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Rapid Plasmid DNA Isolation from Mucoid Gram-Negative Bacteria

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Exopolysaccharides interfere with the isolation and characterization of plasmid DNA from gram-negative bacteria. To repress capsular polysaccharide production, bacteria were cultured in medium containing bismuth nitrate and sodium salicylate. Rapid removal of other contaminating bacterial surface components was achieved by mild acidic zwitterionic detergent extraction. After treatment, bacterial cells were more readily lysed in alkaline detergents. The resulting plasmid preparations contained virtually no capsular polysaccharide and relatively small quantities of lipopolysaccharide and protein, yet they produced yields of nucleic acids similar to those of conventional plasmid preparations. Conventional preparations from encapsulated organisms were largely insoluble and appeared as smears following agarose gel electrophoresis, with indefinite plasmid banding. Plasmids prepared by the new method were highly soluble in conventional buffers and exhibited high-resolution plasmid banding patterns in agarose gels. Plasmids as large as 180 kbp could be isolated and visualized, without apparent nicking, and were readily digested by restriction endonuclease enzymes. The method proved effective with encapsulated or mucoid strains of *Klebsiella pneumoniae*, *Escherichia coli*, *Acinetobacter anitratus*, *Salmonella typhimurium*, and *Enterobacter* species. The complete method for plasmid isolation was not suitable for *Pseudomonas aeruginosa* because of the inhibitory effects of bismuth. Thus, removal of contaminating bacterial surface structures enabled the rapid isolation and characterization of plasmids from mucoid clinical isolates, without the use of organic solvents, CsCl gradients, or expensive, disposable columns.

Many typing methods have been used for identifying bacterial strains. Phenotypic methods have included serotyping, phage typing, antibiotic susceptibility testing, biotyping, bacteriocin production, immunoblotting, outer membrane protein profiling, and isoenzyme analysis (1, 12, 20). More recent advances in typing systems rely on genetic methods, because of the relative instability of phenotype analysis (7).

Plasmid typing, or plasmid fingerprinting, is a relatively new addition to the typing systems used in clinical microbiology. Plasmid DNA content is a unique and relatively stable characteristic of most bacteria, and as such, it has gained importance as an epidemiologic tool (12, 18). Preparing plasmid DNA is a relatively simple, inexpensive procedure, and the same methodology applies to most microorganisms. In many cases, plasmid profiles offer a higher level of sensitivity than do other systems (9, 12, 18). Plasmid analysis has been found to be as specific as phage typing and superior to antimicrobial susceptibility testing in outbreak studies (10). Typically, one or more plasmids can be isolated from the cytoplasm of bacterial cells, especially among the medically important gram-negative bacteria (12).

Plasmids from one strain can be distinguished from those of another on the basis of their molecular sizes. Plasmid size is expressed as the number of kilobase pairs of DNA and is determined by electrophoretic migration in agarose gels. Questionable plasmid profiles can be further distinguished by restriction endonuclease analysis, which cuts large plasmids into smaller, unique fragments and adds greatly to the sensitivity of these tests (7). Similar digestion patterns virtually ensure plasmid identity and increase the probability of a common source (7).

The genetic information encoded on plasmid DNA can define or influence other typing systems used in clinical microbiology, including the biotype, serotype, and antibiotic susceptibility of a particular strain (24). The virulence of *Yersinia enterocolitica* O:3 is largely dependent on the presence of a 72-kbp plasmid (22). Many virulence determinants of *Shigella* species and enteroinvasive *Escherichia coli* are encoded by 180- to 230-kbp plasmids (8). The genes for iron acquisition, adherence to gut epithelium, and the mucoid phenotype of *Klebsiella pneumoniae* are all located on a 180-kbp plasmid (4, 19). To add to their clinical importance, these large plasmids often contain antibiotic resistance determinants (4, 21).

Standard techniques in molecular biology often do not work well when bacteria other than *E. coli* K-12 are used. Rapid methods devised to screen plasmid DNA from clinical bacterial isolates are usually inadequate, particularly when large plasmids are present (12). Capsular polysaccharides (CPSs) and lipopolysaccharides (LPSs) interfere not only with plasmid extraction and purification but also with plasmid banding on agarose gels (5). Recently, two methods that facilitate the purification of macromolecules from encapsulated bacteria have been developed in our laboratory. These include a method to inhibit the production CPS (6) and another to remove outer membrane materials with minimal bacterial lysis (5). In this report, we describe a modification of a rapid plasmid isolation method designed to remove contaminating extracellular substances.

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MATERIALS AND METHODS

Bacteria and media. The strains used in these studies were *K. pneumoniae* 52145 (19) and a nonencapsulated variant, 52145-NCV. *Pseudomonas aeruginosa* 20117 was a gift from the Schering-Plough Research Division (Bloomfield, N.J.). *Salmonella typhimurium* ATCC 14028 was obtained from Difco Laboratories (Detroit, Mich.). Several mucoid gram-negative bacteria isolated in the hospital clinical microbiology section during the study period were screened for their plasmid contents. Clinical isolates were obtained on MacConkey agar and were subcultured on nutrient agar. Select mucoid colonies were cultured overnight in Luria broth (LB) medium at 35°C with gentle rocking. The number of CFU per milliliter was determined by standard plating techniques on nutrient agar medium.

Removal of interfering macromolecules. Bismuth nitrate and sodium salicylate (Sigma Chemical Co., St. Louis, Mo.) were added to LB medium at concentrations of 0.5 and 2.5 mM, respectively, to inhibit the production of CPS (6). Bismuth subsalicylate (Procter and Gamble, Cincinnati, Ohio) was also used. Stock solutions of 100 mM $Bi(NO_3)_3$ were prepared in alkaline (400 mM NaOH) propylene glycol. Stock solutions of 250 mM sodium salicylate (pH 7) were prepared in 50% propylene glycol. Stock solutions were prepared fresh every 3 to 5 days and were kept at 4°C. For plasmid minipreparations, 1 ml of an overnight bacterial culture was transferred to a sterile 1.7-ml microcentrifuge tube. EDTA was added to a final concentration of 5 mM. Stock solutions of 500 mM sodium EDTA (pH 10; Sigma) were prepared in purified water. Tubes were vortexed and allowed to sit at room temperature for 5 min. Samples were centrifuged at 8,000 rpm for 2 min. Supernatants were carefully yet thoroughly removed and discarded. Bacterial pellets were resuspended in 975 μl of saline, and 25 μl of 1% Zwittergent 3-14 detergent (Calbiochem, San Diego, Calif.) in 100 mM citric acid was added. For highly encapsulated strains, 50 μl of detergent was used. Samples were gently mixed and were then incubated for 10 min at 37°C. Samples were centrifuged and the supernatants were discarded as described above. The plasmid isolation method was essentially that of Birnboim and Doly (2) through the potassium acetate step; this was followed by precipitation in 1 volume of isopropanol.

Agarose gel electrophoresis. Plasmid samples were resuspended in TE buffer and were mixed with a 10× loading buffer containing RNase (2 μg/ml; Boerhinger Mannheim, Indianapolis, Ind.), as outlined by Maniatis et al. (17). Samples were loaded onto 0.5 to 0.8% agarose (LE; Sigma) minigels equilibrated with TBE buffer and were electrophoresed at 40 to 60 V. Gels were stained with ethidium bromide; this was followed by two 20-min washes in water. Plasmids were further characterized by digestion with the *Eco*RI restriction endonuclease (Boerhinger Mannheim). Photographs of gels were prepared by using a DNA photographic transilluminator system (Fotodyne, New Berlin, Wis.).

Characterization of plasmid purity. Plasmid preparations from 50 ml of culture were treated briefly with RNase (10 μg/ml) and were placed on a Sepharose 6B gel filtration column (28 by 1.5 cm; Pharmacia, Uppsala, Sweden) equilibrated with 0.05% Zwittergent in 10 mM citrate (pH 4.5) and 25 mM NaCl (5). Flow rates were approximately 0.75 ml/min. Fractions of 3 ml were collected. Column fractions were analyzed for CPS by the uronic acid assay of Blumenkrantz and Asboe-Hansen (3). LPS was estimated by the method of Karkhanis et al. (14), which is a measure of 2-keto-3-deoxyoctulosonic acid. LPS is expressed in equivalents of *E. coli* O55:B5 LPS (Sigma). Nucleic acid content was assessed spectrophotometrically at 260 nm and was visualized on agarose gels. Protein was estimated by the method of Lowry et al. (16).

RESULTS

Removal of CPS. Previous studies demonstrated that addition of bismuth and salicylate to the culture medium resulted in >90% reduction of CPS expression by encapsulated *K. pneumoniae* (6). Although bismuth subsalicylate (0.5 mM) was adequate for these purposes, optimum plasmid preparations were obtained by using 0.5 mM $Bi(NO_3)_3$ and 2.5 mM sodium salicylate. Some bacterial strains were inhibited by bismuth salts at these concentrations, although growth could be restored to near-normal levels with the addition of ferric iron to the culture medium. Iron at 15 μM appeared to counteract the bismuth-mediated growth inhibition, but higher iron concentrations (0.15 to 1 mM) were required to significantly increase the production of CPS (unpublished data).

Cultures of highly encapsulated bacteria are viscous and difficult to sediment by centrifugation. Conversely, overnight growth in Bi^{3+}–salicylate-containing medium enabled bacteria to pellet as readily as *E. coli* K-12 did. However, the pelleted cells were soiled with Bi^{3+}. Before centrifugation, it was necessary to remove the Bi^{3+} that adhered to the bacteria. This was accomplished by adding 5 mM EDTA to cultures for 5 min at room temperature. Plasmids from samples not treated with EDTA showed excessive smearing on agarose gels. Treatment with EDTA also removes LPS and other cell surface components from the plasmid preparations (15).

Removal of other interfering macromolecules. Extraction with an acidic zwitterionic detergent solution prepared bacteria for lysis and plasmid isolation. This procedure removes virtually all of the CPS and much of the LPS and protein, while it leaves the cells largely intact (5). The detergent step greatly facilitated subsequent lysis in alkaline sodium dodecyl sulfate (SDS) and also reduced the amount of precipitate formed after the addition of potassium acetate. Finally, the plasmid pellet formed by isopropanol precipitation was from one-half to one-fifth the size of those produced by conventional methods.

Plasmid DNA analysis. The criteria for plasmid analysis of mucoid bacteria were deciphered by using strain 52145 of *K. pneumoniae*. Plasmid profiles from preparations of treated and untreated *K. pneumoniae* cultures are shown in Fig. 1. Lanes A to D show plasmids from the highly encapsulated 52145 strain, and lanes E to H show plasmids from the unencapsulated strain 52145-NCV. No treatment (lanes A and E) or Bi^{3+}–salicylate–EDTA treatment (lanes B and F) gave profiles without plasmid bands for strain 52145 and smeared profiles with much chromosomal DNA contamination for strain 52145-NCV. However, both the 100- and 180-kbp plasmids were visible after Bi^{3+}–salicylate treatment of 52145-NCV. Acidic zwitterionic detergent treatment without the prior Bi^{3+}–salicylate step (lanes C and G) gave profiles that showed sharp plasmid banding but a low yield for strain 52145 and a good yield for strain 52145-NCV. Plasmid profiles from fully treated cultures (lanes D and H) showed sharp banding and good relative yields in all cases.

The macromolecular compositions of treated and untreated *K. pneumoniae* plasmid preparations, as revealed by

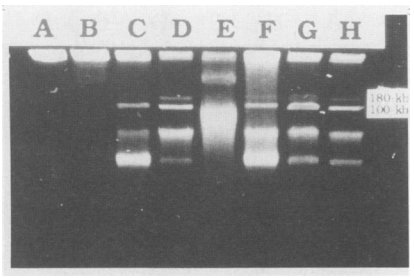

FIG. 1. Plasmid DNA resolution on agarose gels. *K. pneumoniae* 52145 (lanes A to D) and 52145-NCV (lanes E to H) plasmids were electrophoresed in 0.5% agarose. Lanes A and E, conventionally prepared samples (2). Samples were treated with Bi^{3+}–salicylate only (lanes B and F) or Zwittergent only (lanes C and G) or were fully treated (lanes D and H).

gel filtration chromatography, are shown in Fig. 2. While the total amounts of nucleic acid in the two preparations were comparable, most of the LPS, protein, and CPS were removed from the treated preparations. Both samples exhibited a large, digested RNA peak and a minor void volume peak (optical density at 260 nm = 0.5; elution volume = 18 ml) containing most of the plasmid and chromosomal DNAs. Agarose gel profiles of these column fractions are displayed in Fig. 3. Sharp plasmid banding (Fig. 3A, lanes 1 and 2) was seen in treated cultures. Preparations from untreated cultures (Fig. 3B) exhibited nucleic acid smears with no plasmid banding. Furthermore, the RNAs from treated cultures (Fig. 3A, lanes 9 to 11) were digested more thoroughly than were the RNAs from untreated cultures (Fig. 3B, lanes 4 to 7).

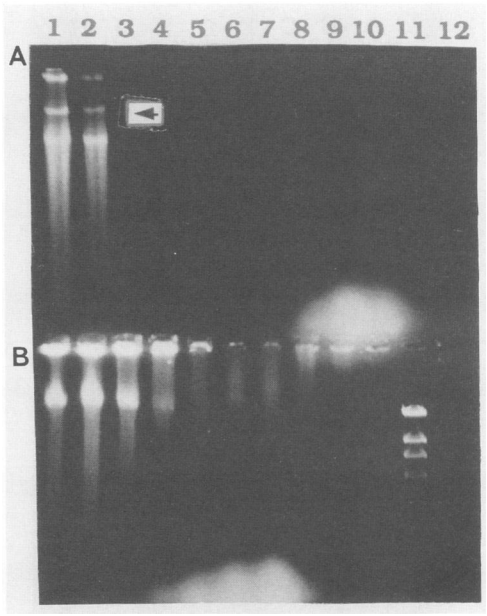

FIG. 3. Agarose gel analysis of gel filtration column fractions. Plasmid samples fractionated by Sepharose 6B gel filtration were electrophoresed on 0.5% agarose. (A) Fractions from samples treated with bismuth-salicylate and acidic zwitterionic detergent. The arrow indicates where large plasmids migrated in the gel. (B) Fractions from conventional plasmid preparations (2). The void volume of the column is represented in lanes 1 to 3. *Hin*dIII-restricted bacteriophage lambda standards are shown in panel B, lane 12.

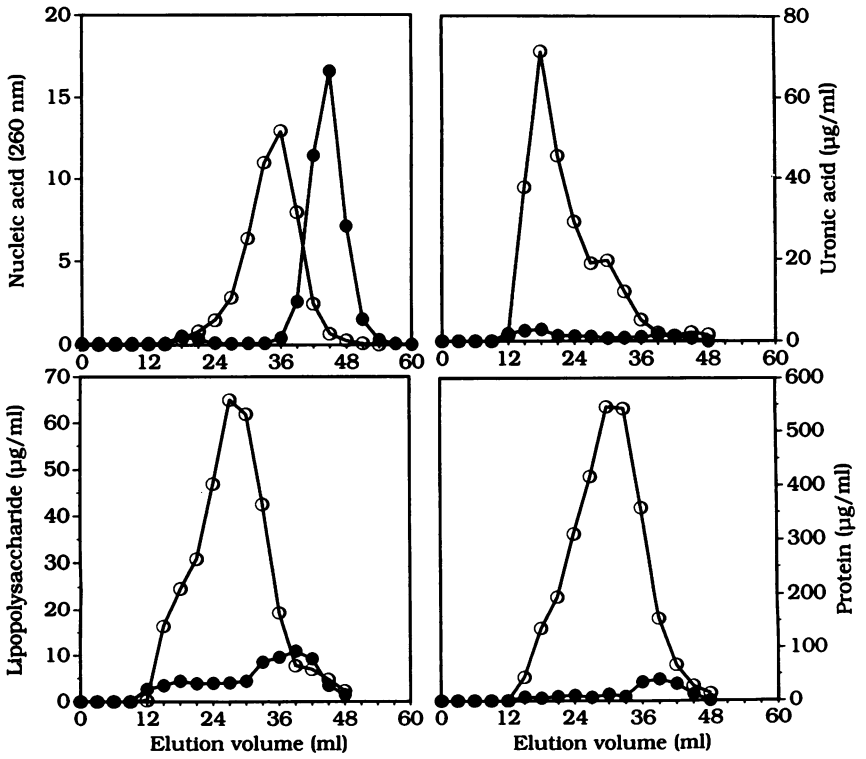

FIG. 2. Macromolecular analysis of plasmid preparations. Plasmids from 40 ml of treated (●) or untreated (○) cultures of *K. pneumoniae* 52145 were fractionated by Sepharose 6B gel filtration. Samples were treated with RNase prior to column loading. Fractions were tested for total nucleic acid, CPS (uronic acid), LPS (2-keto-3-deoxyoctulosonic acid), and protein.

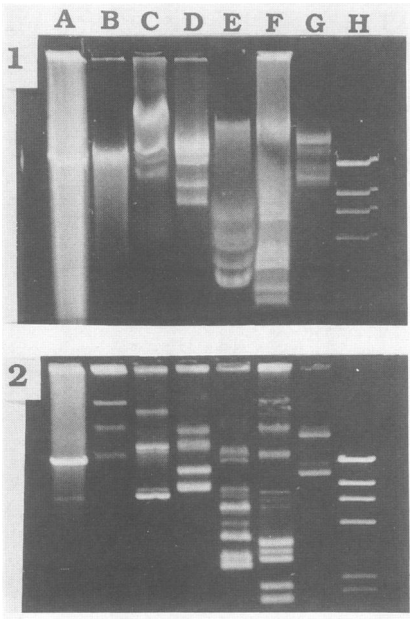

FIG. 4. Plasmids from clinical isolates. Plasmids from *P. aeruginosa* 20117 (lane A); *S. typhimurium* ATCC 14028 (lane B); and mucoid clinical isolates of *A. anitratus* (lane C), *K. pneumoniae* (lane D), *E. coli* (lane E), *E. cloacae* (lane F), and *E. aerogenes* (lane G) were electrophoresed on 0.8% agarose. Lane H, *Hin*dIII-restricted bacteriophage lambda DNA standards. The upper gel (panel 1) shows plasmids produced by conventional methods (2). The lower gel (panel 2) shows plasmids produced by bismuth-salicylate and acidic zwitterionic detergent treatments. No bismuth-salicylate salts were used when culturing *Pseudomonas* strains.

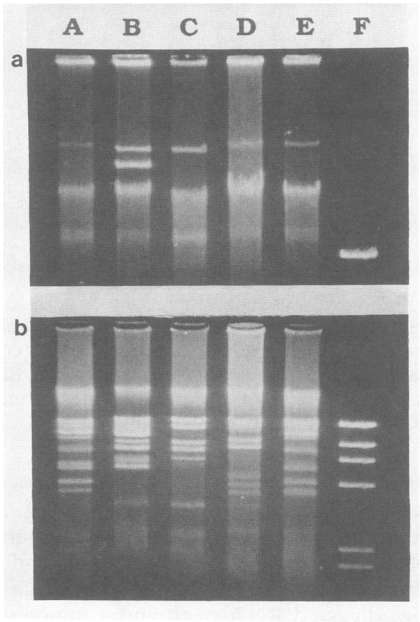

FIG. 5. Plasmid analysis of *E. coli* clinical isolates. Plasmid samples from five mucoid *E. coli* isolates (lanes A to E) were electrophoresed on 0.8% agarose. Plasmids prepared from cultures treated with bismuth-salicylate and acidic zwitterionic detergent are shown in panel a. Restriction endonuclease digests of plasmids with *Eco*RI are shown in panel b. Lane F, *Hin*dIII-restricted bacteriophage lambda DNA standards.

Incomplete RNA digestion was also indicated by comparing gel filtration elution volumes (Fig. 2).

Mucoid clinical isolates. Plasmids from hundreds of clinical bacterial isolates were also examined. A sampling from different genera of gram-negative bacteria is shown in Fig. 4. Except for *Pseudomonas* strains, all cultures were treated with Bi^{3+}–salicylate–EDTA; this was followed by treatment with acidic zwitterionic detergent. No Bi^{3+} or salicylate was added when culturing *Pseudomonas* strains, since Bi^{3+} exacerbated the smearing of plasmids on agarose gels, even when ferric iron was used to counteract Bi^{3+}-mediated effects, and salicylate produced no discernible effect. Nevertheless, agarose gel profiles of *Pseudomonas* plasmids (Fig. 4, lane A) from acidic detergent treatment (lower gel) were superior to those of untreated samples (upper gel). The same held true for plasmids from *S. typhimurium* ATCC 14028 (lane B) and isolates of *A. anitratus* (lane C), *E. coli* (lane D), *K. pneumoniae* (lane E), *Enterobacter cloacae* (lane F), and *Enterobacter aerogenes* (lane G). Of particular note was the complete absence of large plasmids in the upper gel of Fig. 4 and their appearance in several samples in the lower gel.

Restriction endonuclease analysis. Of the numerous isolates obtained from our clinical microbiology section, five mucoid *E. coli* isolates were received in a 1-week span. They originated from different clinical departments and were isolated from various anatomical sites. Plasmid fingerprinting analysis was performed to test for their relatedness. All isolates harbored similar-sized, large plasmids, as seen in Fig. 5a. Plasmid samples produced by conventional alkaline SDS lysis or boiling methods (11, 13, 23) were not visible on agarose gels (data not shown). Strain relatedness was analyzed by using restriction endonucleases. *Eco*RI digestion patterns are shown in Fig. 5b. The similar restriction patterns in lanes A and E suggest that the strains harboring these large plasmids are related.

DISCUSSION

Isolation of plasmids from encapsulated aerobic gram-negative bacilli posed the following difficulties at various stages in the isolation procedure. (i) Bacteria were difficult to sediment because of the viscous nature of CPS; (ii) lysis of bacteria was impeded, as indicated by the lack of clearing when alkaline SDS was added; (iii) the precipitate that formed after the addition of cold potassium acetate was also highly viscous and could not be removed completely by centrifugation, even after successive spins; (iv) plasmid preparations did not dissolve in Tris buffers prior to electrophoresis; and (v) plasmids were not resolved on agarose gels, either from aggregation in gel wells or from being masked or skewed by the amorphous nature of the preparation.

The data indicate that the CPSs in the samples aggregated with plasmid DNA in the agarose gel wells and that other contaminating macromolecules interfered with efficient plasmid analysis. DNA and CPS share similar properties that make it difficult to separate the two macromolecules. Both coelute in gel filtration chromatography because of their high molecular masses. Both precipitate in alcohols. Both are of a strong ionic character that enhances their capacity for interaction with other molecules. It is likely that extensive salt bridging between DNA and CPS causes the massive aggregation and smearing seen in plasmid samples from

encapsulated bacteria. When purified CPS or LPS (5) was added back to clean plasmid preparations from *K. pneumoniae* 52145, only the CPS was shown to smear the resultant agarose gel profiles (data not shown). Other bacterial cell surface structures, such as LPS and protein, appeared to render bacteria refractory to lysis and may have interfered with the reannealing of double-stranded DNA after alkaline denaturation. Removal of these substances overcame the difficulties described above and allowed for reliable and satisfactory analysis of plasmids from mucoid, gram-negative isolates.

The difficulty in isolating large plasmids has been stressed elsewhere (12, 13). In conventional alkaline lysis procedures (2, 17), plasmids are efficiently separated from chromosomal DNA and cell debris during a melting-reannealing step. Because of their relatively small size and proximity, single sister strands of plasmid DNA reanneal rapidly. However, large plasmids do not reanneal as rapidly after melting. Similar to chromosomal DNA, the larger plasmids apparently interact and aggregate with cellular debris in the lysis mixture before they can snap back to their double-stranded state. The result is the loss of such plasmids to precipitation. The isolation of large plasmids is thus improved by removing extracellular cell debris before cell lysis and DNA denaturation. Under these conditions, large plasmids may reanneal more efficiently and, thus, remain in solution as the insoluble cell debris is removed by precipitation.

The difficulty in isolating large plasmids has been considered a problem of low plasmid copy number (12). While small plasmids are carried by bacteria at several copies per cell, large plasmids are usually carried at one or two copies per cell. In our studies, this was noted by the loss of large plasmids after repeated subculture of clinical isolates. However, emphasis must also be placed on the method used to visualize large plasmids. The currently available rapid plasmid isolation methods are too insensitive for the isolation of large plasmids present in low copy number.

Plasmid typing was possible among mucoid clinical isolates only after repression or removal of exopolysaccharides. Other genetic typing methods such as rRNA analysis, chromosomal fingerprinting, or DNA probing should also be easier to develop once these polysaccharides are eliminated. In essence, the techniques outlined in this report make working with clinical isolates as simple as manipulating *E. coli* K-12, without the need for organic solvents, CsCl gradients, or expensive purification columns.

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