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Characterization of Trimethoprim Resistance by Use of Probes Specific for Transposon Tn7

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Transposon Tn7 codes for resistance to trimethoprim and streptomycin. For detection of Tn7 by DNA-DNA hybridization, two recombinant plasmids were constructed. The former contained a 1-kilobase *Bam*HI fragment and the latter contained a 4.3-kilobase *Eco*RI-*Bam*HI fragment of Tn7. These DNA fragments, which did not include the drug resistance genes, were used as probes for detecting Tn7-like sequences in bacterial strains by colony hybridization. They hybridized strongly to bacterial DNA known to carry Tn7 but not to DNA known to carry transposons other than Tn7. These probes were used to study the occurrence of Tn7 in bacterial strains isolated in the Turku City Hospital in Finland. Transposon Tn7 was present in 47.2% of 199 trimethoprim-resistant enterobacteria (MIC ≥ 8 μg/ml). Among the 69 *Proteus mirabilis* strains studied, 75% contained Tn7, although none of these strains transferred trimethoprim resistance in conjugation tests. The reliability of colony hybridization was further confirmed by Southern hybridization to detect the Tn7-specific 2.6-kilobase *Hin*dIII restriction fragment. Colony hybridization proved to be a sensitive and rapid method for detecting Tn7-determined sequences.

Trimethoprim is an antimicrobial agent that inhibits bacterial dihydrofolate reductase (DHFR). It has been widely used in combination with sulfonamides in the treatment of different infections. Trimethoprim as a single agent has been used in the treatment and prophylaxis of urinary tract infections in Finland since 1973 and thereafter in other countries as well (19). In spite of the suspicion about quick development of resistance to trimethoprim, resistance seems to occur in Finland at the same frequency as resistance to other antimicrobial agents commonly used in the treatment of urinary tract infections (15, 17). Trimethoprim resistance is increased in the hospitals with long-term patients (15). In the Turku City Hospital, Finland, trimethoprim resistance (MIC ≥ 8 μg/ml) occurs in nearly 40% of urinary tract isolates studied (15). Among the strains that are highly resistant to trimethoprim (MIC > 1,000 μg/ml), resistance is transferable in 13 to 20%, depending on the species studied (16).

Trimethoprim resistance genes encode DHFRs that are not inhibited by trimethoprim. These genes are located in different transposons, of which transposon Tn7 is the best known (3, 5, 7, 10, 11, 13, 14, 27). Tn7 contains a gene for type I DHFR (10, 11, 27). Another transposon coding trimethoprim resistance is Tn*402*. It contains a gene for type II DHFR (10, 26). Fling et al. (12) have recently found a new resistant enzyme, which is different from the type I and type II DHFRs. Before this, Nugent et al. (23) found a new trimethoprim resistance transposon, Tn*735*, but detailed information about this transposon is not yet available.

Rapid detection and classification of trimethoprim resistance genes will be of primary importance for clinical and epidemiological studies of trimethoprim resistance. For this purpose, we constructed Tn7-specific probes from the known plasmid ColE1::Tn7 (9). These probes contain parts of Tn7 but do not contain the gene for DHFR. We used these probes for detection of Tn7 in certain control strains and routine bacterial isolates by use of colony hybridization and Southern hybridization techniques.

MATERIALS AND METHODS

Bacterial strains. Control bacterial strains and their plasmids are listed in the Table 1. Urinary tract isolates used in this study were collected in the Turku City Hospital (15).

Biochemicals. Restriction endonucleases, T4 DNA ligase (EC 6.5.1.1), nitrocellulose sheets, and nick-translation kits were purchased from Bethesda Research Laboratories, Bethesda, Md., and Boehringer, Mannheim, Federal Republic of Germany. [^{32}P]deoxycytidine triphosphate was purchased from Amersham International, Amersham, U.K.

Isolation of plasmid DNA. Plasmid DNA was purified by a modification of the cleared lysate method of Kahn et al. (18), employing lysozyme and Triton X-100. Cleared lysates were chromatographed in a column (1 by 100 cm) of Sephacryl S-1000 (Pharmacia Fine Chemicals, Uppsala, Sweden) in 50 mM Tris-hydrochloride (pH 8.0) containing 100 mM NaCl (29). Fractions containing plasmid DNA were ethanol precipitated and used for cloning experiments.

For analytical purposes (Southern hybridization), bacterial DNA from control and clinical strains was isolated by the method of Davis et al. (8) with minor modifications. Before ethanol precipitation, the lysates were extracted with phenol-chloroform and dialyzed for 2 days against 10 mM Tris-hydrochloride (pH 7.5) –1 mM EDTA.

Construction of recombinant plasmids pTJ7 and pTJ22. Plasmid ColE1::Tn7 DNA (a gift from Lynn P. Elwell, Burroughs Wellcome) (9) (Fig. 1) was used as the source of Tn7-specific fragments for cloning into the vector plasmid pBR322.

Plasmids ColE1::Tn7 and pBR322 were cut with restriction endonucleases *Eco*RI (EC 3.1.23.13) and *Bam*HI (EC 3.1.23.6). Cut plasmids (1 μg each) were ligated together with T4 DNA ligase (1 U) at 15°C for 16 h. Transformation into *Escherichia coli* HB101 was carried out in 50 mM $CaCl_2$, as described by Mandel and Higa (20). Transformed bacteria containing recombinant plasmids grew on ampicillin plates (12.5 μg/ml) but not on tetracycline plates (25 μg/ml).

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TABLE 1. Colony hybridization of reference strains with nick-translated *Bam*HI and *Eco*RI-*Bam*HI fragments of Tn*7* used as probes

E. coli strains and plasmids	Transposon	Hybridization results with both probes	Source of strains
C600(ColE1::Tn*7*)	Tn*7*	+	L. Elwell (9)
J62:Tn*7*	Tn*7*	+	N. Datta (7)
C600(pBW1)	Tn*7*	+	N. Datta (7)
J62-2(RP4::Tn*78*)	Tn*7*	+	N. Datta (31)
J62-2(RP4::Tn*79*)	Tn*7*	+	N. Datta
J53-2(pHH1268)	Tn*7*	+	N. Datta (24)
J53-2(pHH1269)	Tn*7*	+	N. Datta (24)
J53(R483)	Tn*7*	+	N. Datta (1)
C600(R721)	Tn*7*	+	N. Datta (7)
J62-2(pTH1)	Tn*7*	+	N. Datta (7)
HB101(pTJ1)	Part of Tn*7*	−	This study
J53(R751)	Tn*402*	−	N. Datta (7)
HB101(pFE364)	Tn*402*	−	L. Elwell (9)
116(pHH500)	Tn*735*	−	N. Datta (7)
J62-2(pHH502)	Tn*735*	−	N. Datta (22)
J53(R388)		−	N. Datta (7)
J53-1(R389a)		−	N. Datta (7)
J53-1(R27)		−	N. Datta (7)
HB101(pFE373)		−	L. Elwell (9)
J53(pHH1307)	Tn*733*[a]	−	N. Datta (7)

[a] Transposon Tn*733* contains only a gentamicin resistance gene. Plasmid pHH1307 contains also a trimethoprim resistance gene (7).

Plasmids were isolated as described above and characterized by *Eco*RI, *Bam*HI, and *Hin*dIII (EC 1.3.23.21) digestions followed by agarose gel electrophoresis. Plasmid pTJ7 contained the 1-kilobase (kb) *Bam*HI fragment of Tn*7* (Fig.1) inserted into the *Bam*HI site of pBR322. In plasmid pTJ22, the 4.3-kb *Eco*RI-*Bam*HI fragment of Tn*7* (Fig. 1) was inserted between the *Eco*RI and *Bam*HI sites of pBR322.

Preparation of probes. Plasmid pTJ7 was digested with the restriction endonuclease *Bam*HI, and plasmid pTJ22 was digested with endonucleases *Bam*HI and *Eco*RI as described above. The reaction mixtures were electrophoresed in a 0.75% agarose gel containing ethidium bromide (1 mg/ml). Tn*7*-specific fragments were cut out, and DNAs were electroeluted (21). Isolated DNA fragments were nick-translated to a specific activity of ca. 5×10^7 cpm/μg of DNA, according to the instructions of the manufacturer. Before hybridization, the probes were denatured by heating at 100°C for 10 min.

Colony hybridization. Bacterial strains were grown on Whatman 540 filter paper on nutrient agar plates containing trimethoprim (1,000 μg/ml) for 5 h. As *Proteus mirabilis* strains swarmed on the nutrient agar, filters were inoculated heavily with these cultures and processed immediately. Filters were processed as described by Thayer (30). After prehybridization, the filters were hybridized for 20 h at 65°C with nick-translated ^{32}P-labeled DNA probes (2×10^5 to 5×10^5 cpm) followed by washing.

Southern hybridization. Southern hybridization was performed as described previously (28). The dialyzed and precipitated DNA preparations were digested with endonuclease *Hin*dIII (5 U). Reaction mixtures were electrophoresed in a 0.7% agarose gel and DNA fragments were transferred to nitrocellulose and hybridized with nick-translated probes (2×10^5 to 5×10^5 cpm).

RESULTS

To test the ability of *Eco*RI-*Bam*HI and *Bam*HI probes to detect Tn*7*, 20 *E. coli* control strains were tested by colony hybridization. All the strains containing Tn*7* showed strong hybridization with both probes as expected (Table 1). Bacterial strains carrying transposons other than Tn*7* were clearly negative. Because of identical results obtained with the two probes, the *Bam*HI fragment of Tn*7* was used as a probe in further experiments.

This method was used for detection of Tn*7* in trimethoprim-resistant urine isolates (MIC ≥ 8 μg/ml) collected in the Turku City Hospital from 1980 to 1981 (15, 16). Earlier studies have shown that the frequency of trimethoprim-resistant strains (MIC ≥ 8 μg/ml) in this material is 38.3%, and resistance was transferable in 12.8% of resistant strains (15, 16). All the 199 gram-negative isolates (excluding *Pseudomonas* species) found in this study were tested by colony hybridization with the *Bam*HI fragment of Tn*7* used as a probe. Colony hybridization was positive in 47.2% of the strains studied (Table 2). *E. coli* strains carried Tn*7* in 42% of the 76 strains studied, and *Klebsiella* spp. carried Tn*7* in 12.9% of the 31 strains studied. Other enterobacteria, including *Citrobacter freundii*, *Enterobacter cloacae*, *Enterobacter agglomerans*, *Providencia stuartii*, *Serratia liquefaciens*, *Achromobacter xylosoxidans*, and *Acinetobacter calcoaceticus*, contained Tn*7* in 26.1% of the 23 strains; all Tn*7*-positive bacteria belonged to the first two species. *Proteus mirabilis* showed positive hybridization in 75% of the 69 strains (Fig. 2). All the colony hybridization-positive strains were highly resistant (MIC > 1,000 μg/ml) to trimethoprim. Among the highly resistant isolates, colony hybridization was positive in 69.1% of them.

As a control for the colony hybridization study, Southern hybridizations were carried out. For this purpose, the same 20 control *E. coli* strains as described above (Table 1) and 17

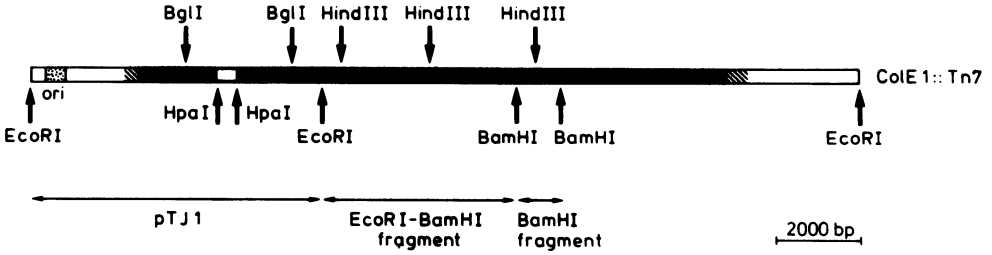

FIG. 1. Restriction maps of plasmids ColE1::Tn*7* and pTJ1. The darkened areas show Tn*7*-specific sequences. The white area between *Hpa*I sites shows the DHFR gene (11, 13, 27). The other *Hpa*I sites are not marked in the picture. Plasmid pTJ1 also contains the gene coding for streptomycin resistance. Plasmid pTJ1 was constructed by digesting ColE1::Tn*7* DNA with restriction endonuclease *Eco*RI followed by religation and transformation. bp, Base pairs.

TABLE 2. Colony hybridization of trimethoprim-resistant gram-negative strains (MIC ≥ 8 μg/ml; excluding *Pseudomonas* species) isolated in the Turku City Hospital during 1980 to 1981 with nick-translated *Bam*HI fragment of Tn*7* used as a probe

Species	Total no. of strains	No. colony hybridization positive (%)[a]
Escherichia coli	76	32 (42.0)
Proteus mirabilis	69	52 (75.4)
Klebsiella spp.	31	4 (12.9)
Citrobacter freundii	7	5 (71.4)
Enterobacter cloacae	3	1 (33.3)
Enterobacter agglomerans	2	0
Providencia stuartii	8	0
Serratia liquefaciens	1	0
Achromobacter xylosoxidans	1	0
Acinetobacter calcoaceticus	1	0

[a] Of the 199 organisms studied, 94 (47.2%) were colony hybridization positive.

colony hybridization-positive and 20 colony hybridization-negative strains were used. In all control strains known to contain Tn*7*, a 2.6-kb *Hin*dIII fragment of Tn*7* was found (data not shown). The control strains negative by colony hybridization were also negative by the Southern hybridization technique (Fig. 3). Among clinical isolates, positive correlation by colony hybridization and Southern hybridization techniques was complete, but a few strains containing the *Hin*dIII fragment of Tn*7* were negative by colony hybridization. The reason for this might be difference in the sensitivity of the Southern and colony hybridization methods or the number of Tn*7* copies, which may vary between different strains.

DISCUSSION

Epidemiological studies on the spread of Tn*7* have not been made before. There are, however, some references to the development of this method for detecting Tn*7* in bacterial strains (5, 7, 9, 12). Datta and co-workers (7) have used the whole ColE1::Tn*7* and Fling and Elwell have used the plasmid pFE506 (12) as probes. These plasmids contain genes for DHFR type I and streptomycin resistance. The DHFR type I gene has sequence homology with the chromosomal DHFR gene of *E. coli* (11, 27). In addition, these contain ColE1 plasmid DNA sequences, which may be present in many naturally occurring plasmids. It is likely that this kind of DNA probe may give false-positive results by the colony hybridization technique. In this study, we used purified restriction fragments of Tn*7* as probes; a similar approach has been used by Fling et al. (12).

The *Eco*RI-*Bam*HI and *Bam*HI fragments, which we used as probes in the colony hybridization test, gave identical results and corresponded to the results of the Southern hybridization test. We found no cross-hybridization of *Bam*HI fragments with other transposons coding for trimethoprim or gentamicin resistance (Fig. 3). In preliminary studies, we attempted to use a DNA fragment containing the DHFR type I gene of Tn*7* as a probe. For that purpose we constructed a recombinant plasmid pTJ1 corresponding to plasmid pFE506 described by Fling and Elwell (10); a 2.5-kb *Bgl*I fragment of pTJ1 containing the DHFR type I gene (11, 13, 27) was used as a probe. Colony hybridization of control strains revealed that this fragment hybridized to DNA of bacterial strains containing Tn*7*, but to a variable extent also to DNA from bacteria known to carry trimethoprim resistance other than that determined by Tn*7*. Recently, it has been shown that the DHFR type I gene has sequence homology with the DHFR gene of chromosomal DNA of *E. coli* (11, 27), which has clarified our results.

The use of the *Bam*HI fragment as a specific probe for Tn*7* requires careful elimination of all pBR322 sequences before nick translation. This was tested by Southern hybridization; if contaminating pBR322 sequences were present, light hybridization to pBR322 on the filter was seen. One of the negative control strains used in colony hybridization filters was *E. coli* HB101(pFE364). Plasmid pFE364 is a pBR322 derivative containing a DNA fragment from Tn*402* (5). These colonies showed either negative or very light positive hybridization.

In Southern hybridization after *Hin*dIII restriction enzyme digestion, two of the colony hybridization-positive colonies

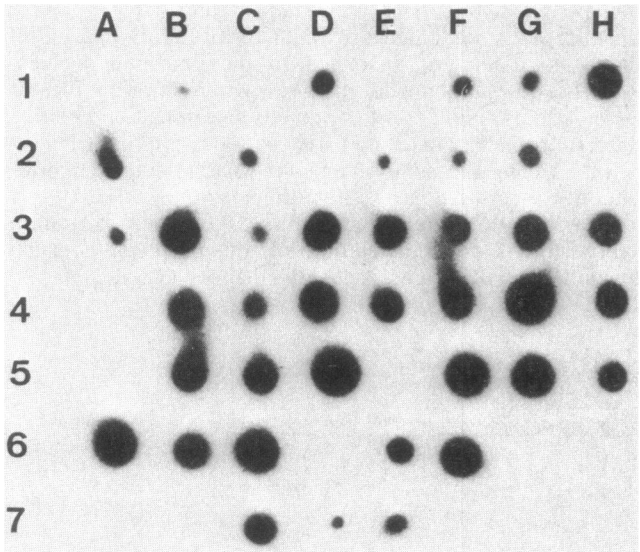

FIG. 2. Colony hybridization of 50 *P. mirabilis* strains with nick-translated *Bam*HI fragment of Tn*7* used as a probe. Hybridization and autoradiography were performed as described in the text. As positive controls, *E. coli* C600(ColE1::Tn*7*) (C7), *E. coli* J62::Tn*7*(D7), and *E. coli* J53(R483) (E7) were used. The negative controls were *E. coli* HB101(pTJ1) (F7), *E. coli* J53(R751) (G7), and *E. coli* HB101(pFE364) (H7).

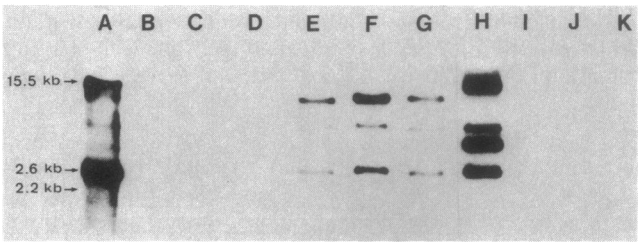

FIG. 3. Southern hybridization of *Hin*dIII-digested DNAs isolated from clinical strains positive (lanes E, F, G, and H) and negative (lanes B, C, and D) by colony hybridization with nick-translated *Bam*HI fragment of Tn*7* used as a probe. As a positive control, ColE1::Tn*7* was used (lane A), and as negative controls R388 (lane I), R751 (lane J), and pHH1307 (lane K) were used. Hybridization and autoradiography were performed as described in the text. The *Hin*dIII fragments of ColE1::Tn*7* as molecular size standards are marked on the left. Note that the 2.2-kb fragment does not hybridize with the probe used; it is seen only in the agarose gel after staining with ethidium bromide.

contained plasmids that exhibited an anomalous hybridization pattern; no 2.6-kb *Hin*dIII fragment was seen. We are currently investigating the exact nature of these plasmids. It is conceivable that these plasmids could be new uncharacterized Tn*7* derivatives. Early studies on Tn*7* have also shown polymorphism of *Hin*dIII restriction sites (2, 3).

When the colony hybridization and the conjugation methods are compared, colony hybridization is more reliable in detecting transposon Tn*7*-mediated trimethoprim resistance. Tn*7*-positive strains were detected by the colony hybridization technique about five times more frequently than by the conjugation test, although transferable transposons other than Tn*7* were also detectable by the conjugation test. An interesting observation is that Tn*7* was detected in 75% of trimethoprim-resistant *P. mirabilis* strains, whereas none of these strains transferred this resistance in the conjugation test (16). In addition to greater sensitivity, colony hybridization can also be used to classify the different transposons. However, it is possible that other transposons or other genes are related to Tn*7*. Thus, using colony hybridization, we can only state that Tn*7*-like sequences are present in the bacteria that show positive hybridization. The actual presence of Tn*7* was supported by Southern hybridization of selected samples and by the high MICs characteristic of transposon-mediated trimethoprim resistance.

In the case of *P. mirabilis* strains, the phase of bacterial growth was eliminated, and the time consumed was shortened by 5 h. This modification did not affect the results, which were revealed by a separate experiment with *E. coli* by using both the regular and heavy inoculum techniques (data not shown).

Nucleic acid hybridization has a number of other applications in microbial diagnostics, e.g., in detection of enterotoxigenic *E. coli* (22) and viruses (4, 6, 32) and in taxonomical studies of bacterial plasmids (25). Detection of transposon-mediated antibiotic resistance is now added to the list. For detection of trimethoprim resistance, transposon Tn*7* colony hybridization provides a rapid and sensitive method that is easily applicable to large numbers of samples, including those from epidemiological studies.

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