Culture-independent approaches to assess microbial diversity in biofilms

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Background

Methods

Conclusion
Background

- **Cultivation in microbiology**
  - Medical microbiology
    - Koch’s postulates (1884)
    - Still the backbone of the FDA diagnostics
      - Few exceptions for a small number of pathogens
      - E.g. Shiga Toxin testing
  - Environmental microbiology
    - Sources:
      - Organic waste, oceans, soils

Terrestrial Bacteria from Agricultural Soils: Versatile Weapons against Aflatoxigenic Fungi
Background

• Cultivation: «Great plate anomaly»
  – Only < 1% of bacteria in natural ecosystems can be cultured (1980s)
  – Negative cultures from biofilms and biofilm infections
    • Otitis media
      – Often negative cultures
      – But positive PCR and confocal microscopy
  • Orthopedic infections
Background

• « Great plate anomaly »
  – Implications:
    • Little correlation between cultures and « real » population
    • Diversity remains largely unknown
    • Medicine: applying the appropriate therapy is difficult
Background

- Cultivation: labour-intensive and time-consuming

Endotracheal tube biofilms

Cayman islands

Pure cultures
Background

• Identification is often slow
  – Molecular: e.g. PCR
  – Biochemical tests
  – Often: results only after 48 h
    • Postponing identification
    • Medical microbiology: postponing treatment

Kligler’s medium

API strips
Background

• Need for faster, unbiased approaches!
• «Cultivation independent methods»
• Directly on DNA or on the biofilm
  – Sequencing: clone libraries and next-gen
  – Fingerprinting: DGGE, TGGE, SSCP, T-RFLP, ARISA
  – Hybridization: microarrays, SIP
  – Probing: FISH
Methods

DNA extraction
Sequencing
Fingerprinting
Hybridization

Directly on the biofilm

Probing
DNA extraction

- DNA extraction directly from the biofilm e.g. floating microbial mats

- DNA extraction from biofilm cells e.g. medical devices
DNA extraction from biofilm cells

- Biofilm removal
- Mechanical disruption of the biofilm without affecting the cells
  - Sonication
  - Vortex
  - Scraping
DNA extraction

• DNA extraction from biofilms
  – No cultivation: no bias
  – But

Extracellular matrix: slimy substances

Dead cells: DNA can persist for weeks (adding propidium monoazide will help)

Microbial diversity: DNA extraction from Gram+, Gram- and yeasts

Host matrix: inhibitory substances
DNA extraction

• Range of methods combining
  ‒ Enzymatic reactions
    • Lysozyme: peptidoglycan bonds
    • Proteinase K: protein degradation
    • RNAse: degradation of RNA
  ‒ Mechanical reactions
    • Bead beating: cell lysis
  ‒ Chemical reactions
    • SDS: denaturation of proteins
    • Chloroform/phenol
    • Ethanol/2-propanol: precipitation of DNA
DNA extraction

• Traditional methods vs commercial kits
  – General DNA extraction kit
  – Biofilm specific DNA extraction kit: PowerBiofilm™ DNA isolation kit
DNA extraction

• Traditional methods vs commercial kits
  – Commercials kits are time-saving but often more expensive
  – Yield, purity and integrity are often comparable

![Degradation](image1)

![No degradation](image2)

• Is there a superior method to extract DNA from biofilms?
  – No!
  – Choice depends on the sample, experience and the lab
Methods

DNA extraction

Sequencing
  Fingerprinting
  Hybridisation

Directly on the biofilm

Probing
Sequencing: clone libraries

1. Amplification of a specific gene e.g. 16S rRNA gene

- DNA extraction
- PCR
- Ligation
- Transformation

Store at -20°C/-80°C

Library of transformed E. coli cells

IDENTIFICATION

Sequencing

Amplification of the insert

Plasmid extraction
Sequencing: clone libraries

2. Shotgun sequencing: a more direct approach

Fragmentation

Mostly F’ plasmid; holding up larger DNA fragments

In silico analysis is more difficult
Sequencing 16S rRNA clone libraries is a popular method (1750 pubmed hits)
Sequencing

• Coverage
  – Coefficient: \( C = 1 - \frac{n}{N} \) (\( n \) = number of unique phylotypes; \( N \) = total library size)
  – Accumulation curves
    • Plotting the cumulative number of species (or OTUs) as a function of the number of sequences
    • Eveness is not examined

• Diversity
  – \( \alpha \) and \( \beta \) diversity
  – Diversity indices: diversity + eveness
  – Shannon diversity index
    • The more different types there are and the more equal that they are distributed, the more difficult to correctly predict the identity of the next sequence
  – Simpson index (D) and the Gini-Simpson index (1-D)
    • D (or 1-D): The probability that 2 members, taken randomly from the same dataset, are identical (or different)
Next-gen sequencing

• Next-generation: after Sanger sequencing (from 2004 on)

• « High-throughput, deep sequencing »
  – High-throughput: multiples of millions nucleotides/second ~ 1 human genome (30x) per day (Illumina, 2012)
  – Deep sequencing: coverage up to 1000x

• Range of methods
  – Sequencing by synthesis
    • Pyrosequencing (454)
    • Illumina sequencing
  – Sequencing by ligation
    • SOLiD sequencing
  – Ion semiconductor sequencing
  – Nanopore sequencing
  – Heliscope sequencing
Next-gen sequencing

- Combining different samples (multiplexing): ↓ costs
- Diversity can be examined by
  - Investigating amplified 16S rRNA genes
  - Whole genomes
Next-gen sequencing

• Millions of short reads
  – Illumina: 50-250 bp and up to 3 billions reads per run
  Need for « next-gen » *in silico* analysis

• 5 steps to identification
  – Undo the multiplexing
  – Remove primers, adapters
  – Quality control
    • Read length, coverage, trimming sequences
  – Alignment
  – Mapping and identification
    – BLAST
Next-gen sequencing

• ∞ ways to analyse
  – Linux, Windows
  – Parameters

  – Software:

  – Open-source:
Next-gen sequencing

• As for every sequencing method:
  – Calculate coverage and diversity indices
  – Construct accumulation curves

If necessary, analyse more sequences!
Next-gen sequencing

Evidence of Uncultivated Bacteria in the Adult Female Bladder


The human microbiota associated with overall health.

Xu X, Wang Z, Zhang X.
College of Light Industry and Food Sciences, South China University of Technology, Guangzhou, China.

Unravelling the bacterial diversity in the atmosphere

Isabella Gandolfi · Valentina Bertolini · Roberto Ambrosini · Giuseppina Bestetti · Andrea Franzetti

Will become (one of) the most used method(s) (528 hits: Next-gen sequencing bacteria)
Methods

DNA extraction
Sequencing
Fingerprinting
Hybridisation

Directly on the biofilm
Probing
Biofilm fingerprinting

• Sequence-based fingerprinting techniques
  – PCR-amplified fragments
    • 16S rRNA genes:
      – Hypervariable regions e.g. V3 region
      – rpoB gene
  – Separation of the amplicons by gel electrophoresis
  – Identification
    • Purification of fragments: sequencing
    • Comparison to reference patterns
Fingerprinting: DGGE

- Denaturing gradient gel electrophoresis

Biofilms

DNA extraction → PCR → Hypervariable V3 region of the 16S rRNA gene

GC-rich clamp at the 5’ primer (40 bp)
Optimizing resolution

Primers: F357-GC and R518
Fingerprinting: DGGE

• Denaturing gradient gel electrophoresis

Polyacrylamide gel with an increasing gradient of chemical denaturants (urea and formamide)

Degree of melting depends on the % GC in the DNA fragment
Fingerprinting: DGGE

- Analysis:
  - Band recognition, normalization, comparison
  - Software: e.g. GelCompar, BioNumerics, Fragment Analysis 1.1

- Indices
  - Pearson product-moment correlation coefficient
  - Diversity indices: Shannon’s diversity index

Diversity and relative abundance
Fingerprinting: DGGE

• Identification is possible
  – Comparing position of bands with reference bands
  – Purifying and sequencing bands

• Problems:
  – Sensitivity: > 1 % of the population
  – Heterogeneity of the 16S rRNA gene
    • 1 species: > 1 band
    • Expensive due to unnecessary sequencing
  – Discriminative power too low for certain species
    • 1 band: > 1 species

  Purify bands and run new DGGE with a narrower gradient of denaturants
Fingerprinting: TGGE

- **Temperature gradient** gel electrophoresis

Polyacrylamide gel with an increasing temperature gradient

Sequence-based separation

Gel electrophoresis
Fingerprinting: TGGE

• Temperature gradient is applied during electrophoresis
  – E.g. 39-48 °C, increasing 0.5°C per hour
  – E.g. 66-70°C, increasing 0.2°C per hour (high % GC organisms)

• Identification/problems: DGGE
**Evaluation of the bacterial distribution within the biofilm by denaturing gradient gel electrophoresis in the rat model of urinary catheters**

Hyun-Sop Choe · Hyun-Jung Kim · Seung-Ju Lee · Ji-Youl Lee · Sang-Seob Lee · Yong-Hyun Cho

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**Topical Effect of a Medically Prescribed Pediatric Antibiotic on Dental Biofilm: A Cross-Over, In Situ Study**

Viviane Santos da Silva Pierro1,*, Dennis de Carvalho Ferreira2, Hugo Emiliano de Jesus2, Alexandre Soares Rosado3, Ronir Raggio Luiz3, Kátia Regina Netto dos Santos3, Lucianne Cople Maia4

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**Dynamic changes in the microbial community composition in microbial fuel cells fed with sucrose**

Nelli J. Beecroft · Feng Zhao · John R. Varcoe · Robert C. T. Slade · Alfred E. Thumser · Claudio Avignone-Rossa

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**Characterization of bacterial communities exposed to Cr(III) and Pb(II) in submerged fixed-bed biofilms for groundwater treatment**

R. Vílchez · C. Gómez-Silván · J. Purswani · J. González-López · B. Rodelas

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DGGE & TGGE

DGGE: more frequently used than TGGE

4228 DGGE hits in Pubmed vs 131 TGGE hits
Fingerprinting: SSCP

- Single Strand Conformation Polymorphism
- PCR-amplified DNA fragments (e.g. 16S rRNA genes)

Chemical denaturation: formamide, urea
Physical denaturation: temperature
Cooled on ice and water
ssDNA: unique 3D folding depending on the sequence
Separation based on folding /sequence
Low concentrations of denaturants can be added
Fingerprinting: CE-SSCP

• Most gels have to run for ± 12-16h
  – ↓ high-throughput
• Capillary electrophoresis
  – Diameter: 20 – 200 µm
Fingerprinting: CE-SSCP

- Detection of the fluorescent fragments
  - Labeled primers
- ↑ High-throughput
- Superior to DGGE
  - Resolution is better
    - Higher diversity is detected
    - Less co-migration of bands
  - Significantly less « gel-to-gel » variation
- Faster
  - Less preparation and analysis time
Fingerprinting: T-RFLP

- Terminal restriction fragment length polymorphism
Fingerprinting: T-RFLP

- **False peaks:**
  - Background (noise)
    - Small, irreproducible peaks
    - Use a certain threshold
  - Pseudo terminal restriction fragments
    - Reproducible peaks, linear to the amount of DNA
    - (partly) Single stranded amplicons: no target for restriction enzymes
    - Use a exonuclease for ssDNA
Fingerprinting: T-RFLP/CE-SSCP

• Semi-quantitative profiles
  – Peak height/area

• Best suited for microbial communities with low to moderate richness
  – Up to 50 distinct organisms

• Identification
  – *In silico* predictions
  – Reference peaks
Homogeneity and Synchronous Dynamics of Microbial Communities in Particulate Biofilms: from Major Populations to Minor Groups

Gaëlle Gévaudan¹, Jérôme Hamelin¹, Patrick Dabert¹*, Jean-Jacques Godon¹*, and Nicolas Bernet¹

Microchip-Based Terminal Restriction Fragment Length Polymorphism for On-Site Analysis of Bacterial Communities in Freshwater

Nobuyasu Yamaguchi,* Syuhei Matsukawa, Yoko Shintome, Tomoaki Ichijo, and Masao Nasu

Graduate School of Pharmaceutical Sciences, Osaka University; 1–6 Yamada-oka, Suita, Osaka 565–0871, Japan. Received February 6, 2013; accepted May 14, 2013

Analysis of the Airway Microbiota of Healthy Individuals and Patients with Chronic Obstructive Pulmonary Disease by T-RFLP and Clone Sequencing

Tetyana Zakharkina¹, Elke Heinzl², Rembert A. Koczulla³, Timm Greulich³, Katharina Rentz¹, Josch K. Pauling⁴, Jan Baumbach⁴a, Mathias Herrmann², Christiane Grünewald⁵, Hendrik Dienemann⁵, Lutz von Müller², Robert Bals¹*
Fingerprinting: ARISA

• « Automatic Ribosomal Intergenic Spacer Analysis »

• Intergenic:
  – Non-coding region between the 16S and 23S rRNA gene
  – Different in length and sequence
  – Discrimination at the species level
  – Higher resolution than T-RFLP
  – Primers with fluorescent labels
Fingerprinting: ARISA

DNA extraction → PCR → Separation of the amplified fragment by CE

Fingerprint-associated identification
In silico prediction
Reference peaks

Works well for communities with low to moderate complexity
Microbial habitat connectivity across spatial scales and hydrothermal temperature gradients at Guaymas Basin

Stefanie Meyer*, Gunter Wegener*, Karen G. Lloyd®, Andreas Teske®, Antje Boetius* and Alban Ramette*

Microbiological Features and Bioactivity of a Fermented Manure Product (Preparation 500) Used in Biodynamic Agriculture

Giannattasio, Matteo¹, Elena Vendramin², Flavio Fornasier², Sara Alberghini¹, Marina Zanardo¹, Fabio Stellin¹, Giuseppe Concheri¹, Piergiorgio Stevanato¹, Andrea Ertani¹, Serenella Nardi¹, Valeria Rizzi³, Pietro Piffanelli³, Riccardo Spaccini³, Pierluigi Mazzei³, Alessandro Piccolo⁴, and Andrea Squartini¹*

Similarity of the ruminal bacteria across individual lactating cows

Elie Jami a,b, Itzhak Mizrahi a,*

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b Department of Molecular Microbiology and Biotechnology, The George S. Wise Faculty of Life Science, Tel Aviv University, Ramat-Aviv 69978, Israel
Methods

DNA extraction

Sequencing
Fingerprinting
Hybridization

Directly on the biofilm

Probing
Hybridization: Microarrays

• Solid surface with tens of thousands spotted probes

• DNA probes:
  – Genes
  – Oligonucleotides
  – cDNA

• Microarrays can be used to:
  – Measure changes in gene expression levels
  – Detect single nucleotide polymorphisms
  – Identify members of a community
Hybridization: Microarrays

PCR-amplified or fragmented Fluorescently labeled Microarray with fixed probes (e.g. 16S rRNA gene fragments)

Detection
Data acquisition and analysis
Statistics

Biofilms
DNA extraction
Hybridization
Hybridization: Microarrays

• Phylogenetic microarrays
  – Identification of microbial communities
  – E.g. PhyloChip: 16S rRNA gene probes
    • G3: 1.1 million DNA probes reflecting 50,000 microbial units including *Bacteria* and *Archaea*
  – E.g. Microbiota array
    • Human gastrointestinal microbiome
Hybridization: Microarrays

• Functional analysis
  – Gene expression
    • StressChip


StressChip as a High-Throughput Tool for Assessing Microbial Community Responses to Environmental Stresses.

• PathoChip


The PathoChip, a functional gene array for assessing pathogenic properties of diverse microbial communities.
Hybridization: Microarrays

• Limitations
  – Unable to detect novel species
  – Design, use and analysis: technically demanding and require extensive testing and validation

• Optimization
  – ~ 50 ng DNA
  – 15 -20 PCR amplification cycles
  – Cross hybridization
    • Leads to over-estimation of the diversity
    • Levels can be determined by validation tests
    • Algorithms to calculate the « true signal »
Hybridization: Microarrays

• Advantages
  – Good taxonomic resolution
  – High-throughput
  – Quantitative
  – High sensitivity (e.g. human intestinal array: 0.00025 %)
  – Moderate cost
  – Fast

• However, it is a good practice to confirm microarray data by other molecular techniques
  – qPCR, sequencing,...
Microarrays

Commonly used method: 994 Pubmed hits (identification only!)
Functional diversity of biofilms

- Study gene expression
- RNA extraction
  - Converted to cDNA
- Transcriptomics
  - Next-gen sequencing
  - Amplicons are compared to reference genomes
- Microarrays
  - PathoChip, StressChip
- qPCR
  - Specific genes
(Functional) diversity: SIP

- Stable isotope probe
- « Method by which specific functional groups of organisms that incorporate particular substrates are identified without cultivation »
- CsCl gradient separation
- Analysis
  - RNA: functional
  - DNA: phylogenetic
(Functional) diversity: SIP

The nitrite-oxidizing community in activated sludge from a municipal wastewater treatment plant determined by fatty acid methyl ester-stable isotope probing

Myriam Kruse\textsuperscript{a}, Sabine Zumbrägel\textsuperscript{b}, Evert Bakker\textsuperscript{c,1}, Eva Spieck\textsuperscript{b}, Till Eggers\textsuperscript{d,2}, André Lipski\textsuperscript{a,*}

Agricultural soil and drilosphere as reservoirs of new and unusual assimilators of 2,4-dichlorophenol carbon

SIP is often used in bioremediation studies!
Methods

DNA extraction

Sequencing
Fingerprinting
Hybridization

Directly on the biofilm

Probing
FISH
Fluorescent in situ Hybridization

Four basic steps:
1. Sample collection and fixation
2. Hybridization
3. Image acquisition
4. Image analysis
FISH

- FISH probes for nearly any microbial taxon
  - EU388: « universal » 16S rRNA gene probe
  - Fluorophores: Cy3, Cy5, Alexa fluor dyes

- FISH = an effective tool for identification, quantification and spatial distribution
  - Physical analysis e.g. cluster of cells

- Assessing diversity?
  - Very limited!
    - Only detecting what is searched for
    - Restricted to non-overlapping spectra of fluorophores
  - However ...

Population dynamics
FISH

• Recent improvements to study diversity of bacterial samples
  – Sequential FISH (July 2013)
    • 6 probes can be detected
  – CLASI-FISH (December 2012)
    • Combinatorial labeling and spectral imaging
    • Fluorophores with overlapping spectra
      – Each probes is labeled with 2 or more fluorophores
      – E.g. 6 fluorophores: 15 possible combinations
    • Spectral analysis: « computational linear unmixing »
      – Spectra of all fluorophores is known
FISH

• Recent improvements to study diversity of bacterial samples
• But still
  – Detection is limited to which probe(s) is(are) used
  – FISH is not suited to unravel/identify the diversity of complex bacterial populations
  – FISH is suited to study
    • The physical shape of biofilms
    • Dynamics of population
    • Specific groups of bacteria in a sample
FISH

Assessment of bacterial and structural dynamics in aerobic granular biofilms

David G. Weissbrod 1,2,*, Thomas R. Neu 4, Ute Kuhlicke 4, Yoan Rappaz 1 and Christof Holliger 1

Neutrophil Extracellular Traps and Bacterial Biofilms in Middle Ear Effusion of Children with Recurrent Acute Otitis Media – A Potential Treatment Target

Ruth B. Thornton 1,2,*, Selma P. Wiertsema 1,2, Lea-Ann S. Kirkham 1,2, Paul J. Rigby 3, Shyan Vijayasekaran 1,4,5, Harvey L. Coates 1,4,5, Peter C. Richmond 1,2

Biofilm formation and microbial activity in a biofilter system in the presence of MTBE, ETBE and TAME

Jessica Purswani a, Belén Juárez a,b, Belén Rodelas a,b, Jesús Gónzalez-López a,b, Clementina Pozo a,b,*
Background

Methods

Conclusion
Culture dependent vs culture independent

• Culture independent methods represent a major improvement to study microbial diversity (of biofilms)

• However, cultivation is still necessary
  – Description of novel species
  – Determination of biochemical traits
    • Antibiotic resistance
  – Processes mediated by bacteria
    • Bioremediation of contaminated soils

Culture dependent: 10274 hits
Culture independent: 5572 hits
Future perspectives

• Culture independent methods
  – New methods (e.g. single cell sequencing)
  – Optimization of the currently used cultivation methods
    • Better understanding of the microbial diversity
    • Specific adjustments

• Next-gen sequencing: the key method to unravel the diversity of microbial populations
Future challenges

• Culture dependent techniques
  – Develop new isolation methods
  – « Cultivating the unculturables »

• Culture independent techniques
  – Fast and high-throughput
  – Computational power
  – Bioinformatics: essential in modern microbiology
Take home message

• Culture independent approaches will never replace cultivation

• A whole range of culture independent techniques are available to study the diversity of a bacterial population
  – Selecting the « best » one depends on the aim of the study and the sample
  – Analyzing data is often challenging due to high requirements for computational power

• Combining culture dependent and independent methods is the best way to study the microbial diversity in biofilms
THANK YOU FOR YOUR ATTENTION