Detection of simple mutations and polymorphisms in large genomic regions

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ABSTRACT

We have developed a novel technology that makes it possible to detect simple nucleotide polymorphisms directly within a sample of total genomic DNA. It allows, in a single Southern blot experiment, the determination of sequence identity of genomic regions with a combined length of hundreds of kilobases. This technology does not require PCR amplification of the target DNA regions, but exploits preparative size-fractionation of restriction-digested genomic DNA and a newly discovered property of the mismatch-specific endonuclease CEL I to cleave heteroduplex DNA with a very high specificity and sensitivity. We have used this technique to detect various simple mutations directly in the genomic DNA of isogenic pairs of recombinant Pseudomonas aeruginosa, Escherichia coli and Salmonella isolates. Also, by using a cosmid DNA library and genomic fractions as hybridization probes, we have compared total genomic DNA of two clinical P.aeruginosa clones isolated from the same patient, but exhibiting divergent phenotypes. The mutation scan correctly detected a GA insertion in the quorum-sensing regulator gene rhlR and, in addition, identified a novel intragenomic polymorphism in rrn operons, indicating very high stability of the bacterial genomes under natural non-mutator conditions.

INTRODUCTION

Spontaneous acquisition of simple mutations (i.e. single or few nucleotide substitutions, insertions/deletions or inversions) by structural or regulatory genes can have pronounced effects on various cellular processes (1,2). In pathogenic bacteria, simple mutations that alter gene expression or modify gene products can provide a selective advantage during the course of a single infection or epidemic spread (3). Such pathogenicity-adaptive (or pathoadaptive) mutations can help bacteria to, for example, avoid immune recognition, optimize host tissue tropism or acquire antibiotic resistance (4). They occur spontaneously in a great variety of genes and in regions that are located in different parts of the genome. Here, we describe an effective method for screening large regions of genomic DNA of bacterial strains to identify the presence and location of simple genetic mutations.

A prominent example of a bacterial pathogen capable of adaptive (micro)evolution in the course of infection is Pseudomonas aeruginosa, the causative agent of chronic lung infections in patients with cystic fibrosis and urinary tract and burn infections in compromised patients (5). Several pathoadaptive mutations have been described in P. aeruginosa including loss of function mutations in the mucA gene that result in the overproduction of an alginate capsule and increased bacterial resistance to host clearance mechanisms and anti-microbial drugs (6,7). Additional mutations are responsible for the loss of flagellar motility, auxotrophy or altered lipopolysaccharide (LPS) structure (8–10) and, in some cases, the adaptive microevolution might be facilitated by a mutator phenotype of the infecting clones (11,12). However, in most cases the nature of the underlying mutations remains unclear. As the pathoadaptive phenotype can result from simple mutations in a great variety of structural genes or regulatory regions, it is very difficult to define the location of such subtle alterations on a genomic level. Genome-wide comparative methods, such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) analysis or genomic microarrays are not suited to search for simple mutational differences between whole genomes. Single nucleotide substitutions and small insertions/deletions can be identified by several methods, including gene sequencing, differential gel mobility of heterologous DNA, oligonucleotide hybridization, and the use of DNA mismatch-cleaving, -modifying or -binding compounds (13–16). However, these techniques, even with automation, usually require PCR amplification of a DNA region containing the mutation and, therefore, are limited to the analysis of a relatively small number of specific regions.

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In this study, we have developed and tested a method that allows screening of large regions of genomic DNA for the detection of various types of nucleotide alterations.

MATERIALS AND METHODS

Bacterial strains and plasmids

*Pseudomonas aeruginosa* strains UPA204 and UPA205 were isolated concurrently from the urine of a patient with a catheterized urinary bladder and symptoms of an acute urinary tract infection. Pyocyanin, elastase and pyoverdine production of these and other clinical isolates of *P. aeruginosa* were determined as described previously (17). *Pseudomonas aeruginosa* PA01 is a standard reference strain for studies on pathogenesis, genetics and gene mapping, and it expresses most of the virulence factors commonly associated with clinical *P. aeruginosa* isolates (18). *Pseudomonas aeruginosa* PA01seq is an isolate of PA01 of which the genome has been recently sequenced and annotated (19). Genomic sequencing has revealed that PA01seq differs from PA01 by a spontaneously occurred CTGA tandem duplication in pilC. *Pseudomonas aeruginosa* PDO300 is PA01 derivative with a G deletion in a poly G strip and an A→G substitution in macA that were described by a homologous exchange (gift of Dr S. Suh, Virginia Commonwealth University, VA, USA). *Escherichia coli* C1845 is a clinical strain isolated from a patient with diarrhea (20). *Escherichia coli* G1148 strain is a derivative of C1845 with an AAT→TAAC replacement in daaP and a downstream C→G substitution that were introduced by a homologous recombination (S. Moseley, unpublished data). *Salmonella enteritidis* TH1966 is a derivative (K. Hughes, unpublished data) of a standard reference strain *Salmonella enterica serovar Typhimurium* LT2 (21). TH1967 is a derivative of TH1966 with a spontaneously occurring A→C substitution in nadC.

Plasmids and cosmids

To test for restoration of pyocyanin production, the clinical isolates were transformed with pMB5, a pLAFR1-based plasmid encoding *rhlABRI* genes (17). As a nadC-specific probe we have used plasmid pKH385 incorporating the 3′ portion of the gene and the downstream region (K. Hughes, unpublished data). Cosmids used in this study as probes for Southern blot hybridization were obtained by cloning 35–45 kb chromosomal regions of *P. aeruginosa* PA01 and were provided by Dr M. Olson, University of Washington (WA, USA).

Pulse-field gel electrophoresis

Pulse-field gel electrophoresis (PFGE) profiles of the UPA204 and UPA205 strains were determined by digesting the chromosomal DNA with SpeI endonuclease for 16 h followed by electrophoretic separation in 0.8% agarose under pulse-field conditions.

PCR amplification

The 1678 bp fragment containing the 730 bp coding sequence of *rhlR* was amplified by using high-fidelity Herculase™ DNA Polymerase (Stratagene) and the following primer pair: 5′-CCTGAAGCGTGCTGCCATAAC-3′ (top) and 5′-CGAAACGGCTGACGACCTCAC-3′ (bottom).

Heteroduplex formation and CEL I treatment

Heteroduplex DNA was obtained by heat-denaturing a sample of either PCR-amplified or fractionated genomic DNA fragments (from two strains being compared) at 95°C for 10 min followed by fast cooling to 85°C at a rate 2°C/s and then a gradual cooling to 40°C at a rate of 3°C/min to allow random re-annealing of the denatured DNA strands. CEL I is a 30 kDa plant nuclease purified from celery as described previously (22). CEL I treatment of the re-annealed DNA samples (10 ng for the purified PCR products and 200–1000 ng for the genomic fractions) was performed at 1:1 to 1:4 w/w enzyme/DNA ratio in a volume of 20 µl of reaction buffer containing 10 mM Tris–HCl, 150 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol, 10% formamide, 9% glycerol, 100 µg/ml BSA (pH 7.8). The reaction mixture was incubated at 40°C for 60 min, put on ice, mixed 1:10 with TBE agarose-loading buffer followed by electrophoretic separation in 0.8% agarose gel.

DNA fractionation

Purified genomic DNA from corresponding bacterial clones (500 µg each) was combined and digested to completion with an appropriate restriction endonuclease followed by size-fractionation in 0.5% agarose using a preparative gel electrophoresis apparatus (Model 491 Prep Cell from Bio-Rad Laboratories) under conditions described previously (23).

Membrane blot hybridization

Alkaline-denatured DNA was transferred from a liquid sample (for a slot blot hybridization) or from the agarose gel (for a Southern blot hybridization) to Nytran Plus nylon membranes (Schleicher & Schuell) under neutral conditions and crosslinked to the membranes by UV light. 32P-labeled hybridization probes were prepared from either PCR-amplified DNA, plasmids, cosmids or genomic fractions (see above) using the Random Primers DNA Labeling System (Gibco BRL). Membrane blot hybridization was performed under high-stringency conditions according to the manufacturer’s instructions.

RESULTS

Characterization of *rhlR* variants of *P. aeruginosa* clinical isolates

The mutation scan was developed using pathogenic isolates of *P. aeruginosa* with a novel type of phenotypic variation. It was found that 15% (50 out of 334 tested) of urinary tract infection isolates of *P. aeruginosa*, when cultured on pseudomonas isolation agar plates, produce a fluorescent pyoverdin pigment but not a blue pyocyanin pigment (M. Brint, unpublished data; presented at the 100th General Meeting of the American Society for Microbiology, Los Angeles, CA, May 2000). This phenotype has also been found among isolates from certain other sources. A urine specimen from one patient with a catheterized bladder yielded a mixture of a pyocyanin-positive isolate, UPA204, and a pyocyanin-negative isolate, UPA205. Each strain showed the same PFGE profile (Fig. 1A). It is
known that production of pyocyanin in *P. aeruginosa*, but not of pyoverdin, requires a transcriptional regulator, RhlR, which is part of a cell density-dependent quorum-sensing cascade (17). When a plasmid encoding *rhlR* was introduced into UPA205, it resumed production of the pigment (Fig. 1B). Similar results were obtained by introducing the *rhlR* plasmid into eight different pyocyanin-negative clinical isolates (not shown). Therefore, the pyocyanin-negative phenotype might be due to a structural mutation of the RhlR, its down regulation, or functional inhibition.

To determine whether UPA205 possesses an altered *rhlR*, the corresponding regions from UPA204 and UPA205 were amplified, the DNA fragments combined, heat-denatured and slowly cooled to allow heteroduplex formation. If the *rhlR* sequences were not identical, the DNA heteroduplexes would contain a mispaired region that could be recognized by a mismatch-specific enzyme. Re-annealed DNA samples were treated with the mismatch-specific endonuclease CEL I (22), then analyzed by agarose gel electrophoresis (Fig. 1C). In samples containing re-annealed DNA from UPA204 and UPA205, two CEL I-cleavage fragments were observed, indicating the presence of a mismatched region in the *rhlR* heteroduplex. [Note: When the amount of enzyme is limiting, CEL I is known only to nick one of the two DNA strands on the 3′ side of the mismatch nucleotide (22). The double-strand breaking activity is due to the use of an ~100-fold higher concentration of CEL I relative to that described previously (22) as well as modified reaction buffer conditions. The mechanism of mismatch-specific DNA cleavage by CEL I is currently under investigation.] Sequence analysis of the PCR products revealed that the *rhlR* of UPA205a has GA insertion in position 140 that leads to a frame-shift and inactivation of the 730 bp long gene (Fig. 1D). Therefore, the pyocyanin-negative phenotype of the UPA205 strain is likely to be a result of the *rhlR* mutation. If the *rhlR* mutation was acquired by UPA205 during evolution within the host, this strain and UPA204 might represent clonal variants of the same strain and, in this case, there should be minimal differences between the genomes.

**Design of the genomic mutation scan**

Although both strains demonstrated identical PFGE profiles, this or other currently available methods of genetic analysis are not suited to examine their isogenicity. To screen large portions of the bacterial genome for the presence of nucleotide polymorphisms, we have designed a strategy for a mutational scan that further exploits the mismatch-cleavage property of the CEL I endonuclease. However, instead of using a PCR-amplified DNA product, the heteroduplex DNA fragments are formed directly from genomic DNA of two bacterial strains. Importantly, before DNA denaturation/re-annealing and CEL I

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**Figure 1.** (A) PFGE profiles of the UPA204 and UPA205 strains. Track 1, UPA204; track 2, UPA205. The chromosomal digest was performed with SpeI endonuclease for 16 h. The top band is at 533.5 kb, the bottom of the first cluster of bands is at 194.0 kb and the bottom band in the second band cluster is at 97.0 kb. (B) Pyocyanin production on pseudomonas isolation agar: plate 1, UPA204; plate 2, UPA205; plate 3, UPA205 transformed with pLARF1 vector; plate 4, UPA205 transformed with an *rhlR*-encoding plasmid pMB5. (C) Agarose gel analysis of CEL I treatment of PCR-amplified *rhlR* gene regions of UPA204 and UPA205. Track 1, the amplified *rhlR* fragments of UPA204 and -205 strains are combined, heat-denatured and re-annealed without CEL I treatment; track 2, same as track 1, but treated with CEL I; track 3, the amplified *rhlR* fragments of UPA204 only, heat-denatured, re-annealed and treated with CEL I; track 4, the *rhlR* fragments of UPA205 only, heat-denatured, re-annealed and treated with CEL I; track 5, the *rhlR* fragments of UPA204 and -205 strains are combined and treated with CEL I without heat-denaturation/re-annealing. On the left the gel is a scale corresponding to a partial 1 kb ladder (Gibco BRL; shown marks correspond to 6.11, 5.09, 4.07, 3.05, 2.04, 1.64, 1.02, 0.51 and 0.22 kb, respectively). (D) *rhlR* and the GA insertion within the PCR-amplified region. Total length of the PCR fragment is 1658 bp (from UPA204). Position of the gene and mutation is marked from the beginning of the PCR fragment.
treatment, total genomic DNA of both strains is digested with a restriction enzyme and size-fractionated (Fig. 2). A CEL I-cleaved heteroduplex within a certain size range would produce at least one DNA fragment of a size smaller than the DNA fragments within that particular fraction, and could be separated from the non-cleaved DNA by gel electrophoresis. We have designated the proposed technology Genomic Identity Review by Annealing of Fractionated Fragments (GIRAFF).

Detection of the rhlR mutation directly in genomic DNA

We have examined whether the rhlR mutation could be detected by GIRAFF directly in restricted genomic DNA of UPA204 and UPA205 strains. Purified genomic DNA from UPA204 and UPA205 (500 µg each) was digested with SalI endonuclease and separated into 30 fractions (Fig. 3A) by preparative gel electrophoresis. Using an rhlR-specific hybridization probe, fractions 36 and 37 were found to contain rhlR (Fig. 3A), which corresponded to the predicted location of the rhlR gene within a 5.2 kb restriction fragment (Fig. 3A) according to the genomic sequence of P. aeruginosa strain PAO1 (19). It is important to note, however, that due to the high complexity of these relatively large genomic fractions, the rhlR-containing fragment is expected to constitute only a minor portion of fractions 36 or 37. DNA from fraction 36 was heat-denatured, re-annealed and treated with CEL I under conditions described in the Materials and Methods and analyzed by agarose gel electrophoresis. Direct visualization of CEL I-cleaved fragments was not possible due to the high complexity of DNA in the fraction, and also because of the background created by non-specific 5′-exonuclease activity of CEL I which results from a high concentration of the enzyme being used (A. Yeung, unpublished observation). Therefore, the agarose-separated DNA was analyzed by Southern blot hybridization using a 32P-labeled rhlR-specific probe (Fig. 3B). Three fragments were detected that corresponded to the original 5.2 kb fragment and two fragments of ~3.2 and 2 kb in size, which agreed with the expected location of the mutation between the sites flanking the rhlR gene. Therefore, CEL I was capable of specifically detecting the rhlR mutation within a highly complex DNA sample.

Detection of various types of simple mutations in different bacterial genomes

To test the ability of the CEL I enzyme to recognize different nucleotide mismatches in the genomes of different bacterial species, we used four pairs of isogenic strains differing by mutations of known nature and location. These included (i) P. aeruginosa PAO1 and PAO1seq which differ by a CTGA tandem duplication in pilC; (ii) P. aeruginosa PAO1 and PDO300 with a G deletion in a poly G strip and an A→G substitution in mucA; (iii) E. coli G1310 and G1148 with an AAT→TAAC replacement in daaP and a downstream C→G substitution; and (iv) S. enteritidis TH1966 and TH1967 with an A→C substitution in nadC (Fig. 3C). Total genomic DNA of the strain pairs was combined, digested with an appropriate restriction enzyme and size-fractionated as described above. The restriction enzyme choice was defined to test the capability of the GIRAFF technology to detect mutations in the genomic restriction fragments of various size. Fractions containing mutated gene regions were identified by gene-specific hybridization probes and subjected to denaturing/re-annealing. CEL I treatment and Southern blot hybridization with specific probes (see Materials and Methods). In all DNA fractions tested the original and novel bands of predicted size were clearly identifiable (Fig. 3C) indicating the feasibility of GIRAFF for detecting various types of nucleotide substitutions or insertions/deletions directly in the genomic restriction fragments of different size and species.

Mutation probing of large genomic regions

To test the ability of GIRAFF to screen larger chromosomal regions, we examined whether it was possible to detect the rhlR mutation by probing with cosmids c750. This cosmid was derived from a cosmid library of P. aeruginosa strain PAO1 (provided by Dr. Maynard Olson, University of Washington Genome Center, Seattle, WA, USA) and carries rhlR within a
46 kb chromosomal region. We also tested whether two to four adjacent genomic fractions can be combined for the analysis to facilitate the hybridization of all genomic fragments on a single membrane blot. We found that probing a blot of total UPA204/UPA205 genomic DNA with labeled cosmid c750 was as efficient in detecting the \textit{rhlR} mutation as the probing of a single fraction with the \textit{rhlR}-specific probe (Fig. 3D). Similarly, it was possible to detect the \textit{pilC}, \textit{mucA}, \textit{daaP} and \textit{nadC} regions within corresponding restriction fragments. The genes are indicated by a solid bar; regions used as the hybridization probes are indicated by a dotted line. (The \textit{pilC}, \textit{mucA}- and \textit{daaP}-specific probes were based on the corresponding PCR-amplified gene regions, while as a \textit{nadC}-specific probe we have used plasmid pKH385 incorporating only the 3' portion of the gene and the downstream region, which explains detection only of the CEL I-cleaved fragment of the lower size). On the right, Southern blot analysis of the CEL I-treated genomic fractions that contain the corresponding restriction fragments. (D) Hybridization of the total genomic blot of UPA204/205 with the \textit{rhlR}-containing cosmid c705. The thin line corresponds to the vector DNA, while the thick line corresponds to the 46 kb of cloned \textit{Pseudomonas aeruginosa} PA01 DNA.

**Figure 3.** (A) Agarose gel analysis of size-fractionated \textit{SalI}-restricted genomic DNA of UPA204/205 strain pair and, underneath the gel, Southern slot blot hybridization of the corresponding fractions with a $^{32}$P-labeled \textit{rhlR} gene-specific probe (even fractions only starting from the fraction 26; L, the loaded restricted DNA before fractionation). Marks of the 1 kb ladder scale on the left correspond to 12.22, 11.20, 10.18, 9.16, 8.14, 7.13, 6.11, 5.09, 4.07, 3.05 (in bold), 2.04, 1.64 and 1.02 kb. Below: the expected position of the GA insertion in \textit{rhlR} (solid bar) within the \textit{SalI} restriction fragment based on the genomic sequence of \textit{Pseudomonas aeruginosa} strain PA01 (dotted line indicates the PCR-amplified region used as the hybridization probe). (B) Agarose gel and Southern blot analysis of the denatured/re-annealed fraction 36 DNA, without (−) and with (+) CEL I treatment. The 1 kb ladder scale as described (A). (C) Expected position of the known mutations in \textit{pilC}, \textit{mucA}, \textit{daaP} and \textit{nadC} regions within corresponding restriction fragments. The genes are indicated by a solid bar; regions used as the hybridization probes are indicated by a dotted line. (The \textit{pilC}, \textit{mucA}- and \textit{daaP}-specific probes were based on the corresponding PCR-amplified gene regions, while as a \textit{nadC}-specific probe we have used plasmid pKH385 incorporating only the 3' portion of the gene and the downstream region, which explains detection only of the CEL I-cleaved fragment of the lower size).
genomes. In contrast, using cosmids as probes for GIRAFF analysis of combined genomic DNA of two unrelated strains the presence of multiple polymorphic sites was always detected (not shown).

Cross-fraction probing of the entire bacterial genome and detection of a novel intragenomic polymorphism

Although the use of cosmid probing is a convenient method for examining genetic polymorphism within large genomic regions, the applicability of this approach for GIRAFF analysis of whole genomes of various bacterial species is obviously dependent on the availability of suitable cosmid clones. To avoid this limitation we tested whether it is feasible to hybridize genomic blots with restriction fractions of the same or related genomes, which were obtained using a different restriction enzyme. Because any two non-overlapping restriction sites are more or less randomly distributed relative to one another, DNA from restriction fractions generated by one enzyme would randomly hybridize across the genomic fractions generated by another restriction enzyme. Two alternative approaches could be used for obtaining hybridization probes for probing fractionated DNA. The first approach is to use the fractionated genomic DNA of a model fully sequenced strain *P. aeruginosa* PA01 strain (19), which provides an opportunity to create a standard set of cross-fraction probes for GIRAFF analysis of various *P. aeruginosa* wild-type strains. The second approach uses genomic DNA of homologous origin, which allows probing of wild-type strain regions that could be unique to the tested isolates.

We have probed *Sal*I-generated UPA204/UPA205 genomic fractions with different individual fractions obtained by *Bam*HI restriction of genomic DNA of UPA205 or *P. aeruginosa* PA01. The cross-fraction probing method detected the cleavage bands corresponding to the *rhlR* mutation (Fig. 4A) demonstrating the suitability of this approach for genome-wide mutation scanning. Using an entire set of the *Bam*HI-generated fractions for cross-fraction probing of the genomic DNA of UPA204 and UPA205 strains resulted in detection of a novel pair of *CEL* I cleavage products in the combined fractions 31–32 (Fig. 4B). We have created a pBluescript library of the *Sal*I fragments from fraction 31. Among 150 clones screened by blot hybridization, selected a plasmid detecting the novel cleavage products (Fig. 4C). It was determined that the restriction fragment susceptible to *CEL* I cleavage represented a...
4073 bp internal region of the \textit{rrn} operon, which is present in four nearly identical copies in the \textit{P. aeruginosa} genome (19). In UPA204 and UPA205 strains the \textit{rrn} operon is also present in four copies (data not shown). The corresponding DNA regions of each strain were amplified by PCR, denatured/annealed and treated with CEL I (Fig. 4D). The PCR-amplified fragments derived from either UPA204 or UPA205 formed identical mismatched heteroduplexes with or without combining with each other indicating the intragenomic origin of the \textit{rrn} mismatch. Sequence analysis revealed that the \textit{rrn} operon copies in both strains possess three adjacent polymorphic sites in a 227 bp Intergenic Transcribed Spacer between alanine tRNA and 23S rRNA genes—a C→G substitution in position 5, an A deletion in position 8 and GA→AG inversion in position 38 (Fig. 4E).

**DISCUSSION**

Our study presents a proof-of-principle for a novel technological approach for the detection of unknown mutations and nucleotide polymorphisms on a level of hundreds of kilobases of genomic DNA. The technology is based on the finding that mismatch-specific endonuclease CEL I is able to recognize and cleave DNA heteroduplexes directly in a sample of genomic restriction fragments of a combined length of ∼0.6 Mb. This makes it feasible, for example, to compare the identity of nucleotide sequence of two entire bacterial genomes.

The technique was able to detect various simple mutations directly in the genomic DNA of isogenic pairs of recombinant \textit{P. aeruginosa}, \textit{E. coli} and \textit{Salmonella} isolates. We have also compared total genomic DNA of two clinical \textit{P. aeruginosa} clones isolated from the same patient, but exhibiting divergent quorum-sensing (RhlR-specific) phenotypes. The mutation scan correctly detected a GA insertion in the \textit{rrn} operon copies in both strains possessing three adjacent polymorphic sites in a 227 bp Intergenic Transcribed spacer between alanine tRNA and 23S rRNA genes—a C→G substitution in position 5, an A deletion in position 8 and GA→AG inversion in position 38 (Fig. 4E).

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