammatically gendered languages (e.g. Spanish, French, etc.), there are words that fall into the feminine gender or masculine gender (and in some languages, there is a neuter gender too). These genders are marked on the words, often in the form of a suffix. My interest in this topic began with the feminization of French profession nouns--specifically how a grammatically gendered language like French can reflect a history of misogynistic norms in the way the language is structured. It started with reading a simple footnote in my French textbook about how there were no officially recognized grammatically feminine forms for certain profession nouns like "author,"

"professor," or "doctor" because those were not jobs traditionally held by women in the history of French society. I then started looking into the history of the French language and other sociolinguistic literature on grammatically gendered languages. Additionally, I reached out to some linguists in the French Department at Berkeley and asked about some of the issues surrounding this topic. Finally, I wrote my findings into a "blog" of sorts, which later turned into a more academically-written paper, which then took me to presenting this work at conferences all over the world. As the feminization of French profession nouns is an ongoing debate in France, I updated my work with what was happening in France with feminist linguists and oppressive language institutions.

I also realized during this personal research project that gender in language went beyond the gender binary, and that many language activists across the world (and across many languages) are now working on ways of making grammatically gendered languages more gender inclusive (e.g. the term "Latinx" in Spanish is used as a gender inclusive term, often used by nonbinary speakers to transcend the binary gender reflected in the grammar of the language). This semester, I was able to expand my interest in language and gender through a research project in Berkeley's Linguistics Department. In the department, there is a research program called the Linguistics Research Apprentice Practicum (LRAP) that graduate students, undergraduate students, and sometimes even faculty are involved in. Recently, a Gender in Language project was created through LRAP, and I now have the chance to use what I learned through my French feminization pet project in research of how gender is marked in Mandarin--and the social and historical implications of linguistic gender. Specifically, we put together a nonbinary grammar and lexicon reference of Mandarin, based on many gender-inclusive movements that are already going on in China, Hong Kong, and Taiwan. Had I never embarked on this personal research journey that began with just a simple interest, I would have never known just how passionate I am about the topic of gender in language--so much so that in the future, I want research the intersection of gender and sociophonetics (which is the subfield of linguistics that deals with how individuals construct, index, and express their identity with the qualities of the sounds in the way they speak).

4) If a personal pet research project isn't for you, you can also cold-email supervisors and ask for their help in creating opportunities! Sometimes, the research that you want to explore might require technical skills or knowledge that you don't quite have yet--and the process to learn what you need might be out of your own control or grasp. In these cases, don't be afraid to reach out to researchers in your department, or even at other schools, to ask for help! Often, researchers are more than happy to chat or send you resources that can help you develop the skills and knowledge base that you are seeking. If you're lucky, they might even have an official or unofficial research position for you (or a project that you can collaborate on). In my own research processes and research exploration, I cold-emailed a good handful of professors and researchers at other universities to ask for insight on various issues, and I received such great resources and help. Specifically, while writing code for my thesis, I had to consider and try out many different computational models on my linguistic data, and talking with researchers whose previous computational work I had read helped me significantly in formatting and analyzing my own data (and of course, in writing code). In one case, one of my cold-emails from a while ago now actually led to me working remotely with a lab at a different university for many months on a very interesting and engaging project. Through that project, I gained computational skills that I'm using not only for my thesis, but for work I want to do in the future.

5) Criticism of your research isn't criticism of you as a person (or as a researcher). In fact, critical responses can often be really helpful in making your research better. In academia, there is an immense (and sometimes unhealthy) amount of pressure on every researcher to continually produce work. While this is actually just a reflection of the toxic internalized capitalism present in academia, the best an individual can do is just to compartmentalize your work and you as a person into separate boxes of your identity. As much as you can be passionate about your work and have your time consumed by it, it's very dangerous to start equating your worth as a person to how well your work does or how much work you produce (again, that's what internalized capitalism will do to you). Instead, you can use criticism of your work as something to improve your research with, because the process of research

in academia will inevitably include critical feedback (on your writing, on the actual content of your work, on your very research question itself, etc.), and your mental health will greatly thank you if you learn to remove your self worth from your role in the institution of academia.

6) Take your time in figuring out who you are and what research you want to do--and when do you figure it out, embrace it! Ultimately, research should be something that brings you joy. At the very least, it should be something that you feel like is worth it, whether that be because you enjoy the specific topic, because you believe in the bigger purpose and impact of your work, or both. As obvious as it sounds, it took me my entire life to know myself as well as I do now, and I know that I'll continue to grow as a person and researcher--and continue to get to know myself better--for the rest of my life. In the process of exploring different types of research in college, I had the misconception that there were certain methodologies that were more "expected," or certain skills that were more "desirable" to have. I thought that I would have to check off a certain list of boxes to be academically marketable as a researcher, when in reality, the most important and "marketable" quality is that one is genuinely passionate about their research, and that they get up after every time they fall down to consistently pursue these passions. Any skill you need along the way of your research, you will no doubt learn and develop as you go. Finally, in the process of learning about yourself, you'll also realize that your growth as a researcher doesn't take away from anyone else's--and similarly, someone else's growth and success will never take away from yours. In a cutthroat, capitalistic institution like academia, it's so, so important to support one another and value compassion and collaboration over competition. Additionally, academia is often inaccessible as an institution, and many communities are excluded in research, discourse, and representation. This needs to change, and personally, I try to use the tools within and related to linguistics to dismantle the inaccessibility in academic spaces. I have come to realize that who I am as an academic is inextricably linked to centering and uplifting communities, such that whatever academic work and research I do will always be for the good of the community I am serving. This, then, becomes reflected in the subfields of linguistics that my research is focused on: sociolinguistics, historical linguistics, and Indigenous language revitalization. While some of these subfields of linguistics is sometimes disregarded by traditional, elitist parts of academia as "not real research" because it serves communities rather than research literature production, I wholeheartedly believe that not only is it real research, it is even more important research because of the greater community implications it has beyond writing an academic paper.

Looking back, I have learned so much through all of the good and bad that comes with research. Academia is not a perfect institution by any means, but I hope that through the researchers who go into this field to make the world a better place, the institution can become a little less flawed as well. I have gained so much from my experiences in undergraduate research, and I would highly encourage everyone to dip their toes in it as well. Who knows? You might just fall in love with it. I will forever be grateful for the Linguistics Department at Berkeley--and for everyone I have been lucky enough to cross paths with-- for making me who I am today and bringing me the best years of my life so far. I want to give a million thank-yous to my family, who have always supported me and believed in me, even when I didn't believe in myself. Finally, I want to thank Lauren Andrews for reaching out to me and giving me this opportunity and platform to share my experiences; it honestly means so much to me, and I'm so humbled to have this space to reflect on the things I've learned.

While that sarcastic post I saw online was meant to be a joke, I think about how lucky I am all the time to be in love with my major, research, and work. Truly, I never could have seen it coming; my reality today is beyond High School Me's wildest dreams, and Five Years Ago Me wouldn't believe you if you told her all the unseen ways she would learn through undergraduate research and grow into a version of herself who has never been happier. I can't even begin to imagine what the next five years will bring.

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