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DNA microarray for discrimination between pathogenic 0157:H7 EDL933 and non-pathogenic *Escherichia coli* strains

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Abstract

The primary technique currently used to detect biological agents is based on immunoassays. Although sensitive and specific, currently employed immunoassays generally rely on the detection of a single epitope, and therefore often cannot discriminate subtle strain-specific differences. Since DNA microarrays can hybridize hundreds to thousands of genomic targets simultaneously and do not rely on phenotypic expression of these genetic features for identification purposes, they have enormous potential to provide inexpensive, flexible and specific strain-specific detection and identification of pathogens. In this study, pathogenic *Escherichia coli* O157:H7-specific genes, non-pathogenic K12-specific genes, common *E. coli* genes, and negative control genes were polymerase chain reaction-amplified and spotted onto the surface of treated glass slides. After labeled bacterial cDNA samples were hybridized with probes on the microarray, specific fluorescence patterns were obtained, enabling identification of pathogenic *E. coli* O157:H7 and non-pathogenic *E. coli* K12. To test the utility of this microarray device to detect genetically engineered bacteria, *E. coli* BL21 (a B strain derivative with antibiotic resistance gene, amp^R) and *E. coli* JM107 (a K12 strain derivative lacking the gene $ompT$) were also employed. The array successfully confirmed the strain genotypes and demonstrated that antibiotic resistance can also be detected. The ability to assess multiple data points makes this array method more efficient and accurate than a typical immunoassay, which detects a single protein product.

Keywords: Microarray; *Escherichia coli*; O157; K12; Pathogen identification

1. Introduction

The enterohemorrhagic *Escherichia coli*, such as strain O157:H7, cause capillary thrombosis, diarrhea, hemorrhagic colitis and hemolytic uremic syndrome by producing Shiga-like toxins (Ryan et al., 1994). Large outbreaks of O157:H7 infection have occurred all over the world including Fukuoka, Okayama, Osaka and Hiroshima in Japan in 1996 (Izumiya et al., 1997), central Scotland in 1996 (Dundas et al., 2001), Germany from 1988 through 1998 (Liesegang et al., 2000) and multistate outbreaks in the US in the past few years (Jaeger and Acheson, 2000; Mohle-Boetani et al., 2001). A reliable and accurate detection method would enable the prevention, diagnosis and treatment of the pathogens. Typically, the presumptive identification of O157:H7 in the clinical laboratory is done by screening bacteria on Sorbitol–MacConkey (SMAC) agar. In this assay, O157:H7 bacteria do not efficiently ferment D-sorbitol and appear as colorless colonies in an otherwise pink population of sorbitol-fermenting organisms (March and Ratnam, 1986). To prevent false identification, a confirmative test is performed by immunoassay against the O157:H7 flagellar antigen (He et al., 1996; Seah and Kwang, 2000) or the Shiga-like toxins (Ludwig

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et al., 2001). However, O157 family members may be H7 or non-H7, and may have only Shiga-like toxin 1, only Shiga-like toxin 2 or both. It was even reported that some O157 strains without Shiga-like toxin genes (*stx*) could still cause severe diarrhea or hemorrhagic colitis (Allerberger et al., 2000; Feng et al., 2001). A fluorescent 4-methylumbelliferyl-β-D-glucuronide cleavage assay has been used to detect the activity of β-glucuronidase which does not appear in most O157 (Thompson et al., 1990), however, a particular O157 (G5101) isolated from a patient with bloody diarrhea was found to be β-glucuronidase positive (Hayes et al., 1995). The pulsed-field gel electrophoresis method has also been used. This technique is based on a comparison ring the endonuclease digestion patterns to identify O157:H7 (Izumiya et al., 1997) but, in some cases, the stx-negative strains were indistinguishable from stx-positive strains (Allerberger et al., 2000). Alternatively, an oligonucleotide probe, PF-27, has been designed to detect a conserved mutation in the *uid*A gene of O157:H7 (Feng, 1993). Since detection of one gene or one specific enzyme product does not produce reliable identification, a combination of the above methods is commonly performed.

The complete genome of O157:H7 EDL933 was recently sequenced and published (Perna et al., 2001). The O157:H7 EDL933 genome (5 528 970 bp) was significantly larger than the K12 MG1655 genome (4 639 221 bp) that was also sequenced previously (Blattner et al., 1997). By comparing the genes of O157:H7 EDL933 with those of the non-pathogenic strain K12 MG1655, strain-specific genes were identified. We selected multiple strain-specific genes, genes common to all *E. coli*, two common antibiotic resistance genes, amp^R and tet^R, and non-bacterial genes (negative controls), as probes to screen the genotypes of sample *E. coli* through simultaneous hybridization. As a platform for this multiplexed assay, DNA microarray technology was used. DNA microarray technology has been commonly applied to the analyses of transcription levels of genes under different environmental conditions (DeLisa et al., 2001; Robles et al., 2001; Varedi et al., 2001), in different disease states (Hata et al., 2001; Sorlie et al., 2001), in the presence of various toxicants (Gerhold et al., 2001), therapeutic compounds, and antibiotics (Marcotte et al., 2001; Gill et al., 2002), and to identify pathogens (Gill et al., 2002; Wilson et al., 2002). The ability of the DNA microarray to simultaneously hybridize low quantity of nucleotides with multiple targets provides a very efficient way of measuring gene expression responses and for confirming genotypes. In this report, we demonstrate that the array successfully confirmed the strain genotypes and detected antibiotic resistance markers. Because the identification is based upon multiple data points, this array method is more efficient and accurate than currently employed immunoassays.

2. Experimental

2.1. Strains and DNA sources

The *E. coli* O157:H7 EDL933, K12 MG1655 and JM107 were obtained from ATCC. The strain K12 ER2267 was obtained from New England Biolabs. Another strain, BL21, carrying a plasmid, pOPH (with ampicillin resistance), was described previously (Wu et al., 2001). The genomic DNAs of these strains were isolated using the Easy-DNA kit (Invitrogen). Control DNA fragments such as *EGFR*, *GAPDH*, and antibiotics resistance genes (amp^R and tet^R) were obtained from vectors, pTRI (Ambion) and pBR322 (Life Technologies).

2.2. Probe selection and amplification

Complete comparison of genomic DNA between O157:H7 EDL933, a pathogenic strain, and K12 MG1655, a common non-pathogenic strain was based on the data published recently (Blattner et al., 1997; Perna et al., 2001). The strain-specific sequences were carefully selected according to the low similarity between each other and to other species, as well as their size (very short or long genes were not easily amplified, recovered and/or hybridized). Thus, the genes were chosen after running the web-based BLAST program (National Center for Biotechnology Information). *E. coli* genes common to multiple strains, such as proteases and DNA restriction enzymes, were also selected. Most selected genes were 0.5–2 kb in length. All polymerase chain reaction (PCR) primers were designed using the freely available web-accessed software, Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi/). The primers were purchased from Ransom Hill Bioscience, Inc. The genes were amplified by the PCR using Platinum PCR Supermix (Life Technologies) or Vent polymerase (NEB) with O157:H7 EDL933 or K12 MG1655 genomic DNA as templates. The thermal cycle parameters were 30 s denaturation at 94 °C, 30 s annealing at 54 °C and 1 min elongation at 72 °C for 40 cycles. The genes were checked on agarose gels to verify the size and quantity. All PCR-amplified products were then purified using a QIAquick multiwell PCR purification kit (Qiagen). The buffer was then exchanged to 50% DMSO (Sigma) using the MultiScreen-PCR, 96-well filtration system (Millipore).

Table 1
The arrangement of genes on the microarray

A1	A3	A5	A7	A9	A11	A2	A4	A6	A8	A10	A12
intH	*z0894*	*z0020*	*z2323*	*z3617*	*z3161*	*z1019*	*sepQ*	*z1866*	*z4183*	*z3206*	*z4326*
C1	C3	C5	C7	C9	C11	C2	C4	C6	C8	C10	C12
tir	*intD*	*feaR*	*rspA*	*b2442*	*atoS*	*yi81_1*	*trs5_3*	*feaB*	*rspB*	*xapB*	*lyxK*
E1	E3	E5		E9	E11	E2	E4	E6	E8	E10	E12
proB	*ampR*	*GAPDH*				*luxS*	*tetR*	*EGFR*			
B1	B3	B5	B7	B9	B11	B2	B4	B6	B8	B10	B12
z5212	*z4810*	*z5692*	*z5337*	*stx1BA*	*eae*	*z4881*	*z5686*	*z5429*	*z5878*	*stx2AB*	*escD*
D1	D3	D5	D7	D9	D11	D2	D4	D6	D8	D10	D12
mcrA	*mcrC*	*lon*	*hsdR*	*endA*	*lacZ*	*mcrB*	*ompT*	*lacI*	*hsdS*	*recA*	*proA*

Genes (a) are 0157:H7 specific, (b) are K12-specific, (c) are common. *E. coli* genes, and purple are negative controls. Each quadrant of this table was printed in triplicate on the actual array.

2.3. Microarray printing and processing

CMT-GAPS II slides (Corning) were used as substrates for the arrays. The samples were loaded in 96-well plates (Corning) and printed on coated slides using an Affymetrix 417 arrayer. The distance between each spot was 500 μm and each gene was spotted in triplicate. The temperature was 22–23 °C and the humidity was 70% during the printing process. The DNA was immobilized on the surface by baking the slides at 80 °C for 3 h.

2.4. Genomic DNA labeling

Instead of labeling RNA of *E. coli*, the most commonly employed protocol, we labeled the *E. coli* genomic DNA (2–3 μg) directly using the BioPrime DNA labeling system (Life Technologies). The O157:H7 EDL933 DNA was labeled with Cy5-dCTP and K12 ER2267 DNA was labeled with Cy3-dCTP. In the comparison experiments, the DNA from BL21 carrying pOPH was labeled with Cy3-dCTP and the DNA from JM107 was labeled with Cy5-dCTP with the same protocol. Previously, reverse labeling was carried out, with results not significantly different than those obtained using these labeling scenarios. The labeling preparations were incubated at 37 °C for 2 h and then purified with Microcon YM-30 filters (Millipore). The final volume of the mixed (cy3 and cy5 labeled sample) solution was concentrated to less than 10 μl and stored in the dark at 4 °C until hybridization.

2.5. Hybridization

The GMS GAPS II slide (Corning) was incubated in 50% formamide (Sigma), 5 × SSC (diluted from 20 × SSC, 0.3 M sodium citrate and 3 M NaCl) (Sigma) and 0.1% SDS (Sigma) at 42 °C for 40 min, washed in distilled water, and fixed in isopropyl alcohol (Sigma). The labeled and mixed DNA sample was combined with 15 μl formamide, 7.5 μl 20 × SSC, 0.3 μl 10% SDS, 1 μl 10 mg/ml salmon sperm DNA (Life Technologies), and 1 μl 4 mg/ml yeast tRNA (Sigma) and boiled for 5 min. The solution was then centrifuged and applied to the spotted area on a pre-hybridized slide. The microarray was covered by Hybri-Slip (Sigma) and placed in the hybridization chamber overnight at 42 °C. When the hybridization was finished, the microarray was washed in successive steps: (1) 2 × SSC, (2) 0.1 × SSC plus 0.1% SDS and (3) 0.1 × SSC. Finally, the microarray was dried at 500 rpm for 5 min using Eppendorf 5810 centrifuge.

2.6. Scanning and analysis

The hybridized slide was scanned using an Affymetrix 428 scanner (Affymetrix). False color images of Cy5 and Cy3 signals were produced with the free software, Scanalyze, (obtained from the Eisen Lab; http://rana.lbl.gov/eisensoftware.htm).

3. Results and discussion

Tables 1 and 2 list the spot locations and genes chosen for this array. Spots A1–A12, B1–B12 and C1 contained O157:H7 specific genes (printed in red in Table 1). Spots C2–C12 contained K12-specific genes (printed in green in Table 1). Spots D1–D12, E1 and E2 contained common *E. coli* genes (Printed in orange in Table 1). Many of these common genes such as *mcr*ABC, *hsd*RS, *omp*T, *rec*A and *pro*AB were selectively mutated or deleted in different strains. By using these probes, the detailed identification of *E. coli* strains was made possible. Spots E3–E6 (printed in purple in Table 1) contained control genes that do not appear in *E. coli*. Detection of B9 (*stx*1) and B10 (*stx*2) was critical in identifying pathogenic O157:H7 because these genes encode Shiga-like toxin subunits (AE005442, AE005296). B11 (*eae*) is also an important marker for

Table 2
Function (if known) of genes printed on the array

O157 EDL933-specific genes		
A1	*intH*	integrase
A2	*z1019*	secreted effector
A3	*z0894*	glutamate mutase
A4	*sepQ*	transportation
A5	*z0020*	unknown
A6	*z1866*	integrase
A7	*z2323*	unknown
A8	*z4183*	unknown
A9	*z3617*	unknown
A10	*z3206*	degradation
A11	*z3161*	unknown
A12	*z4326*	enterotoxin
B1	*z5212*	unknown
B2	*z4881*	adolase
B3	*z4810*	unknown
B4	*z5686*	kinase
B5	*z5692*	kinase
B6	*z5429*	unknown
B7	*z5337*	unknown
B8	*z5878*	integrase
B9	*stx1BA*	toxin
B10	*stx2AB*	toxin
B11	*eae*	intimin adherence protein
B12	*escD*	peptide secretion
C1	*tir*	translocated receptor
K12 MG1655-specific genes		
C2	*yi81_1*	transposon-related
C3	*intD*	integrase
C4	*trs5_3*	transposes
C5	*feaR*	regulatory protein for the 2-phenylethylamine catabolism
C6	*feaB*	phenylacetaldehyde dehydrogenase
C7	*rspA*	starvation sensing protein
C8	*rspB*	starvation sensing protein
C9	*b2442*	integrase
C10	*xapB*	xanthosine permease
C11	*atoS*	sensor protein for degradation regulator atoC
C12	*lyxK*	L-xylulose kinase
Common E. coli genes		
D1	*mcrA*	degradation of DNA
D2	*mcrB*	degradation of DNA
D3	*mcrC*	degradation of DNA
D4	*ompT*	protease
D5	*lon*	protease
D6	*lacI*	transcriptional repressor
D7	*hsdR*	DNA restriction
D8	*hsdS*	DNA restriction
D9	*endA*	endonuclease
D10	*recA*	recombination
D11	*lacZ*	beta-D-galactosidase
D12	*proA*	glutamylphosphate reductase
E1	*proB*	glutamate kinase
E2	*luxS*	AI-2 synthase
Control genes		
E3	*ampR*	ampicillin resistance
E4	*tetR*	tetracycline resistance
E5	*GAPDH*	dehydrogenase
E6	*EGFR*	growth factor receptor

O157:H7 since it encodes the intimin adherence protein that helpes bacteria attach to intestinal cells (AE005595). The gene spotted at C1 (*tir*) produces a translocated intimin receptor which is a characteristic for enteropathogenic and enterohaemorrhagic *E. coli* (Frankel et al., 2001). Although the functions of many genes are still unknown, based on the BLAST comparison, they appeared to be good strain-specific markers.

Fig. 1 illustrates the hybridization patterns of *E. coli* O157:H7 EDL933 and K12 ER2267. As seen in Fig. 1(a), significant signals appeared in spots A1–A12, C1, C2, E1–E2, B1–B12, D4, D5, D6, D9, D10, D11 and D12. Due to the good labeling efficiency and correct hybridization of the O157:H7-specific spots (A1–A12, B1–B12 and C1), the genes comprising these spots proved to be excellent probes for detecting O157:H7 EDL933. No K12-specific gene probes (C3–C12) were cross-reactive with the O157:H7 DNA except C2 (*yi81_1*), which expresses a hypothetical protein with transposon-related functions (AE000112). This is most likely due to an unusually high non-specific binding of this gene. The common genes at spots D5 (*lon*), D9 (*end*A), D11 (*lac*Z), D4 (*omp*T), D6 (*lac*I) demonstrated that each *E. coli* species was properly detected. Finally, the genes that do not exist in O157:H7 EDL933 were examined. Indeed, no signals from D1 (*mcr*A), D2 (*mcr*B), D3 (*mcr*C), D7 (*hsd*R), D8 (*hsd*S), E3 (*amp*R), E4 (*tet*R), E5 (*GAPDH*) and E6 (*EGFR*) were detected when assayed by O157:H7 EDL933 DNA. Overall, the hybridization results demonstrated that the microarray accurately detects the 'fingerprint' of O157:H7 EDL933.

Fig. 1(b) illustrates the pattern for the non-pathogenic K12 ER2267. Clearly, the locations of hybridization signals are different from those of the O157:H7 strain. None of the O157:H7-specific dots had significant signals. In contrast, almost all of the K12-specific genes (in the second row of the image: C2–C12) produced strong signals (except C9 (*b2442*)), indicating correct identification of K12 sample DNA. Although b2442 exists in K12 MG1655, we suspect that it is missing in K12 ER2267 because of the minimal signal. The strong hybridization of these K12-specific spots demonstrates that these genes are excellent probes in distinguishing O157:H7 from K12. In the case of the common *E. coli* gene group (in spots D1–D12 and E1–E2), both positive and negative hybridizations were observed. D1 (*mcr*A) is known to be absent in K12 ER2267 (*New England Biolabs catalog and technical reference*) and our results were confirmatory. Also K12 ER2267 has large deletions on *mcr*BC (D2 and D3), as well as on *hsd*RMS restriction systems (D7 and D8) (*New England Biolabs catalog and technical reference*). Thus, it was reasonable to see null signals on these spots. The proteases, *omp*T (D4) and *lon* (D5), were present in K12 and were easily detected. In addition, the K12 ER2267 strain, reported to be positive for *lac*I, *pro*A and *pro*B (*New England Biolabs catalog and technical reference*), is confirmed by signals on these spots (D6 (*lacI*), D12 (*proA*) and E1

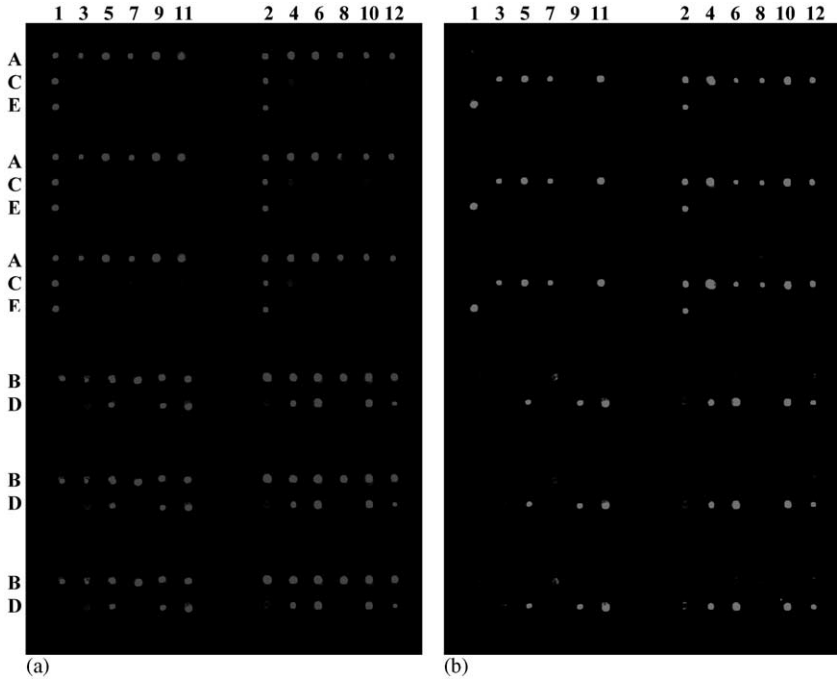

Fig. 1. Hybridization patterns of (a) Cy5-labeled (red) O157:H7 EDL933 genomic DNA and (b) Cy3-labeled (green) K12 ER2267 genomic DNA.

(*proB*)). Three spots, D9 (*end*A), D10 (*rec*A) and D11 (*lac*Z) also expressed strong signals, although the activities of these gene products were reported to be abolished (*New England Biolabs catalog and technical reference*). A reasonable assumption is that these sequences were mutated by frame shift, point mutation or a similar method instead of a large deletion, thus these genes could still be detected on the cDNA microarray. Feng (1993) reported a similar case in that O157:H7 had negative β-glucuronidase activity due to the fact that it possessed several base mutations in *uid*A. Finally, as expected, negative control spots, E3 (*amp*R), E4 (*tet*R), E5 (*GAPDH*) and E6 (*EGFR*) did not produce any signal.

Overall, at least 90% of the selected genes in our microarray solidly confirmed the genomes of our tested O157:H7 EDL933 and K12 ER2267 strains. By comparing the hybridization patterns, the pathogenic and non-pathogenic *E. coli* could be readily distinguished.

A complete overlapping image with both O157:H7 EDL933 and K12 ER2267 signals is shown in Fig. 2. In this figure, red spots confirm that all O157-specific genes are printed in the first rows in all four quadrangles as well as in the C1 position. The green spots confirm that all K12-specific genes are printed from C2–C12. Exceptions include C2, which was somewhat yellow due to nonspecific binding of O157:H7 DNA, and C9 (*b2442*), which was presumed absent in K12 ER2267. Common *E. coli* genes, E1 (*pro*B), E2 (*lux*S), D4 (*omp*T), D5 (*lon*), D6 (*lac*I), D9 (*end*A), D10 (*rec*A), D11 (*lac*Z) and D12 (*pro*A) are seen as yellow signals among all others. This color confirmed their appearance in both O157 and

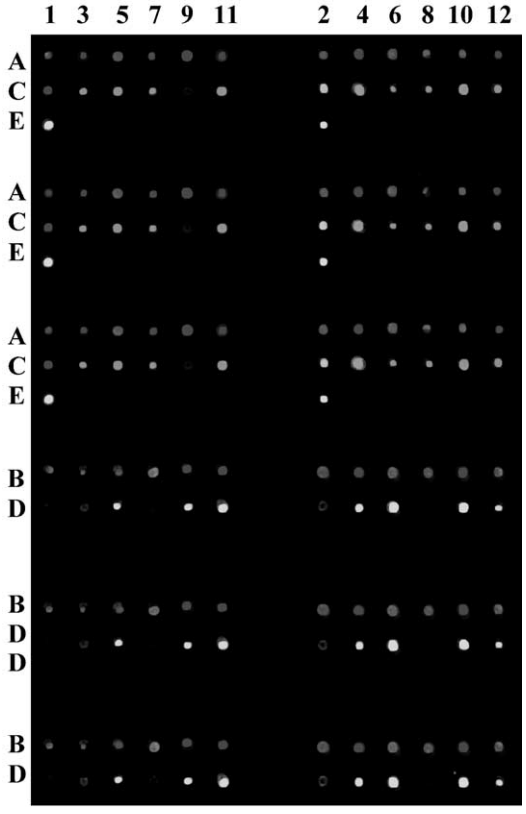

Fig. 2. Microarray image generated by overlapping both O157:H7 EDL933 and K12 ER2267 signals.

K12. Dark spots at D1 (*mcr*A), D2 (*mcr*B), D3 (*mcr*C), D7 (*hsd*R) and D8 (*hsd*S) confirm that these genes are absent in both strains. Spots E3–E6 were also dark,

demonstrating correct lack of hybridization at these negative control spots. The noise from non-specific binding shown in Fig. 1 was significantly reduced after overlapping the two images. Although slight cross reactivity was still observed (e.g. D2 and D3), it was relatively weak compared to the true hybridization in other spots. Overlapping two images generated a more definite result since the noise was normalized twice (by each color).

Most laboratory *E. coli* strains are derivatives of the K12 or B strains (*New England Biolabs catalog and technical reference*). Thus, we also tested *E. coli* BL21 (B strain derivative) and JM107 (K12 derivative) to investigate whether their patterns were still distinguishable from the pathogenic O157:H7, and to show that their genetically engineered properties could be correctly identified. We further transformed the BL21 with an ampicillin resistant plasmid to test whether the microarray can correctly detect a genetically engineered drug-resistant strain. The results are shown in Fig. 3. Fig. 3(a) shows that non-pathogenic BL21 possessed K12-specific genes (C2–C12 except *b2442*) but not O157 specific genes (row A, row B and C1). An exception was A8 (*z4183*), which showed some signal. BL21 is an *ompT*— strain and no signal at spot D4 (*ompT*) confirmed this fact. Importantly, spot E3 (amp^R) correctly detected the ampicillin resistance gene on the plasmid. As expected, JM107 (Fig. 3(b)), showed that 10 out of 11 K12-specific genes were conserved in the strain (except *b2442*), and no O157:H7 specific spots produced signals except A8 (*z4183*). We are not sure whether the hybridization of A8 (*z4183*) was due to nonspecific binding; however, more than 30 definite results in row A, B and C clearly identified the non-pathogenic strain. Lastly, JM107 is known to be $proA+proB+mcrA-lacI^q$ (ATCC) and the hybridization results at D12, E1, D1 and D6 confirmed this genotype. Two curious negative results, however, are noted here: First, *E. coli* B strain should be devoid of Lon activity (*New England Biolabs catalog and technical reference*), however, D5 (*lon*) in Fig. 3(a) produced signal. Second, the *lacZ* mutation in JM107 was known to be a large deletion but there was still signal at D11 (*lacZ*) in Fig. 3(b). We hypothesize that if these genes were not completely absent (i.e. partial deletion), one might still detect hybridization. Nevertheless, correct signals from more than 90% of the tested genes provided solid identification.

By choosing to label genomic DNA from *E. coli* samples, we were afforded two distinct advantages: (1) less starting material was necessary; and (2) the higher stability of DNA. Specifically, only 2–3 μg of DNA was sufficient for good labeling. In comparison, ~80 μg of

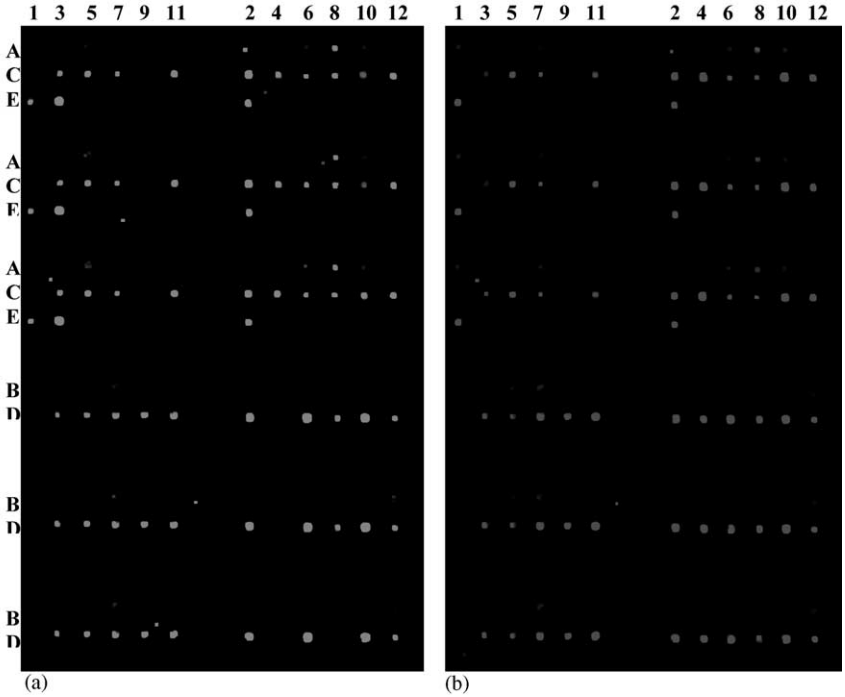

Fig. 3. Hybridization patterns of (a) *E. coli* BL21 genomic DNA labeled with Cy3-dCTP (green), (b) *E. coli* JM107 genomic DNA labeled with Cy5-dCTP (red).

RNA was required, as we previously noted (DeLisa et al., 2001). Also, although not reported, RNA was more susceptible to degradation.

4. Conclusion

In summary, the selected genes are appropriate targets for accurately distinguishing both pathogenic O157:H7 and the non-pathogenic *E. coli* strains considered in this work. Since the microarray can hybridize many multiple genes simultaneously, the detection assay accuracy and efficiency is significantly enhanced in comparison to many traditional methods. Most importantly, a diversity of strains including not only wild-type, but also genetically modified variants can be distinguished. This approach has great potential as a high throughput screening process to recognize particular bacterial or viral identities with confidence.

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