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Review article

Detection of bacterial pathogens in environmental samples using DNA microarrays

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Abstract

Polymerase chain reaction (PCR) is an important tool for pathogen detection, but historically, it has not been possible to accurately identify PCR products without sequencing, Southern blots, or dot-blots. Microarrays can be coupled with PCR where they serve as a set of parallel dot-blots to enhance product detection and identification. Microarrays are composed of many discretely located probes on a solid substrate such as glass. Each probe is composed of a sequence that is complimentary to a pathogen-specific gene sequence. PCR is used to amplify one or more genes and the products are then hybridized to the array to identify species-specific polymorphism within one or more genes. We illustrate this type of array using 16S rDNA probes suitable for distinguishing between several salmonid pathogens. We also describe the use of microarrays for direct detection of either RNA or DNA without the aid of PCR, although the sensitivity of these systems currently limits their application for pathogen detection. Finally, microarrays can also be used to ''fingerprint'' bacterial isolates and they can be used to identify diagnostic markers suitable for developing new PCR-based detection assays. We illustrate this type of array for subtyping an important food-borne pathogen, *Listeria monocytogenes*.

Keywords: Microarray; Comparative genomics; DNA hybridization; *Listeria*; *Renibacterium*

1. Introduction

Bacterial pathogens pose a significant threat to human, animal, and agricultural health. Consequently, considerable effort has been devoted to developing rapid, sensitive, and specific assays for these organisms. In this context, polymerase chain reaction (PCR) is the most important molecular tool because of the potential for detecting less than 10 copies of a specific gene within a complex sample. Conventional PCR assays incorporate a pair of oligonucleotide primers to amplify a specific gene that is then detected using agarose gel electrophoresis combined with an intercalating dye (e.g., ethidium bromide) and UV light. Electrophoresis makes it possible to estimate the length of the PCR product and length often serves as a proxy for product identification. More than one set of primers can be used in the PCR reaction to simultaneously detect several genes. These ''multi-

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plex'' assays, however, usually do not exceed a total of six primer sets because of limitations in the ability to resolve many fragments in agarose and because of the potential for generating nonspecific products that make interpretation difficult. If necessary, Southern blots, dot-blots, or sequencing can be used to positively identify specific PCR products. Other variations on this theme include detecting PCR products with automated sequencers (e.g., Keim et al., 2000) or using real-time PCR with fluorescent probes (e.g., Oberst et al., 1998). Both of these approaches are still limited by the number of primer sets that can be used in the reaction. One way around this limitation is to run a series of parallel, multiplex PCR assays to boost the number of genes that can be identified (e.g., Johnson and Stell, 2000).

DNA microarrays offer another means to enhance the detection capabilities of PCR. In this context, microarrays serve as a series of parallel dot-blots for rapid detection of sequence variation within a given locus (e.g., 16S rDNA) and for detection of multiple products resulting from multiplex PCR. Because product length is not a requirement for identification, PCR can be used to generate short products and because nonspecific products will not be detected with the array, more primers can be used in a multiplex reaction. This review will focus on using microarrays to detect and identify PCR products. Microarrays can also be used to directly detect nucleic acids without PCR, or to subtype (''fingerprint'') bacterial isolates, or to generate new diagnostic markers for PCR. We will briefly highlight these other applications because of their importance in development of pathogen assays.

2. Overview of DNA microarrays

Ye et al. (2001) provide a detailed overview of microarrays and applications in microbiology. In essence, DNA microarrays are reverse dot-blots for which sequence-specific ''probes'' are attached to a substrate in a lattice pattern (Schena, 2000). Probes appear as ''spots'' in the final image where each spot represents a unique probe sequence and spots are usually 100–200 μm in size and located within 200–500 μm of each other. ''Targets'' are applied to the array and targets that hybridize to complementary probes are detected using some type of reporter molecule. Probes are either polymerase chain reaction (PCR) products or oligonucleotides, the latter being either deposited mechanically on the substrate or constructed in situ using lithographic techniques (Lockhart et al., 1996). PCR products or oligonucleotides can be deposited on any number of substrates, with modified glass being the most common. There are a myriad of approaches to modifying slides and to attaching probes to the slide surfaces (Belosludtsev et al., 2001; Chrisey et al., 1996; Lindroos et al., 2001), but in many cases, simple adsorption of either PCR products or oligonucleotides is sufficient for array fabrication (Call et al., 2001a). With adsorption, probes do not require special modification (e.g., terminal amine), and consequently, the cost of array manufacture can be reduced considerably. Probes are typically deposited on glass surfaces using a contact printing system such as quill pins, solid pins, or ring-and-pins (Schena, 2000).

Once microarrays have been printed, targets are prepared for hybridization. Depending on the objective, targets may be PCR products, genomic DNA, total RNA, aRNA, cDNA, plasmid DNA, or oligonucleotides. In most cases, the targets incorporate either a fluorescent label or some other moiety such as biotin that permits subsequent detection with a secondary label. Direct or chemical labeling with Cy-3 and Cy-5 fluorescent dyes is the most common means for detecting targets on microarrays, particularly when competitive hybridizations are employed in expression analysis. There are many alternative fluorescent dyes suitable for target detection (e.g., Panchuk-Voloshina et al., 1999), and when only one channel is required for detection purposes, then conjugates of streptavidin with phycoerythrin or AlexaFluor 546 (Molecular Probes, Eugene, OR) have been employed to detect biotin-labeled targets. Biotin–streptavidin chemistry or similar hapten labeling schemes are much less expensive compared with incorporating conjugated nucleotides in the PCR or reverse transcriptase reactions (Alexandre et al., 2001), although indirect detection methods add additional steps to the detection process.

If signal from target-probe duplexes is too weak for direct detection methods, then enzymatic signal amplification can be used to boost array sensitivity. Two enzymatic systems that have been employed are enzyme-linked fluorescence (ELF-97; Molecular

probes) (Belosludtsev et al., 2001; Call et al., 2001a,b; Small et al., 2001) and tyramide signal amplification (TSA) (Karsten et al., 2002). For expression arrays, sensitivity can be increased enzymatically by amplifying the number of targets that are hybridized to the array (Eberwine, 1996; Puskás et al., 2002).

Once post-hybridization steps are completed, then arrays are imaged using a high-resolution scanner. These are laser- or filter-based systems that use specific light spectra to excite fluorescent molecules and collect the subsequent emission spectra using CCD cameras. Spatial resolution of these systems can be as low as of 3–5 μm, although 10 μm of resolution is sufficient for conventional microarrays. ELF-97 is an exception because this compound produces an exquisitely bright crystal that can be excited with a broad spectrum UV source and imaged with only minor adaptations to a CCD-based gel documentation camera (Call et al., 2001a). Single-channel imaging systems are adequate for detecting the presence of specific bacterial targets. Digital images can be analyzed with any number of commercial or freeware computer programs to ascertain spot intensities (see http://ihome.cuhk.edu.hk/~b400559/arraysoft.html for a useful collation of many freeware and commercial image processing and database products).

3. Pathogen detection

Any PCR-based detection system, while potentially very sensitive, must still confront a number of front-end challenges inherent with sampling complex substrates and this is true regardless of the method used to identify the PCR products (Chandler, 1998; Loge et al., 2002). For example, when using PCR to detect pathogens in water, a number of factors will reduce the sensitivity of the assay. A positive detection in this context is fairly simple to interpret but knowledge of assay sensitivity, which varies from sample to sample, is critical to interpreting negative results. That is, if sampling conditions adversely impact the assay, then a negative result may lack the sensitivity to detect a given pathogen at the minimum concentration that is known to present a health risk. Failure to account for this lack of sensitivity may result an incorrect conclusion about the health risks associated with a particular environment (e.g., water supply). Thus, sampling designs need to consider how much sample is processed, the efficiency of pathogen isolation, the efficiency of nucleic acid extraction, and the effect of coprecipitating factors that inhibit PCR (Loge et al., 2002). Several of these variables may also impact direct RNA and DNA detection methods described below (Sections 3.3 and 3.4). When proper controls are included in the overall assay design, it should be possible to develop fully automated, field deployable detectors that are capable of working with a variety of sample substrates (Bavykin et al., 2001; Chandler et al., 2000).

3.1. Microarrays as end-point detectors for PCR

When PCR is used to amplify DNA from environmental samples, then microarrays can be used to identify the amplified products (Fig. 1). For example, if highly conserved primer sequences are present (e.g., universal 16S rDNA primers), then PCR can be used to amplify bacterial DNA followed by hybridization to an array composed of pathogen-specific probes. The probes themselves are located within the polymorphic region that is flanked by the conserved primer sequences. In this way, a single PCR reaction

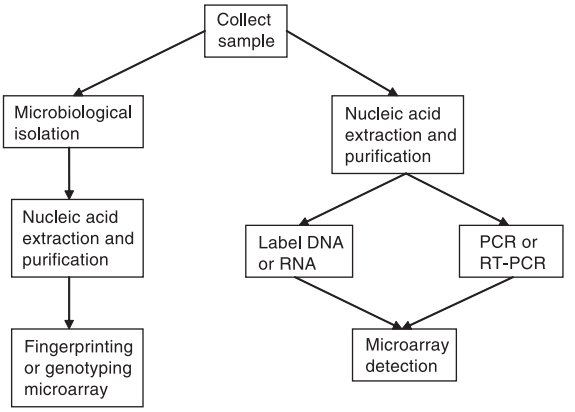

Fig. 1. Illustration of the multi-faceted role that DNA microarrays can have in pathogen detection. After sample collection, bacteria can be isolated using conventional microbiology and characterized using microarrays, or nucleic acids can be extracted from an environmental sample and PCR used to amplify targets for microarray detection. Mixed genome arrays can be used to fingerprint isolates and to discover new markers suitable for development of PCR assays. As with any detection scheme, sampling issues need to be considered with microarray detectors.

can be used to simultaneously detect different pathogens. Additionally, while conventional gel electrophoresis is limited by the number of multiplexed PCR primers that can be used (usually six sets), many more primer sets can be used when the reaction products are evaluated using a microarray detector (Call et al., 2001b). This is because microarrays are not limited to identification by product length. Indeed, shorter products hybridize more efficiently to the arrays and they are produced more efficiently by PCR. Spurious PCR products caused by mispriming are not a significant problem because only those sequences specific to the microarray probes will be detected. Spurious products, however, may reduce overall efficiency of the PCR reaction. It is also important to point out that the pathogen-specific sequences must be known before the array is designed. If a PCR product is present but does not hybridize to any existing probes, then sequencing will be needed to identify the product. Furthermore, as with all assays, microarray detectors should undergo validation experiments to confirm probe specificity. Fortunately, these experiments can be conducted fairly swiftly because each hybridization involving known strains of bacteria will produce specificity data for every probe on the array. Extensive testing against a panel of unknowns will require more time.

Guo et al. (1994) were the first to couple PCR amplification and microarray detection. They used oligonucleotide probes to identify five single nucleotide polymorphisms from the human tyrosinase gene. Rudi et al. (2000) fabricated a membrane-bound array of oligonucleotides for the 16S rDNA gene. After probe validation, the array was used to estimate relative concentrations of cyanobacteria from lake water. Call et al. (2001b) developed an oligonucleotide microarray suitable for detecting multiplex PCR products from enterohemorrhagic *Escherichia coli* (EHEC). A combination of immunomagnetic cell capture, PCR, and the microarray provided sufficient sensitivity to detect <100 CFU ml^{-1} in chicken rinsate. The array was also suitable for genotyping EHEC using a multiplex reaction for four virulence determinants. Chizhikov et al. (2001) produced a similar array for multiplexed PCR and six genes associated with food-borne pathogens. Their results highlighted both the utility of this format and demonstrated that detection of multiplexed products using gel electrophoresis can produce ambiguous results. In some instances, the presence of nonspecific PCR products made gel interpretation difficult, whereas microarray results were less likely to be confounded by the presence of nonspecific PCR products.

3.2. Example of 16s rDNA pathogen detection

In order to further evaluate the potential for microarrays as pathogen detectors, we are developing an array that identifies fish pathogens based on species-specific polymorphisms for the 16S rDNA gene. We chose fish pathogens as a model system because conventional diagnostic procedures can be very challenging for these fastidious organisms. The limitations of non-molecular methods not only affect veterinary diagnostics but also severely limit our ability to study the ecology of these economically important pathogens.

Our initial array was composed of 25-mer oligonucleotide probes that are specific for 16S rDNA sequences for several fish pathogens (Fig. 2). Unmodified probes were deposited onto positively charged, Teflon-masked, immunohistochemistry slides (Erie Scientific, Portsmouth, NH). The masking permitted simultaneous printing and use of up to 12 independent arrays/slide. Probes were resuspended in an alkaline buffer and deposited within masked wells using an Affymetrix 417 arrayer (Call et al., 2001b). For this example, universal PCR primers EubB (Mauel et al., 1996) and 517r (Murray et al., 1996) were biotinylated (5-prime terminus) and used to amplify a 529-bp segment of the 16S rDNA sequence. Reaction conditions included 1X reaction buffer (Fisher Scientific, Pittsburgh, PA), 1 unit *Taq* polymerase, 2 mM $MgCl_2$, 400 uM each dNTP, and 400 nM each primer. Boiled lysate (1 colony in 200 μl of water) was prepared for *Renibacterium salmoninarum* (ATCC 33209) and 5 μl of lysate was added to the PCR reaction for a 25-μl reaction volume. Cycle conditions included 4 min denature at 94 °C followed by 30 cycles of 94 °C (30 s), 60 °C (30 s) and 72 °C (30 s) with a final incubation for 10 min at 72 °C. After PCR was complete, 5 μl of PCR mix was added directly to 30 μl of hybridization buffer composed of 4X SSC (60 mM NaCl, 0.6 mM Na–citrate, pH 7.0) and 5X Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin). This mix was heat denatured (95 °C) for 5 min before being

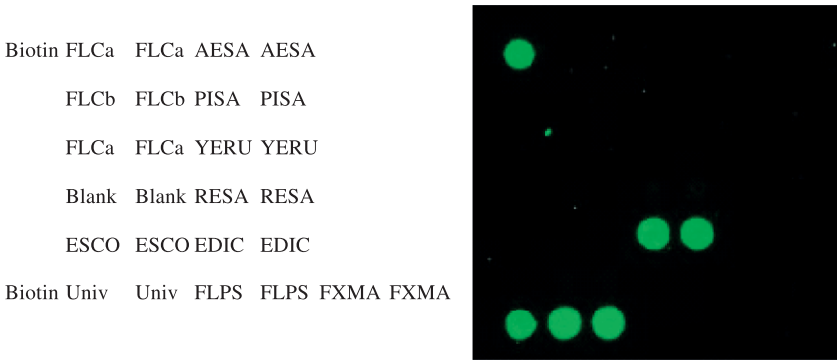

Fig. 2. PCR products from the *Renibacterium salmoninarum* (RESA) 16S rDNA gene were hybridized to an array composed of 12 probes (printed in duplicate). The left panel shows the relative position of different probes. Biotin spots serve as orientation points. The PCR product hybridized to both the universal 16S (Univ) and RESA probes (right panel). Other probes on the array include *Aeromonas salmonicida* (AESA), *Edwardsiella ictaluri* (EDIC), *Escherichia coli* (ESCO), *Flavobacterium columnare* (FLCa,b,c), *Flavobacterium psychrophilum* (FLPS), *Yersinia ruckeri* (YERU), *Piscirickettsia salmonis* (PISA), and *Flexibacterium maritmus* (FXMA).

placed on the microarray. The slide was placed in a humidified 50-ml tube which was then submerged in a water bath (58 °C) overnight. Hybridized products from these types of experiments can be detected using a streptavidin conjugated with a fluorescent reporter molecule. In this case, however, we wanted to maximize detection sensitivity and identify any nonspecific hybridization between our *R. salmoninarum* target and other array probes. To do this, we used tyramide signal amplification (TSA Biotin System, Perkin-Elmer, Boston MA). Slides were pre-blocked at 23 °C for 30 min with TNB buffer (100 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% blocking reagent; TSA reagents). Slides were washed according to Hegde et al. (2000) and remaining steps followed manufacturer's instructions from the TSA Biotin kit. After washing biotinyl tyramide from the slide, 2 μg/ml of streptavidin conjugated to AlexaFluor 546 (Molecular Probes, Eugene OR) was added in 1X SSC and 5X Denhardt's solution. Slides were given a final wash followed by drying and imaging using an Applied Precision ArrayWoRx scanner (Issaquah, WA).

Fig. 2 illustrates results from hybridizing the *R. salmoninarum* 16S PCR products to our array. In this case, we are able to detect species-specific PCR products with a high degree of specificity and this has now been demonstrated for six additional microbes using identical hybridization conditions. The array will be expanded to include additional 16S probes and tests are planned for extensive probe validation, sensitivity, and application to detection of fish pathogens both in tissue and environmental samples.

3.3. Direct detection of 16s rRNA

Detection of nucleic acids in complex substrates (e.g., soil) is complicated by co-extraction of compounds that frequently interfere with enzymatic manipulations. Furthermore, if PCR amplification is used, rare transcripts may be underrepresented in the final analysis (Chandler, 1998). One way to circumvent these problems is to directly detect rRNA from pathogens of interest. Direct detection of rRNA also provides an indication of pathogen viability and if enzyme manipulation is not required, the assay should be much less susceptible to inhibitors commonly found in soil extracts. There is a tradeoff, however, because direct detection will have significant sensitivity limitations compared with PCR-based assays.

Small et al. (2001) developed a glass-based microarray suitable for direct detection of 16S rRNA from soil extracts. The array was composed of oligonucleotide probes specific for several species of soil bacteria. Total RNA extractions were hybridized directly to the array without reliance on enzymatic manipulations such as PCR. 16S rRNA did not readily hybridize to the oligonucleotide probes, presumably because secondary structure prevented probes from efficiently interacting with complementary rRNA. Addition of

biotinylated oligonucleotides (“chaperones”) in the hybridization buffer appeared to relax structural interference and permit hybridization and detection. Importantly, these chaperone sequences were positioned proximal to probe sequences and consequently, multiple chaperones were needed to detect different species of rRNA. This method was limited by overall sensitivity (10^6 CFU g m^{-1} soil), although more sophisticated detection chemistry and scanning systems might enhance detection sensitivity by several orders of magnitude. Guschin et al. (1997) developed a polyacrylamide gel-pad microarray that was also suitable for direct detection of 16S rRNA. In this case, the RNA was chemically labeled with fluorescein and the arrays could be reused 20–30 times. This system was suitable for total cellular RNA, although in vitro amplified 16S rRNA produced the most discriminatory results.

3.4. Direct detection of DNA

Direct detection of DNA might also circumscribe biases from PCR and assay inhibition, depending on how targets are labeled. Although viability of bacteria cannot be ascertained from DNA hybridizations, DNA is more stable than RNA and consequently may better reflect the composition of microbial communities.

Wu et al. (2001) fabricated a PCR-based array for genes related to nitrogen cycling and thoroughly evaluated the performance of the array relative to sensitivity, specificity, and probe design. Using direct DNA hybridizations, the array was suitable for detecting genes from a mixed community when a minimum of 25 ng of genomic DNA was applied to the array. While seemingly sensitive, the authors noted that this level of sensitivity may be inadequate for some applications. Assuming an average cell yields 4.5×10^{-15} g of genomic DNA, then the 25-ng limit was equivalent to 5.6×10^6 cells. Not surprisingly, specificity was a function of hybridization stringency and genes having less than 80–85% sequence identity were differentiated with the array. Longer probes (up to 1500 bp) produced more intense signal compared with shorter probes.

Direct detection of DNA (or RNA) from environmental samples is challenging unless microbe populations are quite large. Furthermore, if the research objectives include enumerating the relative abundance of different genes in a mixed population, then it may be difficult to relate the intensity of probe signal with target abundance. That is, if there is significant sequence divergence within the target population (i.e., many homologous but not identical sequences present), then signal intensity is confounded by copy number and sequence divergence (Wu et al., 2001).

3.5. Genotyping with microarrays

Pathogen detection is only the first step. Enumeration and isolate characterization generally follow detection. Microarrays are less useful for pathogen quantification because they are limited to relative comparisons unless coupled with carefully controlled experimental conditions (Loge et al., 2002). In the latter case, accuracy may be limited to order of magnitude differences. It may be possible to incorporate relative intensity standards within the microarray assays, but this will likely be limited to enumeration of direct nucleic acid detection rather than PCR products (real-time PCR would be much more accurate for the latter).

If pathogens can be isolated using conventional microbiological techniques, then it becomes possible to further characterize each isolate by genotyping (e.g., Call et al., 2001b; Chizhikov et al., 2001; Johnson and Stell, 2000) or fingerprinting (Borucki et al., in press). Fingerprinting usually involves techniques such as pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphisms (AFLP), randomly amplified polymorphic DNA (RAPD), and repetitive element PCR (Box, REP, ERIC). These methods rely on electrophoresis to resolve DNA fragments having different lengths. When fragments (“bands”) are identical in size between two samples, they are usually considered identical. A similarity index is then used to generate a quantitative estimate of genetic relatedness between samples based on the proportion of shared bands. These fingerprinting methods are very useful in part because no *a priori* knowledge is required for the genome of interest. As a consequence, however, very limited information can be gained about the genome using these methods. There is no simple means to identify the sequences encoded by these bands short of laborious excision, cloning, and sequencing.

Microarrays offer an alternative means to characterize pathogenic isolates. For example, genomic DNA

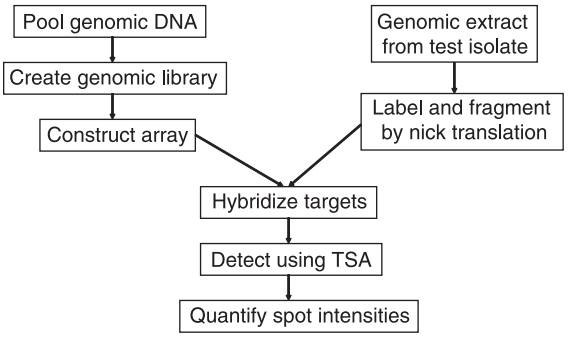

Fig. 3. Procedure outline for developing and implementing a mixed genome microarray.

can be extracted from a pathogen and hybridized directly to an array composed of gene sequences (Fig. 1). Differential spotting patterns could be compared between isolates as a "fingerprint", with the advantage that specific genes can be identified. For example, Dorrell et al. (2001) developed a whole genome microarray from a single reference strain of *Campylobacter jejuni*. When genomic DNA from different strains was hybridized to the array, 21% of genes in the reference strain was either absent or highly divergent in one or more of 11 strains tested on the array. This type of array provides valuable information about the classes of genes that we might expect to be "dispensable" within a species, and this type of array could be used to fingerprint strains relative to the reference strain. Hakenbeck et al. (2001) used a similar type of whole genome microarray and found that up to 10% of *Streptococcus pneumoniae* genes appeared altered between test isolates and a single reference strain. Again, these polymorphic probes would be useful for fingerprinting different clones. Similar findings have been reported for *Staphylococcus aureus* (Fitzgerald et al., 2001) and for *Pseudomonas aeruginosa* (Liang et al., 2001).

Microarrays that are used to characterize genomic DNA incorporate species-specific gene sequences, but they do not necessarily require *a priori* knowledge about the genome of interest. For example, Cho and Tiedje (2001) made random genomic libraries for four species of *Pseudomonas*. Genomic DNA from test isolates was hybridized to the array, providing an assessment of overall phylogenetic relationships between the isolates. Inclusion of gene fragments from multiple libraries goes a lot further to inform us what is unique about each strain (as opposed to simply what is missing relative to a single reference strain). A logical extension of these methods is to use DNA from more than one reference strain to make a single genomic library. Such a "mixed-genome" microarray is constructed by mixing genomes from multiple strains, generating a random genomic library and constructing a subsequent array using randomly selected clones (Fig. 3). This type of array can serve both as a fingerprinting tool and as a tool for identifying new genetic markers that may be amenable to PCR-based assays.

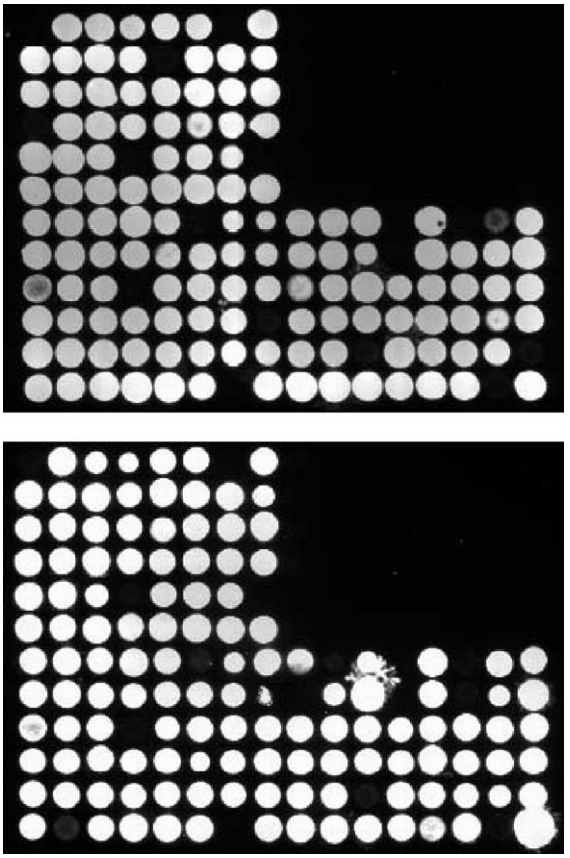

Fig. 4. Example of a mixed genome microarray for *Listeria monocytogenes*. Genomic DNA was extracted from two serotypes of *L. monocytogenes* and hybridized to identical arrays. Each panel represents identical portions of a larger microarray after detection using tyramide signal amplification (TSA). Visible spots represent sequences that are present in the sample genomes. Missing spots are sequences absent from the sample genome. In this example, there are eight spot differences between the two serotypes (e.g., column 1, row 4).

Borucki et al. (in press) developed a mixed genome microarray to identify gene sequences that differentiate different serotypes of *Listeria monocytogenes*. An initial genomic library was constructed followed by microarray fabrication using PCR products from this library. For each of 29 test strains, genomic DNA was extracted, labeled, and hybridized to the mixed genome microarray. Using this method, shared probe sequences could be identified as well as sequences differentiating less genetically related isolates (Fig. 4). Overall genetic similarity was assessed (number of shared probe sequences) and two previously identified phylogenetic divisions (serotypes 1/2a and 1/2c vs. 1/2b and 4b) were clearly identified along with 10 probe sequences that may serve as useful markers for differentiating serogroups. Over 8% of probe sequences was either absent or highly divergent in one or more test isolates. When compared with a fingerprinting tool such as pulsed-field gel electrophoresis (PFGE), this *L. monocytogenes* microarray provided a comparable level of discrimination even though the array had only 585 probe sequences. An expanded array could yield more genetic markers and even greater discriminatory power than PFGE.

4. Conclusion

Although relatively simple in concept, DNA microarrays are powerful tools for pathogen detection and characterization. Direct detection of nucleic acids from bacteria is feasible, but may lack the level of sensitivity needed for routine screening of environmental samples. When the amount of nucleic acid is not limiting, however, microarrays may prove very valuable as a fingerprinting tool and as a tool for marker discovery. When coupled to PCR, microarrays have detection sensitivity equal to conventional methods with the added flexibility needed for discriminating multiple PCR reactions and for pathogen detection based on 16S rDNA sequences.

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