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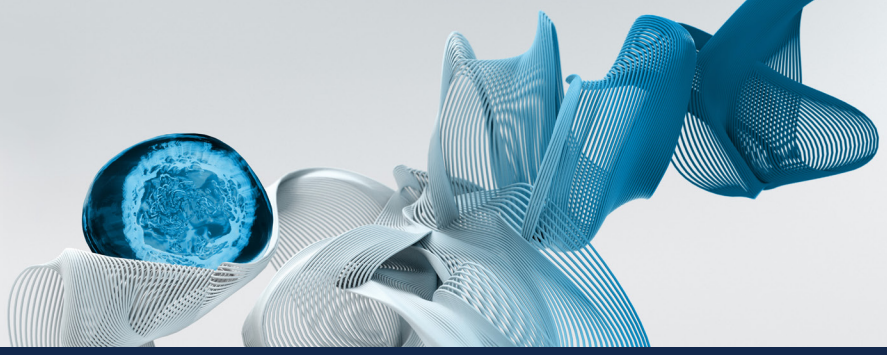

Technical Note

ResolveDNA Microbiome

Cells explored. Answers revealed.

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Genomic Analysis of Single Bacteria using ResolveDNA Microbiome

Bacteria make up the bulk of life on earth, and it is estimated that humans are aware of only a small percentage of microbes. Even with our limited knowledge of bacterial diversity around us, ground breaking scientific discoveries have been made possible because of the bacteria around us, including Taq polymerase, restriction endonucleases, and CRISPR gene editing technology. Additionally, the more we learn about the symbiotic relationship between humans and bacteria, the more we appreciate the profound impact bacteria have on human health.

Major advances have been made studying bacterial populations and selected strains. However, for technical reasons, it previously has been difficult to investigate bacterial heterogeneity on a whole genome scale. BioSkryb Genomics has developed a novel product, ResolveDNA Microbiome, which utilizes Primary Template-directed Amplification (PTA) technology to amplify DNA from a single bacterium with quality rivaling bulk DNA. This technology unlocks the near-complete genome of a single bacterium, enabling myriad new applications, including, but not limited to, understanding diversity within bacterial strains, within environments, and novel discovery of difficult-to-culture specimens (Figure 1).

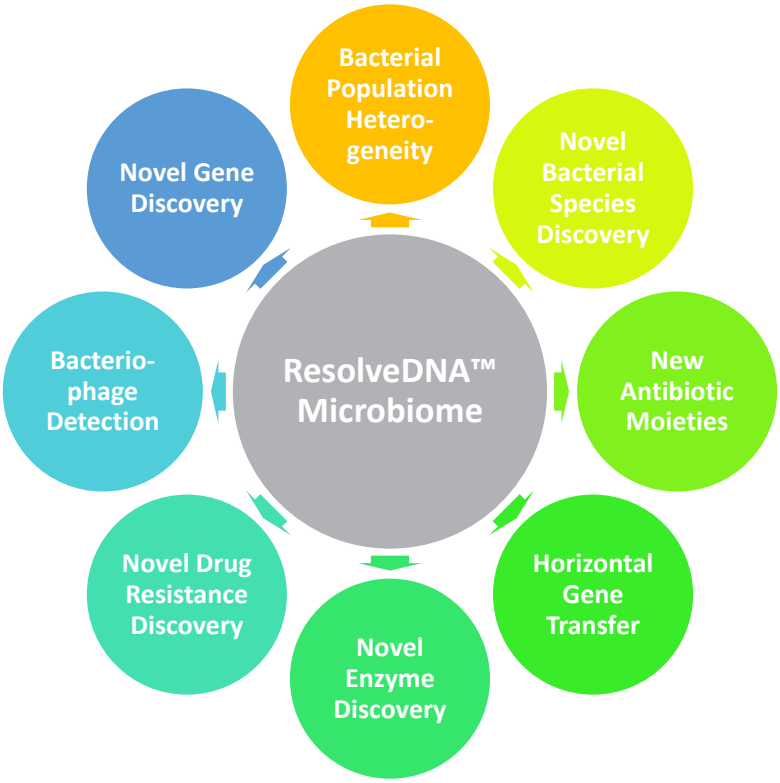


Figure 1. ResolveDNA Microbiome: Bacteria populate virtually every ecosystem known. Only a small fraction of bacterial species are characterized. The diversity of bacteria influence and shape their environments, and have signficant human health impacts. ResolveDNA Microbiome can address a diverse set of biological questions that can lead to significant insights and impact.

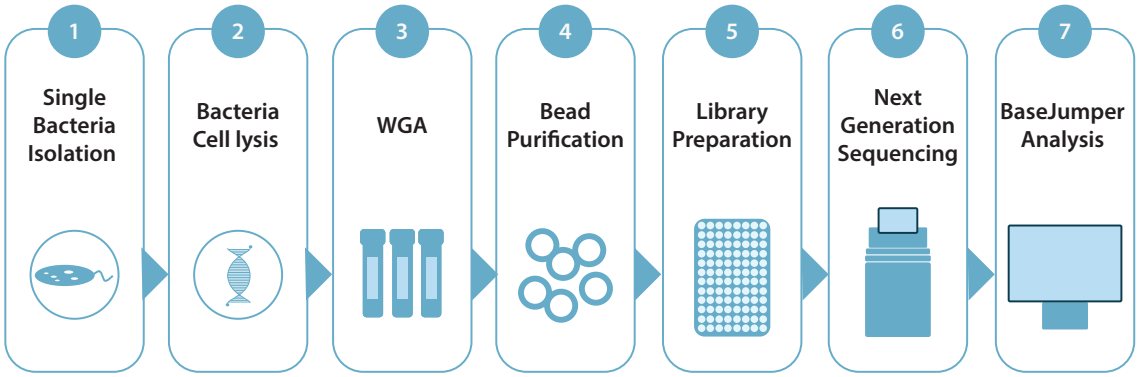


Figure 2. ResolveDNA Microbiome workflow. Single cells are first isolated by FACS, followed by efficient lysis of either gram positive or gram negative bacteria. Lysed cells, containing ~5-10 fg of genomic DNA then undergo Primary Template-directed Amplification. Amplified DNA from the reaction is then purified and quantified, followed by ligation or tagmentation-based library preparation, sequencing, and analysis with BaseJumper.

Introduction

Microorganisms, the most diverse group of life on Earth[1] play crucial roles in the biochemical cycling on earth. They were the first life forms to appear on Earth, and are present in virtually all habitats on the planet. Bacteria grow in soil, acidic hot springs, radioactive waste, water, and deep in the Earth's crust. In addition, they grow in organic matter and the live bodies of plants and animals, and scientists are increasingly appreciating both the symbiosis and pathogenesis between bacteria and human health.

Beyond understanding our environment and the microbes that inhabit it, microbiology has a long and storied history of driving our understanding of fundamental biology, and of developing biological tools. Virtually all of molecular biology owes the development of its manipulatory methods to microbiology research, from restriction endonucleases to PCR to CRISPR. Taken together, it is clear that delineating microbial biodiversity[2] is key to not only predicting ecosystem responses to environmental changes[3], but also improving bioprocesses, discovering novel biological mechanisms and tools, understanding human health, and the elucidation of new therapeutic biomolecules.

In the eukaryotic realm, the development of single-cell analysis methodology over the past decade has revolutionized our understanding of the heterogeneity and interconnectedness of the cellular microenvironment[4-6]. For largely technical reasons, this revolution has not extended in the bacterial realm to the same degree. Since the crux of each cell is its genome, the capability to further resolve the bacterial diversity relies on the ability to decipher the individual genomes of these cells. Up until now, a bottleneck was the keystone technology to faithfully prepare the single bacterial cell for next generation sequencing analysis. To enable this capability we have developed the ResolveDNA microbiome system to allow amplification, library creation and sequencing analysis of individual bacterial cells (Figure 2). The ability to interrogate the genomes of individual bacteria allows a wide expansion of application development in the discovery of new functional elements of the microbiome[7-8].

Materials and Methods

Single Bacteria Isolation. Three sample types were isolated 1) gram negative bacteria (*E.coli*), 2) gram positive bacteria (*B. subtilis*), and 3) a mixture of these species, to evaluate the performance of the ResolveDNA Microbiome WGA Kit.

Bacterial stocks of each species were grown in LB broth for 24 hours at 37°C. Afterwards, cultures were filtered through a 20 µm filter to remove large cell clusters and counted by OD measurement, then an aliquot of each sample was mixed to create a 1:1 cell ratio. Samples were centrifuged, resuspended in 1X dPBS, and live/dead stained using Syto9/PI. Single-cell bacteria were sorted into 96-well plates containing 1 µL ResolveDNA Cell Buffer using a Sony SH800 sorter equipped with a 130um sorting chip. Sorted plates were briefly vortexed and flash frozen and stored at -80C until ready to perform PTA WGA.

DNA Amplification with the ResolveDNA Microbiome WGA Kit. The ResolveDNA Microbiome protocol was followed, resulting in amplified bacterial DNA. This DNA was then purified with the ResolveDNA bead purification kit. We found individual bacteria typically yielded ~100-200 ng of amplified DNA (Figure 3A) and had an average size range of 900 bases (Figure 3B). Purified amplified DNA was then transformed into sequencing libraries using the ResolveDNA Library Preparation kit. Sequencing libraries were again purified and analyzed by TapeStation 4200 (Agilent) which demonstrated optimal size of PTA amplified single bacterial genome libraries (~500bp) (Figure 3C). These libraries were then sequenced using the Illumina MiniSeq Platform. Raw sequencing data were analyzed for quality, contig assembly, and alignment.

Results

Analysis of single bacteria demonstrated the ability to detect nearly the entire bacterial genome from each individual cell. We found both bacteria species tested approached the theoretical maximum genome size (Figure 4 Column A&B). For this analysis approximately 20 of each bacteria were sequenced (5 representative shown - figure 4). We noted minor differences in coverage, single cell genome of *E. coli* typically covered ~ 90-95% of the genome, where single *B. subtilis* detected up to

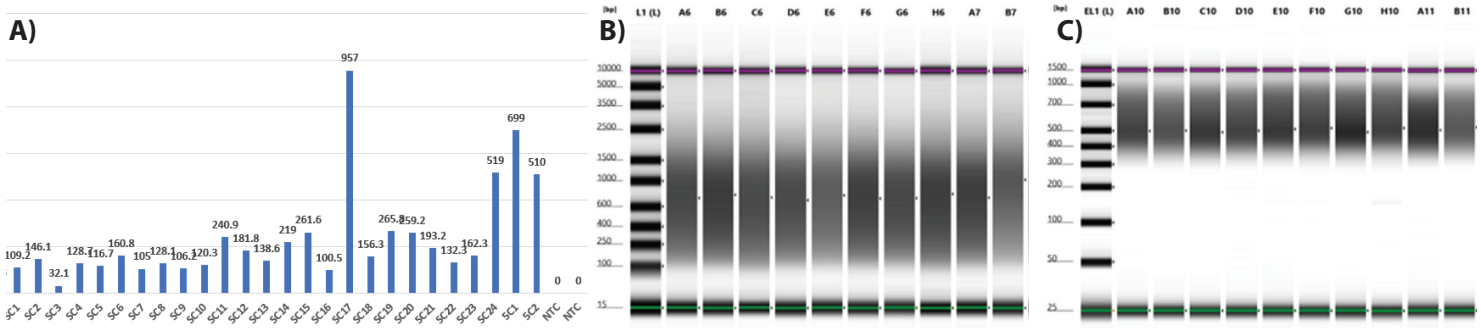
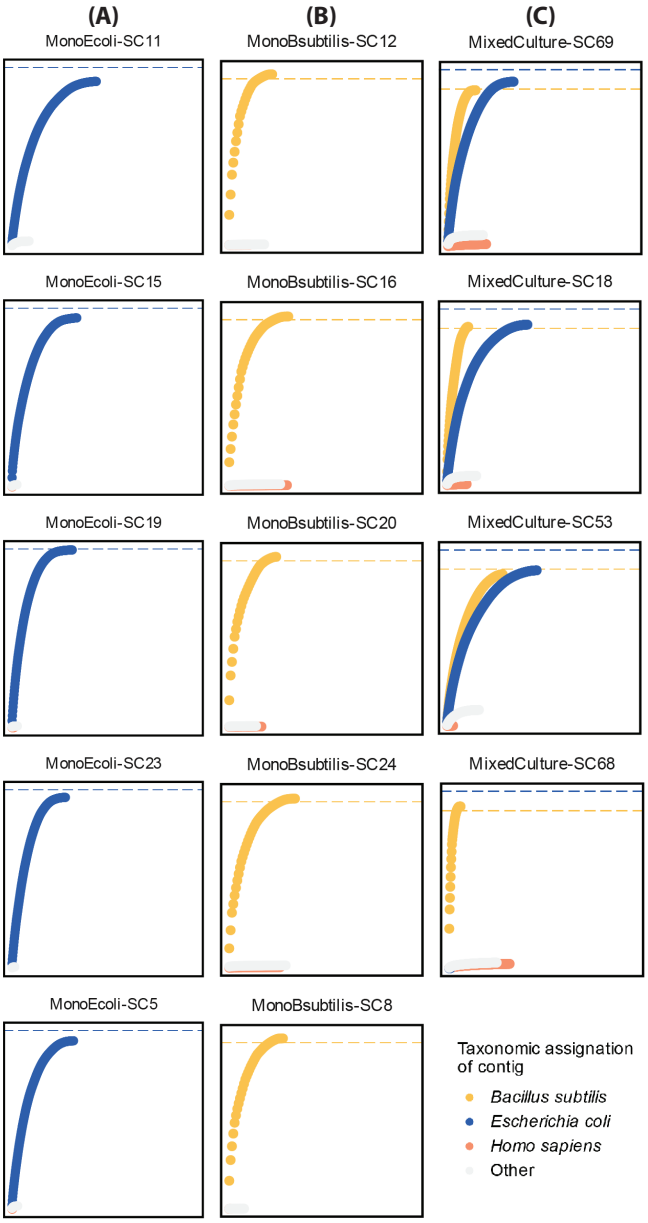


Figure 3. ResolveDNA Microbiome single bacterial cell amplification and library preparation: isolated single bacteria were amplified, followed by bead based amplified DNA purification using the ResolveDNA bead purification system. We found the majority of single bacterial cells generated approximately 150 ng of amplified genomic DNA(A). To determine the size range of the amplification products were subjected to D5000 tapestation analysis (B). We found the range of amplification products centered ~900 bp, while the overall range appeared to extend from 100 to ~1500 BP, which is slightly smaller than observed for eukaryotic cells. Amplified DNA (~ 100ng) were then converted to sequencing libraries using th ResolveDNA library preparation kit. Insert size was then verified by TapeStation using the D1000 tape cassette. Libraries were then subjected to NGS analysis using the Illumina MiniSeq using a high output 300 cycle kit, generating 150 Bp, paired end reads.



100% of the theoretical genome. The data further detected minor components of other (unidentified) contigs, however these are considered to be minor contaminants, which based on the data do not affect the ability to identify the species/genus of origin.

Having sequenced these individual bacteria, a third set of samples was prepared where a combination of both the *E. coli* and *B. subtilis* were sorted into wells (Figure 4C). Again, greater than 20 samples were processed (4 representative samples shown). We found both bacterial families were detectable, with cumulative contigs the length of the respective genomes. In many cases it appears we dispensed more than one cell into each well, as both *E. coli* and *B. subtilis* genomes were detected, however, in the last panel only *B. subtilis* was detected, suggesting assay specificity. Trace amounts of human DNA and unidentified species DNA were detected in these samples, however the short contigs make these reads easy to identify and remove.

Collectively, the data provide evidence that minute numbers of bacteria can be separated, amplified and prepared for NGS analysis. Moreover, we demonstrate in a single well, multiple bacteria and their respective genomes can be differentiated using the ResolveDNA next generation sequencing system.

Conclusions

BioSkryb has developed a powerful approach to investigating diversity in the bacterial kingdom. Using the ResolveDNA Bacterial WGA kits, microbial diversity can be routinely assessed with the highest degree of resolution, the single bacterial cell. This enables the capability to delineate mixed populations and recover the entire genomes of these cells, to generate complete genomic profiles of unculturable bacteria, and to reveal the unique biology of the diversity of bacterial life.

Figure 4. ResolveDNA Microbiome performance. Individual bacterial cells were isolated by FACS, and amplified using the ResloveDNA Microbiome system. Amplified products were converted to sequencing libraries, and sequenced on an Illumina MiniSeq @ ~1 million reads/cell. The data demonstrate that > 90% of the single cell genomes of (A) E. coli and (B) B. subtilis cells are able to be reproducibly detected. In addition, mixed species experiments (C) further demonstrate the ability to discriminate multiple bacterial species in the same well.

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