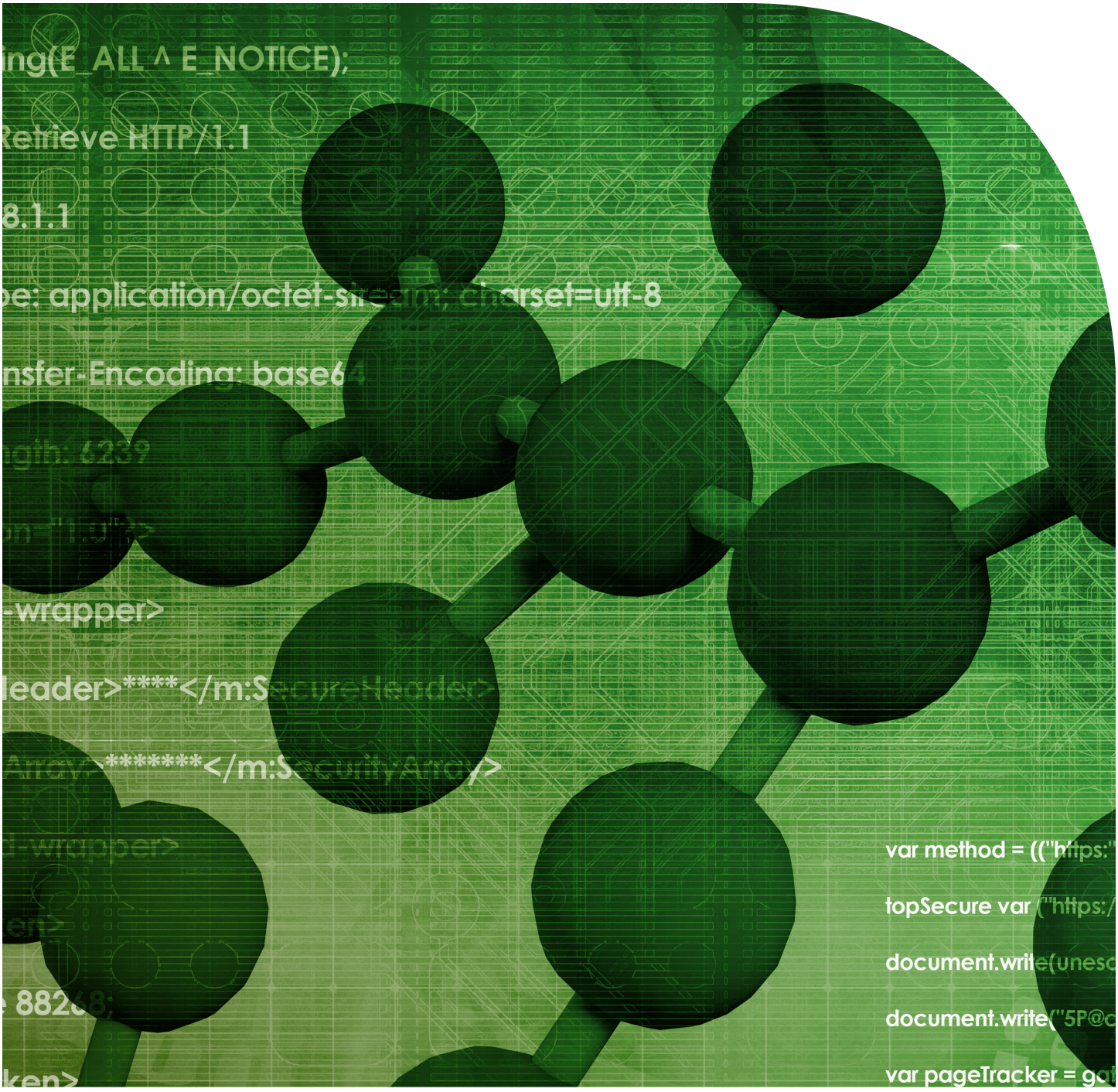


Ribotyping Easily Fills in for Whole Genome Sequencing to Characterize Food-borne Pathogens

David Sistanich
Technical Support Specialist
Hygiena, LLC

Whole genome sequencing (WGS) has become the ultimate test for determining the genetic profile of an organism. Its ability to display the order of every base pair in an entire length of DNA (the whole genome) promises to easily and flawlessly present every part of the genome for analysis, and to determine an organism's exact identification and function (including the ability to cause disease).

However, this promise remains elusive, because:

- We don't yet know the function of every nucleotide base pair of DNA in a genome, so identifying the order of DNA with unknown function isn't helpful for identifying pathogens.
- The cost of WGS (including data storage and analysis) is still beyond the reach of most facilities.
- It requires advanced training and expertise in sequencing and data analysis, which most food testing labs do not have or cannot afford.
- Few genomes are mapped this precisely, so false-negative results from attempts to match with known reference pathogens are still likely.
- Analysis can require submission to databases shared with regulatory agencies, to which food processors may have some sensitivity.

Largely because of these limitations, WGS has not been adopted by most companies to monitor food safety.

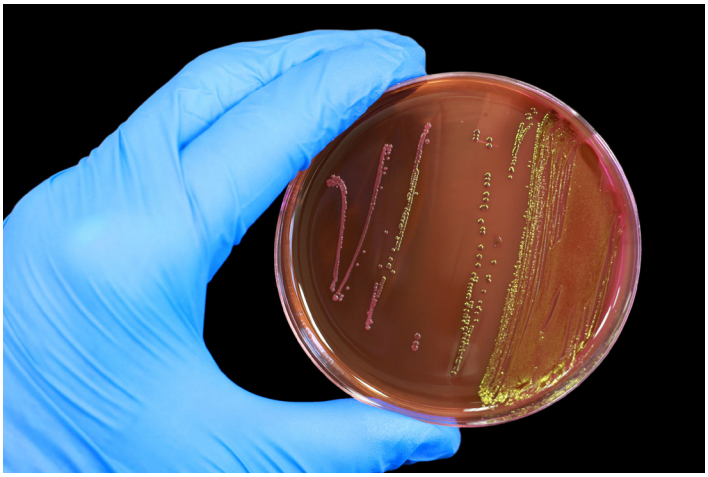
The US Food and Drug Administration (FDA) and the US Department of Agriculture's Food Safety and Inspection Service (USDA/FSIS) have begun using WGS in their own labs, to "perform foodborne pathogen identification during foodborne illness outbreaks," and "identify

pathogens isolated from food or environmental samples," the FDA explains on its website, as well as to identify genes resistant to antibiotics and heat. The agencies have also developed an online system, called GenomeTrakr, a national network of government and academic laboratories that collects information about pathogens and checks other databases to look for links between outbreaks.

So far, no US agency requires that food processors adopt WGS, but, the agencies suggest that food processors and retail outlets use some type of microbial analysis method that can rapidly identify the genomic background of a potential foodborne pathogen. The United Nations' Food and Agriculture Organization (FAO), meanwhile, also advocates WGS, but acknowledges its challenges: "While WGS can significantly contribute to improving food safety management, it still relies on the appropriate interpretation of laboratory data in the context of epidemiological evidence; WGS alone will not suffice," the organization wrote in a 2016 technical background paper.

What does WGS do?

When "next-generation sequencing" was introduced around 2009, most laboratories focused on "exome" sequencing, which concentrated on the much smaller sequences of the genome (known as exons) that express proteins. Today, whole genome sequencing is the ultimate goal of "next-generation sequencing," in which DNA from an organism is rapidly analyzed and the order of the base pairs determined. Sequencing is now automated, and large volumes of genomic data are produced to look for normal sequences, mutations and other genotype anomalies. These data can then be compared to other databases worldwide to determine a pathogen's exact origin, strain and potential for causing severe illness. By tracing foodborne illness back to the source in this way, outbreaks and recalls can be stifled if not prevented completely.



What's the value to food processors?

While food processors, federal investigators, and epidemiologists share the same goal in maintaining a safe food supply, they don't necessarily serve the same functions or share the same needs. Food processors may not need to see the entire genome of an organism to make food safety decisions. The most practical application of WGS for a food processor could be tracking strains of contaminating microorganisms in the plant and differentiating resident strains from transient ones. This also gives another tool to manage incoming ingredients providing more detailed information about the strain. Knowing where and when a particular strain may have been encountered previously equips the food processor with powerful information that can potentially expedite what might otherwise be a lengthy and costly investigation.

What alternatives to WGS are available?

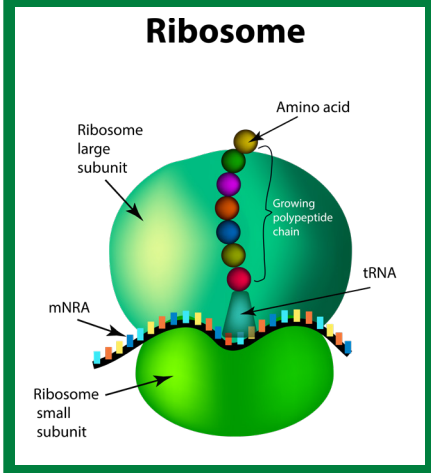
Non-WGS techniques have been recommended by the FDA and FAO. One technique, called pulsed-field gel electrophoresis (PFGE), is mentioned frequently in FDA and USDA literature, but the technique doesn't always discriminate for some strains of bacteria like *Salmonella* species (spp.).

Another popular technique is "DNA fingerprinting," or "automated ribotyping," using DNA fragments from the region of the genome that encodes for rRNA (ribosomal RNA) production to make a precise determination of gene sequences that identify organisms down to the strain level. Instruments like Hygiena's RiboPrinter® System analyzes 5s, 16s, and 23s DNA sequences from the rRNA operon of the genome and the flanking areas, in order to identify (and also rule out) organisms with strain level differentiation. While the RiboPrinter® System targets a smaller section of the genome than PFGE, PFGE techniques are highly manual and time-consuming, opening the window to error and less reproducibility.

Ribotyping combines several traditional microbiology techniques, automated processes and data analysis, and molecular genetics of bacteria:

Ribosomal RNA (rRNA) Operon Analysis (ribotyping)

A cell's ribosome is where proteins are created. These structures, which resemble a clenched fist, are shaped by ribosomal RNA (rRNA), which also enable the assembly of proteins. Bacteria rely on three distinct genes that encode for rRNA production: 5s, 16s, and 23s. The advantage for genetics analysis is that the DNA making up these three rRNA sequences is conserved–it has been around for hundreds of millions of years, and most of the nucleotide sequences are the same within a specific genus and species (particularly the 16s gene). Other DNA sequences located between those coding for rRNA, known as flanking or spacer sequences, are much more variable between bacterial species. By comparing DNA fragments generated from conserved and variable parts of a genome, it becomes much easier to precisely determine the species and strain (also known as a subtype) of bacteria.



Cell Culture

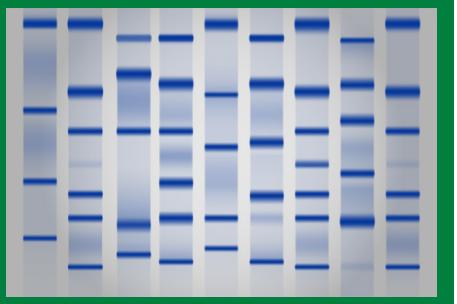
The first step taken toward ribotyping is an age-old, tried and true procedure; cell culture, and more specifically an isolation streak to ensure pure culture. Samples are taken from a target surface, and allowed to grow to create colonies of bacteria, in culture in a lab. For the RiboPrinter® System, this is the only step that takes place outside the system. The technique is simple and easy to learn. Loading the instrument is intuitive and guided by the onboard software.

Digestion, Restriction Enzymes

Performed automatically in the RiboPrinter® System, colonies are suspended in a buffer solution and broken apart (lysed), which frees DNA from the cells. The freed DNA is then treated with restriction enzymes, such as *Eco*RI or *Pvu*II, which target extremely specific points along a DNA sequence and break up (digest) the DNA strands. These DNA strands are then entered into a gel for electrophoresis.

Gel Electrophoresis and Southern Blots

Once digestion is complete, fragments of DNA are collected and transferred through the gel by electrophoresis. An electric charge is run through the gel, which separate the DNA fragments based on size.



The DNA is run completely through the gel and transferred directly to a nylon membrane that is moving at the same speed as the gel fragments, then it is visualized by a Southern Blot. The Southern blot is a traditional technique named after its inventor and has been used for more than 50 years. The membrane is washed in a probe that is specific to the gene fragments of the rRNA Operon and flanking regions. It is chemically labeled so that a conjugate will stick to it that we will use to visualize the DNA. Unlike traditional Southern blots which use radioactivity to detect DNA fragments, the RiboPrinter® System uses non-radioactive, chemiluminescent detection. This blot creates the familiar band patterns that can be used to compare bacterial samples against each other. This entire process also is automated in the RiboPrinter® System.

Database comparison

Whole genome sequencing can produce copious amounts of data, usually requiring sophisticated (and currently, expensive) analysis by well-trained laboratory personnel. Databases like PulseNet and GenomeTrakr are valuable for providing references against which samples can be compared; however, other voluntarily (and anonymous) databases around the world, like Cornell University's Food Microbe Tracker, are equally gathering vital information about food pathogens.

The RiboPrinter® System also can be compared against thousands of samples of pathogenic organisms. For each sample, a RiboPrint™ pattern is extracted from image data. This pattern is compared to other RiboPrint™ patterns and stored in the system to help identify the sample. The onboard database can compare a sample against 8,500 other patterns, covering 1,700 species. The RiboPrinter® System software also compares all of a user's samples to each other, clustering the user's own data into subspecies or strain level patterns, which the software calls "Ribogroups." Thus, a historical strain-level database is created that allows users to track and trend isolate data over time, much like a personal reference database.

Source	Number	Label	RiboPrint™ Pattern 1 kbp 5 10 15 50
End Product	DUP-16836	Bacillus subtilis	
Hands	DUP-12545	Bacillus subtilis	
Discharge Line	DUP-18405	Bacillus subtilis	
ATCC 6051	DUP-9501	Bacillus subtilis	
Raw material 473	DUP-18116	Bacillus subtilis	
Raw material 811	DUP-16836	Bacillus subtilis	
Bench surface 92	DUP-18513	Bacillus subtilis	

ID Label	Sample Comment	Isolated From	Similarity to RiboGroup Environmental 999-281-S-2	RiboPrint™ Pattern 1 kbp 5 10 15 50
Bacillus thuringiensis	Sterility Failure	Final Product	0.99	
Bacillus thuringiensis	EM Isolate	Fill Line	0.99	
Bacillus thuringiensis	Raw Material	LN# 903	0.81	

Conclusion

Whole genome sequencing is a powerful technique. It can address a wider range of microbes and genetic changes than can any other technology, and is best for conducting epidemiological studies aimed at determining the source of a widespread outbreak. But a September 2017 poll of 100 food processors by *Food Safety Magazine* showed that 93 percent of responding companies said they would not be using WGS. One reason for this reluctance is cost, another is the expertise needed not only to run the sequencing test but also to analyze the data, using current bioinformatics techniques. A third reason is that WGS may be too successful at identification. Any data uncovered would be legally discoverable and reportable to the FDA, whether it ultimately ends up pointing to a pathogen or not.

Ribotyping using the RiboPrinter® System is simple and automated enough that users do not need expertise in its underlying techniques. It is a powerful, cost-effective and labor saving addition to any microbial analysis and could provide a valuable alternative to a WGS laboratory as an important component of an overall food safety plan to protect the world's food supply against pathogens. These ever-evolving technologies are allowing government agencies to do their job of identifying potential worldwide outbreaks, and for food processors to do their job of protecting their supply chains, improve their food safety plans and ultimately protect consumers.

David Sistanich is a technical support specialist for Hygiena. He has more than 15 years' experience as a molecular and microbiologist, quality control scientist and sales support specialist.