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Genomics tools in microbial food safety

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ABSTRACT

Genomics tools, and specifically whole-genome sequencing (WGS) methods, are rapidly transforming food safety, specifically through improved outbreak detection. In the U.S., whole genome sequencing of all human clinical *Listeria monocytogenes* isolates has been performed since 2013; isolates from foods and food processing plants obtained by regulatory agencies are also routinely characterized by WGS. Routine application of WGS appears to increase the number of listeriosis outbreaks that are detected and traced back to specific food sources. While rapidly improving WGS technologies have driven use of omics tools in food safety over the last 5 to 7 years, improved analytic methods will drive effective application of omics tools including genomics, transcriptomics, proteomics, and metabolomics over the next few years.

Introduction to omics approaches in microbial food safety.

For food safety, the term “omics” encompasses a cornucopia of different methodologies including genomics, transcriptomics, proteomics, and metabolomics that, through advanced bioinformatics, provide insights relevant to reducing microbial food safety hazards [1]. Previous reviews have summarized application of these omics tools in food related applications using the term “foodomics” [2,3]. While many omics approaches are having a major impact on the methods of food safety research, genomics and whole genome sequencing (WGS) have been the most widely applied [1]. Specifically, WGS of bacterial isolates has started to replace other molecular methods, such as Pulse Field Gel Electrophoresis (PFGE), as subtyping method of choice. It appears that we are at the cusp of rapid and widespread application of WGS-based tools by regulatory and public health agencies as well as researchers. There is, however, an urgent need to facilitate use of omics tools, as well as the information generated by these tools, by the food industry and food safety professionals. This review is aimed to provide an overview of recent trends in the use of omics tools in microbial food safety (Figure 1), with an emphasis on genomics applications to bacterial foodborne pathogens.

Evolution and current status of whole-genome sequencing tools.

Whole-genome sequencing technology has progressed far beyond Sanger sequencing that produced the first complete bacterial genome sequence, of *Haemophilus influenza*, reported in 1995 [4], and the first human genome [5], reported in 2004 which cost almost 3 billion dollars and 13 years of work. Now a variety of next-generation sequencing tools exist that use alternatives to chain-terminating (Sanger) sequencing chemistry for high-throughput nucleotide sequence determination (reviewed in [6]), mainly (i) amplified template sequencing, which generate relatively shorter read lengths and (ii) single-molecule sequencing, which generate relatively longer read lengths. Each generates dramatically larger volumes

of raw sequence data at a reduced per base cost. Choice of a particular sequencing platform for a project depends on the specific advantages and disadvantages of each platform.

Amplified template platforms, e.g. Illumina sequencers, are well-suited for experiments involving many strains and resequencing applications (when a closely related reference genome can guide analysis). These platforms generate millions of small (in the 100s of base-pairs) sequence reads. They have proven popular for improved molecular subtyping of foodborne pathogens [1]. These platforms provide economies of scale for centralized sequencing of small (< 10 Mb), bacterial genomes. In retrospective sequencing of nearly 200 *L. monocytogenes* isolates from U.S. retail deli operations (M Stasiewicz, PhD thesis, Cornell University, 2015), high-output sequencing with multiplexing reagents for library preparation allows many (up to 96+) isolates to be sequenced in parallel, generating WGS for <\$100 per isolate. Costs will likely drop further; custom protocols reduce costs of multiplexed library preparation from >\$50 to <\$10 per sample [7].

Single molecule platforms, particularly Single-Molecule Real-Time (SMRT) and nanopore-based sequencing platforms, e.g. PacBio and MinION sequencers respectively, are well-suited for comparative genomics questions that rely on finished genomes [8]. Using sequencing reads in the 10 of thousands of base pairs, these platforms allow for production of finished, closed, bacterial genomes through hybrid analysis along with short-read data [9]. Finished genomes can also be constructed from single-molecule data alone [10,11], including for a *Salmonella* Typhimurium isolated from chicken breast [12]. Illustrating value beyond genome assembly, a USB-operated nanopore sequencer has been shown to resolve genomic artifacts from short-read data, such as the structure and insertion site of an antimicrobial resistance island in *Salmonella* Typhi [13].

Evolution and current status of bioinformatics tools used to analyze bacterial WGS data.

Bioinformatics tools for bacterial WGS data perform the same basic tasks as were necessary before high-throughput WGS, e.g. genome assembly, comparison, and subtyping. The challenge is to adapt the tools to (i) primary data that are large collections of much shorter genome sequence fragments, and (ii) comparisons between increasingly larger numbers of whole genomes where manual, genome-by-genome, comparisons are impractical. These adaptations pose both computational and user-interface challenges (see introductory tutorials [14,15]).

Currently, two main strategies exist for the analysis of bacterial WGS data, including (i) whole genome Single Nucleotide Polymorphism (SNP) analysis, and (ii) whole genome, gene-based analyses such as whole-genome Multi Locus Sequence Typing (wgMLST). Typical workflows for each involve (i) sequencing quality control, (ii) possible genome assembly, (iii) variant calling and (iv) subtyping based on variants. Quality control tools can be automated by in-house pipelines [16], allowing the biologist to focus on downstream analysis. Whole genome mapping [17] and *de novo* assembly [18] tools are increasing in flexibility and speed. But phylogenetic and subtyping results can be estimated without the need for these time consuming steps, using tools including: k-mer based core- and pan-genome SNP detection (e.g., kSNP [19]); de Bruijn graph approaches to SNP, indel, and larger variant detection (e.g., Cortex [20]); and read mapping to only targets of interest for *in silico* MLST and gene detection (e.g., SRST2 [21]) and niche applications such as *Salmonella* serotype determination [22]. Whole-genome MLST, implemented in the Bacterial Isolate Genome Sequence Database (BIGSdb) [23] and applied to *Campylobacter* [24], provides an intermediate between highly discriminatory, less standardized whole-genome SNP-based phylogenetics and highly standardized, less discriminatory MLST. Other comparative genomics functions that have been scaled to the analysis of multiple microbial genomes include: annotation [25,26], mobile element detection [27,28] and recombination detection [29-31].

To facilitate adoption of modern bioinformatics tools, graphical, often web-based, user interfaces are allowing biologists without extensive programming skills to apply modern algorithms to their data.

Examples include: the web-based generalist software Galaxy (<http://galaxyproject.org/>, [15,32]) specific bacterial WGS analyses hosted by the Center for Genomic Epidemiology (<http://www.genomicepidemiology.org/>), tools from sequencer companies (e.g. Basespace from Illumina, <http://basespace.illumina.com/>) and programs from software companies (e.g. Geneious <http://www.geneious.com> and Bionumerics <http://www.applied-maths.com/bionumerics>). While user interfaces are appropriate for analysis of single or small numbers of genomes, they are not yet well suited to high-throughput analyses due to challenges of moving large amounts of data between servers and the lack of analysis flexibility. Still, it is likely that cloud-based data storage and computationally less expensive analysis pipelines for food safety will be developed in the near future, as has been envisioned for clinical molecular diagnostics [33].

Outbreak detection and surveillance using WGS.

Retrospective and pilot studies on use of WGS to characterize bacterial foodborne pathogens associated with outbreaks or surveillance efforts have been published since approximately 2011 [34]. WGS-based subtyping provides for improved subtype discrimination over PFGE and other subtyping methods, which is particularly important for clonal pathogens. Work is ongoing to adapt WGS analyses to real-time outbreak detection and surveillance.

Two studies published in 2014 [35,36] specifically explored the use of WGS to provide improved subtype discrimination for *Salmonella enterica* serotype Enteritidis. This serotype represents a particularly important target for WGS-based subtyping as approx. 50% of human serotype Enteritidis isolates in the U.S. represent a single XbaI PFGE type with 85% of isolates classified into 5 PFGE types [36]. The study by den Bakker et al. [36] specifically showed that WGS will allow for improved detection of serotype Enteritidis outbreaks. Future, broad based implementation of WGS-based subtyping for *Salmonella* and

specifically *Salmonella* Enteritidis, is thus likely to substantially increase the number of *Salmonella* outbreaks that are detected and recognized.

WGS also addresses the issue of “overdiscrimination” by PFGE; isolates that share a very recent common ancestor can show different PFGE patterns, which typically are caused by gain or loss of mobile elements such as prophage or plasmids (M Stasiewicz, PhD thesis, Cornell University, 2015). For example, isolates with two distinct PFGE patterns were linked to a listeriosis outbreak that occurred in Canada in 2008; WGS showed that isolates with these two PFGE types were closely related, likely shared a recent common ancestor, and a prophage accounted for the difference in PFGE type [37]. Similarly, the difference of PFGE type between two *L. monocytogenes* isolates linked to a listeriosis outbreak from cantaloupe was likely related to the gain or loss of a comK prophage [38]. Finally, variation in the presence or absence of distinct plasmids was related to variation in PFGE types among isolates from two listeriosis outbreaks linked to turkey deli meat, one in 1998-1999 and one in 2002 [39].

Routine use of WGS for surveillance has been implemented in 2013; in September of that year the U.S. CDC announced that all human *L. monocytogenes* isolates collected in the U.S. would be characterized by WGS [40]. Since implementation of routine WGS of human *L. monocytogenes* isolates, a number of small and medium size listeriosis outbreaks have been detected, ostensibly facilitated by use of WGS [41-45]. Two important features emerge from these outbreaks. First, retrospective WGS of isolates from 5 listeriosis cases from up to 5 years prior to a 2015 outbreak in ice cream was able to confirm those cases were related to the ongoing outbreak [42]. Second, both an outbreak linked to soft cheese [45] and one linked to bean sprouts [44] were initially identified not by classical epidemiological approaches but by comparing the WGS of *L. monocytogenes* from food samples collected during routine FDA inspection, and subsequent re-inspection, to routinely collected WGS of clinical *L. monocytogenes* isolates.

Epidemiologic surveillance of foodborne pathogens using WGS will continue to expand. While the U.S. FDA GenomeTrakr project

(<http://www.fda.gov/Food/FoodScienceResearch/WholeGenomeSequencingProgramWGS/>) began sequencing *L. monocytogenes* and *Salmonella* isolates obtained by regulatory agencies from foods and food processing plants, current efforts are expanding the network to cover (i) additional foodborne pathogens, *E. coli*, *Campylobacter*, *Vibrio*, *Cronobacter*, etc., and (ii) international scope, with labs in Argentina, Ireland, the U.K., Turkey, Belgium, and Mexico (as of 5/10/15, <http://www.ncbi.nlm.nih.gov/bioproject> query 'genometrakr').

WGS beyond bacterial foodborne pathogens

While the majority of applications of WGS in food safety have focused on bacterial pathogens, some work has been reported on application of WGS beyond bacterial foodborne pathogens. For example, WGS of *Cryptosporidium parvum*, *C. hominis* and *C. muris* have been reported [46-48]. Routine WGS of foodborne parasites, however, is not yet used for surveillance or outbreak investigations [1]. That previous review, published in 2014 [1], also summarized applications of WGS for detection and characterization of viral pathogens; in this case WGS is often used in metagenomics applications, including sequencing of total viral nucleic acids. In one outbreak, WGS of norovirus cDNA not only allowed successful delineation of the scope of a nosocomial outbreak, but phylogenetic analyses also allowed for establishment of the likely direction of transmission between two patients [49]. Continued development of whole-genome deep-sequencing methods for noroviruses and other viruses will provide approaches that allow for routine WGS-based virus surveillance [50], similar to WGS-based surveillance systems for bacterial pathogens.

Other omics approaches in food safety

Omics approaches other than genomics and WGS also are important tools and technology platforms in food safety, but are more typically used in research applications and have not yet moved to routine application to the same extent as WGS. As recently reviewed [1], metagenomics-based characterization of microbial communities provides a very promising and powerful approach to food safety testing, which allows for culture-independent identification, from food and environmental samples and clinical specimens, of non-culturable and difficult to detect foodborne disease causing agents [51]. Of note, the term metagenomics is often used to refer to two distinct approaches, including (i) 16S rRNA gene amplicon microbiome sequencing as well as (ii) “true” metagenomic sequencing where all DNA in a sample is sequenced. Potential concerns associated with the use of metagenomic approaches to detection of foodborne pathogens include the possibility of positive signals for pathogens due to presence of DNA in the absence of life organisms. In addition, 16S rRNA sequencing may not have the discriminatory power to reliably differentiate closely related pathogens and non-pathogens (e.g., *L. monocytogenes* and *L. innocua* [52]) and “true” metagenomic sequence assembly pipelines and analysis tools may also lead to misleading identification of organisms and pathogens that are not truly represent in a sample (see <http://nickloman.github.io/2015/02/11/metagenomics-best-hit-analysis-caveat-emptor/>). While metagenomics tools thus show considerable potential for food safety applications, further development efforts are needed to fully realize the potential of these methods.

Transcriptomics, proteomics, and metabolomics approaches also have potential for food safety applications [1,2], but food safety applications are largely limited to research on foodborne pathogen biology, including research on development and discovery of new control strategies. For further details on metabolomics and transcriptomics tools and their application to food safety, see reviews by Castro-Puyana and Herrero [53] and Valdés et al. [54], respectively. These approaches have the potential though to allow for characterization of the physiological state of pathogens in foods, which may lead to paradigm shifting approaches to food safety, where assessment of the physiological state of a pathogen

in a food is considered when assessing to human health hazard posed by an organism present in a given food.

Conclusion

Whole genome sequencing has already transformed foodborne disease outbreak detection. Improved subtyping methods are able to detect increasingly smaller outbreaks and collaborations between clinical (CDC) and regulatory (FDA) agencies have allowed for outbreaks to be identified through retrospective analysis of isolates collected during regulatory actions. Further improvements in genome sequencing technology and bioinformatics tools will allow food safety researchers to concentrate on asking critical biological questions rather than grappling with complex data sets. Omics approaches in food safety will continue to move beyond descriptive questions, such as how closely related one pathogen to another, to functional questions, such as how pathogens interact with their microbial and food matrix environment and how that knowledge can drive improvements in food safety.

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Figure Legend

Figure 1. Flow diagram of current omics approaches in food safety, with an emphasis on whole genome sequencing of bacterial foodborne pathogens for improved surveillance. Each box groups a major class

of inputs or outputs, with specific elements of those classes listed in smaller text. Information flows between elements are indicated by directional arrows.

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Figure