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Plasmid-Borne or Chromosomally Mediated Resistance by Tn7 Is the Most Common Response to Ubiquitous Use of Trimethoprim

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The folic acid analog trimethoprim has been in clinical use for more than 10 years. The use of it in Sweden has doubled in the last 6 to 7 years, and from the distribution statistics it can be calculated that during 1 year 4 to 5% of the population in Sweden are given this drug. The bacterial resistance mechanisms to be found in response to such a selection pressure were investigated in a relatively isolated population in northern Sweden (the county of Jämtland), in which one centrally located bacteriological laboratory serves the area. Trimethoprim-resistant strains were collected during an 8-month period from consecutive specimens of bacteria from the urinary tracts of patients. Among the highly resistant strains of enteric bacteria, trimethoprim resistance mediated by transposon-borne dihydrofolate reductase of type I was found to dominate. The corresponding Tn7-like transposon was found to be localized both on the chromosome of isolated *Escherichia coli* strains and also on a 50-kilobase *Inc*I transferable plasmid which was found in several different serotypes of *E. coli*. In two enterobacterial strains, resistance to more than 10^3 μg of trimethoprim per ml was furthermore found to be caused by a ca. 80-fold increase in the formation of chromosomal dihydrofolate reductase.

The folic acid analog trimethoprim has been used as an antimicrobial agent for more than a decade. A few years after the introduction of trimethoprim for clinical use, R plasmid-mediated resistance to this drug was reported (13). This plasmid-borne resistance also demonstrated a new principle regarding bacterial insensitivity, in that a drug-resistant variation of the target enzyme, dihydrofolate reductase, was found to be expressed from the plasmid (1, 22). Several types of plasmid-borne, drug-resistant dihydrofolate reductases have been subsequently discerned, and at least one of them was shown to be located on a transposon, Tn7 (3, 4). In later years, trimethoprim has been used extensively for clinical purposes, and resistance to it seems to have increased in frequency (7, 9, 25).

The use of trimethoprim in Sweden can be defined quantitatively by data from the computerized Swedish Drug Information System (Department of Drugs, National Board of Health and Welfare). From these data it could thus be seen that more than 0.15% of the population receive trimethoprim each day or that during 1 year 4 to 5% of the population are given this drug. It was interesting to investigate the types of bacterial resistance mechanisms that could be found in response to such a selection pressure in a relatively isolated population in northern Sweden (the county of Jämtland), in which one centrally located bacteriological laboratory serves the area. During an 8-month period, trimethoprim-resistant strains were collected from consecutive specimens of bacteria from the urinary tracts of patients. Among highly resistant bacteria, transposon Tn7 was found to be common, either located chromosomally or borne on a conjugative *Inc*I plasmid, 50 kilobases (kb) of size, which seemed to be endemic to the area. Two cases of marked overproduction of chromosomal dihydrofolate reductase which caused high resistance to trimethoprim were also found.

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MATERIALS AND METHODS

Bacterial strains and plasmids. Laboratory strains of bacteria and plasmids are listed in Table 1. Clinically isolated enterobacterial strains are listed in Table 2, and their collection is described in the text. Testing for antibiotic resistance was performed by the agar diffusion method with antibiotics-containing paper disks from AB Biodisk, Solna, Sweden. The serotyping of *Escherichia* strains was kindly performed by I. and F. Örskov, International *Escherichia* and *Klebsiella* Centre, Copenhagen.

Media. The rich medium LB or the mineral salts medium M9, supplemented with Casamino Acids (0.05% [wt/vol]), was used throughout (19).

Transfer of R plasmids. The transfer of R plasmids was performed as described earlier (21) and also on agar plates, and recombinants were selected on M9 agar plates containing the appropriate antibiotics.

Plasmid DNA preparation. The procedure described earlier for the isolation of small plasmids was used (17). For larger R plasmids, the method described by Hansen and Olsen was used (12).

Restriction endonuclease cleavage. Plasmid DNA (0.5 to 1.0 μg) was incubated in Tris-hydrochloride (pH 7.2, 6 or 86 mM [for *Eco*RI])–NaCl (50 mM)–$MgCl_2$ (6 mM)–dithiothreitol (1 mM)–bovine serum albumin (0.1 mg/ml) in a total volume of 24 μl.

Transformation. The transformation procedure described by Maniatis et al. (17) was used. Transformants were selected on M9 agar plates supplemented with Casamino Acids (0.05% [wt/vol]) and appropriate antibiotics (tetracycline [35 μg/ml], trimethoprim [35 μg/ml]).

Electrophoresis. Samples were run horizontally on 0.75% agarose gels. Electrophoresis buffer was Tris-acetate (40 mM, pH 7.8)–sodium acetate (28 mM)–sodium EDTA (2 mM). Samples were mixed with 0.05% bromphenol blue in 6 M urea. Electrophoresis was performed at 200 V for 3 h or at 25 V overnight. Fragments of DNA were visualized under UV light by staining for 30 min in a water solution of

TABLE 1. Bacterial strains and plasmids

Designation	Relevant characteristics[a]	Source or reference
Strain		
C600	*E. coli* K-12 *leu thr thi*	2
C600::Tn*7*	Sm^r Spc^r Tp^r	This study
EC1005	*E. coli* K-12 Nal^r *met*	K. Nordström
Plasmid		
R2258	Sm^r Spc^r Tp^r *IncI*	This study
R483	Sm^r Spc^r Tp^r *IncI*	6
R721	Sm^r Spc^r Tp^r *IncI*	3
R144	Tc^r *IncI*	18
pLGL1	Tc^r	15
pLGL1::Tn*7*	Sm^r Spc^r Tc^r Tp^r	15
ColE1::Tn*7*	Sm^r Spc^r Tp^r	15
pBR322	Ap^r Tc^r	5
pRSS021	Ap^r Tc^r Tp^r	This study

[a] Abbreviations: Ap, ampicillin; Nal, nalidixic acid; Sm, streptomycin; Spc, spectinomycin; Su, sulfonamide; Tc, tetracycline; Tp, trimethoprim; and *Inc*, incompatibility group.

ethidium bromide (1 μg/ml). For size determinations, an *Eco*RI digest of phage λ was included in the run.

Isolation of DNA fragments and nick translation. DNA fragments were recovered by cutting the electrophoresis gel and electrophoretically eluting the DNA in a glass tube. DNA was recovered in a dialysis bag tied to the bottom of the glass tube, precipitated with ethanol, and finally dissolved in 10 mM Tris-hydrochloride (pH 7.5) containing 1 mM EDTA. Isolated fragments were labeled with [^{32}P]dTTP as described by Maniatis et al. (17).

DNA-DNA hybridization. DNA fragments were transferred from agarose gels to nitrocellulose filters by the method of Southern (23). The DNA-DNA hybridization was performed as described by Maniatis et al. (17).

Enzyme preparation and assay. Dihydrofolate reductase was extracted and partially purified as described by Tennhammar-Ekman and Sköld (24). The final step in this procedure was ion-exchange chromatography on DEAE-Sephacel (Pharmacia Fine Chemicals, Uppsala, Sweden). Material from a 700-ml bacterial culture was chromatographed on a column (0.9 by 15 cm) and eluted at a rate of 12 ml/h by a linear gradient of 0.05 to 0.50 M KCl in 0.05 M Tris-chloride buffer (pH 7.2) containing 1 mM dithiothreitol. Enzyme activity was determined as described by Warner and Lewis (26), and tests for inhibition by trimethoprim were performed by adding the drug to the indicated final concentration 2 min before the addition of dihydrofolate, which started the reaction. Enzyme assays on crude extracts were performed several times and agreed to within 10%.

Colony hybridization. Colony hybridization was performed as described by Grunstein and Hogness (11), except that a Schleicher and Schüll Membranfilter BA 85 was used.

Isolation of *Escherichia coli* C600 with Tn*7* on the chromosome. Tn*7* originally from R483 but borne on plasmid ColE1 was inserted into the chromosome of *E. coli* C600 by using the incompatibility between ColE1::Tn*7* and pBR322. The chromosomal location of Tn*7* could be detected as a decrease in dihydrofolate reductase activity, because the single copy of Tn*7* on the chromosome corresponded to a much lower gene dosage than ColE1::Tn*7* with its high copy number.

Chemicals. Cesium chloride was purchased from Kock-Light Laboratories Ltd., Hoverhill, Suffolk, United Kingdom. Ethidium bromide was from Calbiochem-Behring, La Jolla, Calif. Restriction endonucleases and T4 ligase were from New England Biolabs, Beverly, Mass. Lysozyme, Triton X-100, and calf thymus DNA were from Sigma Chemical Co., St. Louis, Mo. Agarose (electrophoresis grade) was from Bio-Rad Laboratories, Richmond, Calif. [^{32}P]dTTP (600 Ci/mmol) was purchased from New England Nuclear, Dreieichenhain, Federal Republic of Germany. Nitrocellulose paper was from Schleicher and Schüll, Dassel, Federal Republic of Germany. Trimethoprim lactate was a gift from Wellcome Research Laboratories, Beckenham, England; ampicillin was from Astra Läkemedel AB, Södertälje, Sweden. Tetracycline was from Lederle Laboratories, Pearl River, N.Y., and chloramphenicol was from Parke-Davis, Pontypool, United Kingdom.

RESULTS

Characterization of trimethoprim-resistant strains. During an 8-month period, bacterial strains resistant to more than 8 μg of trimethoprim per ml were collected consecutively at the central bacteriological laboratory serving the studied area, which is a rather isolated county in which the distribution of antibiotics is well known (Sköld et al., submitted for publication). The collected strains were of bacteria infecting the urinary tract of both inpatients and outpatients (more than 10^5 bacteria per ml of urine). A total of 153 strains were isolated from a total of ca. 2×10^3 specimens. Of these, 60 were identified as *Pseudomonas* spp., 35 were identified as *E. coli*, 34 were identified as *Proteus* spp., 21 were identified as *Klebsiella* spp., and 3 were identified as unspecified coliforms. Fifteen of the strains, mostly *Proteus* spp., were resistant to only 10 μg of trimethoprim per ml. All others were resistant to well over 100 μg/ml, and 43 were resistant to 10^3 μg/ml. In the latter group there were 19 *E. coli*, 22 *Pseudomonas* spp., and 2 *Proteus* spp.

The highly resistant strains were analyzed for the occurrence of Tn*7* by colony hybridization. A Tn*7*-specific probe was generated by digesting plasmids ColE1::Tn*7* and pBR322 (Table 1) with restriction endonuclease *Ava*I. After the ligation of this fragment mixture, recombinants of pBR322 expressing resistance to trimethoprim were sought. One recombinant plasmid of 6.9 kb, which mediated resistance to trimethoprim, tetracycline, and ampicillin, was isolated. Upon redigestion with *Ava*I, this plasmid, pRSS021, was found to contain a 2.6-kb fragment from which the trimethoprim resistance of Tn*7* is thus expressed. This fragment is located completely within the Tn*7* borders, according to the restriction enzyme digestion map (10). Of the 43 strains that were highly resistant to trimethoprim, 15 were shown to react with the probe derived from Tn*7*. All of these were *E. coli*. There was no hybridization to any of the *Pseudomonas* or *Proteus* strains or to controls containing R388 and R751 which represented the type II enzyme. A list of *E. coli* strains shown to contain Tn*7* DNA is given in Table 2, in which serotypes and other resistance markers observed in these strains are also noted. It can be seen that 10 different serotypes were found and that the resistance pattern was quite variable, although all of the strains were resistant to sulfonamide.

All collected strains were tested for the transfer of trimethoprim resistance by conjugation with a plasmid-free *E. coli* K-12 strain, EC1005. Six donors were found which contained different plasmid patterns in electrophoresis. They were all *E. coli*, resistant to 10^3 μg of trimethoprim per ml, and showed DNA hybridization with the probe derived from Tn*7* (Table 2). Along with trimethoprim resistance, low-level resistance to streptomycin and spectinomycin was also

TABLE 2. Consecutive isolates of enteric bacteria showing high resistance to trimethoprim[a]

Consecutive strain no.	Serotype	Resistance markers	Reaction to:		
			Hybridization to Tn7 probe	Transferable Tp	Over-production of DHFR[b]
E. coli					
T2	O15:H18:K2	Tp Su Sm Ap	+	−	−
T4	NT	Tp Su Sm Ap Tc	+	+	−
T21	O126:H45	Tp Su Sm	+	+	−
T44	O86:H10	Tp Su Sm Ap Tc Nf	+	+	−
T55	O86:H10	Tp Su Sm Ap	+	+	−
T59	NT	Tp Su	−	−	−
T75	O6:H10	Tp Su Sm	+	−	−
T81	O92:H33:K$^-$	Tp Su Sm	+	−	−
T86	O6:H10	Tp Su Sm Nf	+	+	−
T89	O6:H10	Tp Su Sm	+	−	−
T91	O114:H10:K$^-$	Tp Su Sm Nf	+	−	−
T95	O6:H10	Tp Su Sm Tc Nf Er	+	−	−
T96	O6:H10	Tp Su Sm Ap	+	+	−
T114	O6:H10	Tp Su Nf	−	−	−
T118	R:H$^-$:K55	Tp Su Ap Nf Nal	−	−	+
T121	K$^-$	Tp Su Sm	+	−	−
T125	O106:H18:K7	Tp Su Sm Nf Er	+	−	−
T127	O6:H1:K13	Tp Su Sm	+	−	−
T131	NT	Tp Su Nf	−	−	−
P. vulgaris					
T117	NT	Tp Su Ap Tc Nal Er	−	−	−
T142	NT	Tp Su Tc Nf Nal Er	−	−	+

[a] Abbreviations: Ap, ampicillin; Er, erythromycin; Nal, nalidixic acid; Nf, nitrofurantoin; Sm, streptomycin; Su, sulfonamide; Tc, tetracycline; Tp, trimethoprim; and NT, not tested.
[b] Chromosomal dihydrofolate reductase (DHFR) was increased ca. 80-fold over average crude extract levels in *E. coli* and *P. vulgaris*.

transferred, but there were no other resistance traits. Analysis of the isolated plasmid by electrophoresis and restriction enzyme digestion showed that the transferred trimethoprim resistance was in all six cases mediated by the same plasmid which was called R2258. The fragment pattern after digestion with *Hpa*I is shown in Fig. 1. The size of R2258 was calculated to be 50 kb, and this plasmid occurred in *E. coli* of at least three different serotypes (Table 2). Furthermore, the original strains were isolated from several geographically separated locations. Table 2 lists all enterobacteria occurring among the collected strains, and it can be seen that although drug-resistant enzymes of type I (found in Tn7) dominated as the mechanism of resistance to trimethoprim, there were also two cases of increased production of indigenous chromosomal dihydrofolate reductase represented by one *E. coli* and one *Proteus vulgaris* strain. Crude extracts from cells of these strains showed dihydrofolate reductase activities which were ca. 80-fold higher than in similar extracts from *E. coli* K-12 (Table 3). Partially purified enzyme from these extracts showed only one increased peak of activity at the chromatographic position of chromosomal dihydrofolate reductase, which is distinct from the corre-

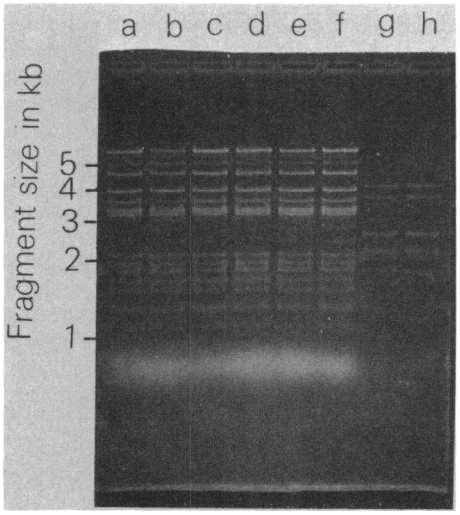

FIG. 1. Digestion with *Hpa*I of plasmid DNA from six transconjugants expressing trimethoprim resistance (lanes a to f). *Ava*I digestion of DNA from pLGL1::Tn7 with Tn7 from R483 (lane g) or from R2258 (lane h). The fragments were separated electrophoretically on a 0.75% agarose gel.

TABLE 3. Dihydrofolate reductase activities in crude extracts of cells from different strains

Bacterial strain	Amt of enzyme activity (nmol of DHFR reduced/min per 10^9 bacteria)
T118 (see Table 2)	38.9
T142 (see Table 2)	41.0
C600 (R483)	8.1
C600 (R2258)	16.9
C600 (R721)	13.9
C600::Tn7 (from R2258)	2.5
C600::Tn7 (from R483)	2.4
C600[a]	0.5

[a] Only chromosomal dihydrofolate reductase (DHFR) occurred in this strain.

sponding position of type I enzyme. This activity was furthermore inhibited 70 to 80% at 1 μM trimethoprim, a concentration which does not interfere with the Tn7-mediated activity. Overproduction of chromosomal dihydrofolate reductase as a mechanism of trimethoprim resistance in a clinical isolate has been described previously (8).

Characterization of the transferable plasmid R2258. Purified DNA of R2258 was introduced by transformation into the *E. coli* strain C600 containing the *Inc*I plasmid R144 (Table 1). Transformants were selected on plates containing trimethoprim and tetracycline, picked and grown on drug-free plates, and finally replica plated on medium containing trimethoprim or tetracycline.

Of 100 colonies tested from each type of plate, 92 of those that grew in the presence of tetracycline were also trimethoprim resistant, whereas only 12 of those from the trimethoprim replica plate were also resistant to tetracycline. These data are interpreted to reflect incompatibility between R144 and R2258, i.e., that the latter belongs to compatibility group *Inc*I. The large number of trimethoprim-resistant colonies observed on the tetracycline plate could be explained by the transposition of the Tn7-like transposon from R2258 to the host chromosome. This interpretation was supported by the observations that none of these doubly resistant strains was able to transfer trimethoprim resistance by conjugation with a plasmid-free recipient strain and that the expression of dihydrofolate reductase was lower (Table 3).

That the Tn7-like transposon readily transposes from R2258 was shown in an experiment in which R2258 was transformed into *E. coli* C600 harboring plasmid pLGL1 (Table 1) which contains a fragment of host chromosomal DNA, which in turn contains the specific chromosomal receptor site for Tn7 (15). In four of six electrophoretically analyzed transformants, a plasmid of 20 kb which was distinct from both R2258 and pLGL1 was found. During the transformation of strain C600, this plasmid was found to specify trimethoprim as well as streptomycin and spectinomycin resistance. Restriction enzyme digestion analysis furthermore showed this plasmid to be identical with pLGL1::Tn7 (15) (Fig. 1).

Other plasmids, such as R483 and R721 (Table 1), have previously been shown to contain Tn7. Lichtenstein and Brenner (15, 16) demonstrated that Tn7 has a unique insertion site on the chromosome of *E. coli* K-12. A 1-kb *Eco*RI fragment containing this site was isolated by the same authors and inserted into plasmid pACYC184 to give hybrid plasmid pLGL1 (15).

This small chromosomal *Eco*RI fragment was labeled with ^{32}P as described above and used as a probe in DNA-DNA hybridization of electrophoretically separated *Eco*RI fragments from plasmids R2258, R721, and R483. No hybridization was observed, which indicated that there are no extensive homologies between the chromosomal and the plasmid insertion sites for Tn7, as could be expected when the unique insertion site of Tn7 observed in the bacterial chromosome (15, 16) also occurred in plasmids.

Expression of dihydrofolate reductase from plasmid R2258. The dihydrofolate reductase activity expressed from R2258 is twice that expressed from R483 (Table 3). When enzyme expression from the chromosomally located Tn7-like transposon derived from R2258 was compared with the analogous expression from Tn7 transposed from R483, however, the activities were about the same (Table 3). This is interpreted as demonstrating that there exists a constitutive expression of dihydrofolate reductase from Tn7 and that the mentioned difference between cells containing R2258 and those containing R483 was due to different plasmid copy numbers.

DISCUSSION

The large consumption of trimethoprim as an antibacterial drug could be expected to evoke a response of resistance among pathogenic bacteria. It was interesting to investigate this response regarding resistance mechanisms among human urinary tract pathogens in a geographical area in which representative bacterial specimens could be obtained. The county of Jämtland in Sweden has a population of 128,000, which is served by a centrally located bacteriological laboratory.

Almost a third of the 153 consecutively collected trimethoprim-resistant strains showed high-level resistance (MIC, $>10^3$ μg/ml) to the drug. Fifteen of the 21 enterobacteria belonging to this highly resistant group of strains hybridized to a probe that represented the trimethoprim resistance expressing part of transposon Tn7. Since the serotypes and also the resistance patterns of these strains, which were all *E. coli*, were quite variable, this observation indicates that the gene expressing the trimethoprim-resistant dihydrofolate reductase of type I and previously found to be mediated by transposon Tn7 is efficiently spread among enterobacterial strains in this area.

Six of the highly resistant enterobacterial strains that hybridized to the Tn7-derived probe could transfer their trimethoprim resistance to a susceptible recipient. In all six cases, the transfer was mediated by the same 50-kb *Inc*I plasmid. This was determined from the identity of fragment patterns during gel electrophoresis of purified plasmid DNA from the different strains after digestion with the restriction enzyme *Hpa*I. The bacterial strains in which the plasmid was originally demonstrated were of at least three different serotypes and were furthermore isolated from several geographically separated areas. This could be interpreted as the occurrence of a plasmid endemic to the area which efficiently transferred Tn7-mediated trimethoprim resistance. This transferable plasmid was shown to be able to transpose a piece of DNA corresponding to the size (14 kb) of Tn7 to plasmid pLGL1, which contains the Tn7-specific receptor from the *E. coli* chromosome (16). The resulting plasmid was indistinguishable from pLGL1::Tn7 during gel electrophoresis after digestion with the restriction enzyme *Ava*I (Fig. 1, lanes g and h). Nine of the isolated strains of *E. coli* reacted with the Tn7-specific probe at colony hybridization but did not transfer trimethoprim resistance at conjugation. Since transposon Tn7 is known to transpose readily to the chromosome of *E. coli*, these strains most likely have trimethoprim resistance genes (identified by the probe) borne on a Tn7-like transposon on the chromosome.

It was proposed earlier that the overproduction of chromosomal dihydrofolate reductase ought to be an unlikely cause of resistance to trimethoprim among bacteria because of the presumed harmful metabolic effects of large intracellular amounts of this enzyme (14). In contrast to this proposition, we found that in two enterobacterial strains, resistance to more than 10^3 μg of trimethoprim per ml was caused by a ca. 80-fold increase in the formation of chromosomal dihydrofolate reductase. These two cases, together with one studied earlier (8), thus seem to establish the overproduction of chromosomal dihydrofolate reductase as a mechanism of antifolate resistance among pathogenic enterobacteria.

In summary, the dissemination of transposon Tn7, both plasmid borne and chromosomally located, seems to be the

most common resistance response among enterobacteria to the ubiquitous use of trimethoprim. This is in agreement with similar, recently published results from Finland (20).

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