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Regulatory Changes in the Formation of Chromosomal Dihydrofolate Reductase Causing Resistance to Trimethoprim

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High resistance to trimethoprim mediated by the several hundredfold overproduction of the drug target enzyme, dihyrofolate reductase, in a clinically isolated *Escherichia coli* strain, 1810, was cloned onto several vector plasmids and seemed to be comprised of a single dihydrofolate reductase gene, which by DNA-DNA hybridization and restriction enzyme digestion mapping was very similar to the corresponding gene of *E. coli* K-12. Determination of mRNA formation in the originally isolated resistant strain and strains with cloned trimethoprim resistance determinant demonstrated an about 15-fold increase in production of dihydrofolate reductase mRNA compared with that in *E. coli* K-12. This was explained by the occurrence of a promoter up mutation in the resistant isolate accompanied by changes in the restriction enzyme digestion pattern found by comparison with the corresponding pattern from *E. coli* K-12.

Resistance to the folic acid analog trimethoprim [2,4-diamino-5-(3',4',5'-trimethoxybenzyl)pyrimidine] used as an antimicrobial agent is nowadays a rather common phenomenon among clinically isolated bacteria, and it is usually mediated by plasmids. Extrachromosomal resistance results in highly resistant bacteria (minimal inhibitory concentration, >1,000 μg/ml) and is caused by the occurrence of plasmid-encoded, drug-resistant dihydrofolate reductases, which supplement the chromosomally produced reductase (15, 19).

Chromosomally located, low-level resistance to trimethoprim was induced in the laboratory by mutagenesis of *Escherichia coli*. It was due either to a mutationally altered dihydrofolate reductase with a decreased sensitivity for trimethoprim or to a mutational change in regulation leading to increased levels of the chromosomal enzyme (18).

In a clinical survey (27), several bacterial strains were found that showed high resistance to trimethoprim, and this resistance was not plasmid borne. These strains had the common property of overproducing dihydrofolate reductase. One of them is *E. coli* 1810. This strain showed an approximately 200-fold overproduction of dihydrofolate reductase activity. The enzyme from strain 1810 has been found to differ from the *E. coli* K-12 dihydrofolate reductase in the following properties: the electrophoretic mobility in a nondenaturing polyacrylamide gel is somewhat slower, and the K_i value for trimethoprim is 3 times higher (27). Furthermore, the turnover number was by methotrexate titration found to be 1.4 times higher than that of the *E. coli* K-12 enzyme (B. Tennhammar-Ekman, Ph.D. thesis, University of Uppsala, Uppsala, 1982).

In the present work, the mechanism of the overproduction of enzyme in strain 1810 was investigated by the cloning of the responsible genetic area onto several vector plasmids and detailed restriction enzyme digestion mapping. The close relationship to the corresponding part of the *E. coli* K-12 chromosome was established by DNA-DNA hybridization. The massive enzyme formation could be only partly accounted for by increased mRNA levels.

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MATERIALS AND METHODS

Bacteria and plasmids. The strains of *E. coli* and the plasmids used are listed in Table 1.

Growth media. The rich LB medium (13) and the mineral salts medium M9 (13) supplemented with the required amino acids (50 μg/ml) and thiamine hydrochloride (1 μg/ml) or with uracil (50 μg/ml) were used. Antibiotics were added at the following concentrations: trimethoprim (5 or 200 μg/ml), ampicillin (50 μg/ml), tetracycline (25 μg/ml), streptomycin (100 μg/ml), or rifampin (50 μg/ml).

Mating procedure and transformation. Conjugation was performed on solid media by cross-streaking donor and recipient strains. Competent cells were transformed as described by Mandel and Higa (11). Transformed cells were grown in LB medium for 3 h, and trimethoprim-resistant transformants were then selected on M9 agar supplemented with 200 μg of trimethoprim per ml.

Isolation of plasmid DNA. Plasmid DNA from strain SN01(pJF01) was isolated by the method of Hansen and Olsen (7) and purified by cesium chloride-ethidium bromide gradient centrifugation in a Beckman type 40 rotor at 36,000 rpm for 60 h. Plasmid DNA produced under chloramphenicol amplification (8) was isolated as described by Davis et al. (5).

Restriction enzyme digestion of plasmid DNA and agarose gel electrophoresis. The conditions for restriction endonuclease reactions were those suggested by the manufacturers. Analysis of plasmids and DNA fragments was performed by electrophoresis in 0.5, 0.75, or 2.0% (wt/vol) horizontal agarose gels at 200 V for 3 h or at 50 V overnight and in 40 mM Tris-acetate (pH 7.8)–28 mM sodium acetate–2 mM disodium EDTA. To isolate fragments of DNA and plasmids from agarose gels, the corresponding gel slices were put in dialysis bags together with 1 ml of electrophoresis buffer and eluted electrophoretically at 50 V overnight. The solution of electrophoretically extracted DNA was applied to a DE52 (Whatman) anion-exchange column previously equilibrated with 10 mM Tris-hydrochloride (pH 8.0)–1 mM disodium EDTA. The DNA was eluted with 0.6 M NaCl in the same buffer and finally precipitated with 95% ethanol at −20°C.

Ligation. The ligation procedure used was described earlier (26).

DNA-DNA hybridization. DNA fragments were trans-

TABLE 1. Plasmids and strains

Plasmid or strain	Relevant phenotypic properties[a]	Size (kb)	Reference
Plasmids			
pBR322	Amp^r Tc^r	4.4	3
pBEU1	Amp^r	17.4	29
pCT398	Amp^r Tc^r	12.0	28
pCV27	Amp^r Tp^r	12.7	21
pJF76	Tc^r Tp^r	12.2	This study
pJF60	Amp^r Tp^r	25.2	This study
pJF70	Amp^r Tp^r	25.2	This study
pJF80	Tc^r Tp^r	19.8	This study
KLF4	Pro$^+$ Leu$^+$ Thr$^+$	>210	10
pJF01	Pro$^+$ Leu$^+$ Thr$^+$ Tp^r	>210	This study
E. coli strains			
1810	Tp^r		27
OS01	*leu thr* Tp^r		This study
AB2463	*leu pro thr recA*		1
C600	*leu thr*		1
HB101	*leu pro rpsL recA*		4
SN01	*leu thr ura rpoA rpsL recA*		14

[a] Abbreviations: Amp^r, ampicillin resistance; Tc^r, tetracycline resistance; Tp^r, trimethoprim resistance.

ferred from agarose gels to nitrocellulose filters by the method of Southern (24). Nick translation of DNA was performed as described by Maniatis et al. (12). The hybridization was carried out in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as described by Swedberg and Sköld (26).

Radioactive labeling of RNA. Radioactive labeling of intracellular RNA was performed by the addition of [^{14}C]uridine (50 μCi/ml; 0.03 μM) to exponentially growing bacteria at 2 × 10^8 cells per ml 2 min before sampling (13). After extraction with hot phenol (13) and precipitation, the RNA was dissolved in a 100 mM sodium acetate buffer (pH 7.7) containing 10 mM Mg^{2+} and finally incubated at 37°C with 20 μg of electrophoretically purified DNase I per ml as described by Smith and Calvo (20). After a second phenol extraction and cold ethanol precipitation, the RNA was finally dissolved in 2× SSC.

Determination of *fol* mRNA by RNA-DNA hybridization. To nitrocellulose filters (type BA85, 24-mm diameter; Schleicher & Schuell Co.), 5 μg of a denatured 1.1-kilobase (kb) *Pst*I-*Sal*I DNA fragment from plasmid pCV27 containing the entire *fol* gene from *E. coli* K-12 (21) was added. After baking at 80°C for 2 h the filters were incubated with different amounts of ^{14}C-labeled RNA in 800 μl of 2× SSC at 67°C for 20 h (13). After hybridization the filters were

TABLE 2. Trimethoprim resistance and dihydrofolate reductase activities in strains with the two *fol* gene types

Strain	MIC[a] (μg/ml)	Dihydrofolate reductase activity[b]
HB101	1	0.5
1810	>1,000	248
OS01	>1,000	153
SN01(pJF01)	>1,000	168
HB101(pBR322)	1	0.5
HB101(pJF76)	>1,000	1,433
HB101(pCV27)	10	12.5
HB101(pCT398)	1	0.5
HB101(pJF80)	>1,000	1,608
HB101(pBEU1)	1	0.5
HB101(pJF60)	>1,000	974
HB101(pJF70)	>1,000	942

[a] The minimal inhibitory concentration (MIC) was determined by plating on appropriately supplemented M9 agar medium containing different drug concentrations.

[b] Expressed as nanomoles of dihydrofolate reduced per minute per milligram of protein.

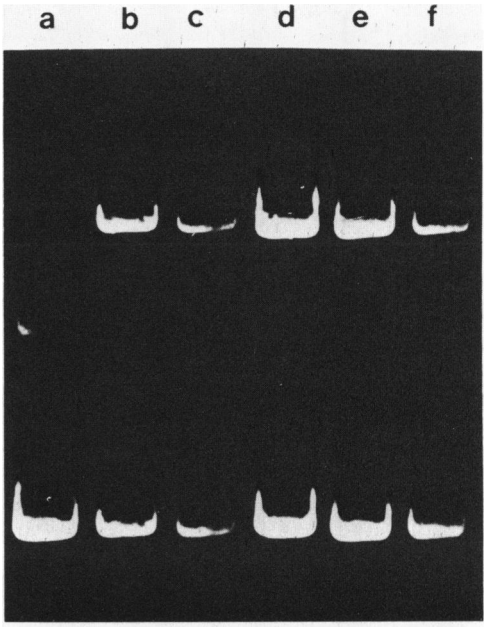

FIG. 1. Electrophoretic separation on 0.75% agarose gel of plasmid DNA from clones obtained by transforming bacterial strain HB101 with the purified hybrid plasmid pJF76 isolated from an agarose gel. Lanes: a, pBR322 as a marker; b through e, plasmid DNA isolated from HB101 transformed in four successive cycles with electrophoretically purified pJF76; f, pJF76 purified by CsCl gradient centrifugation.

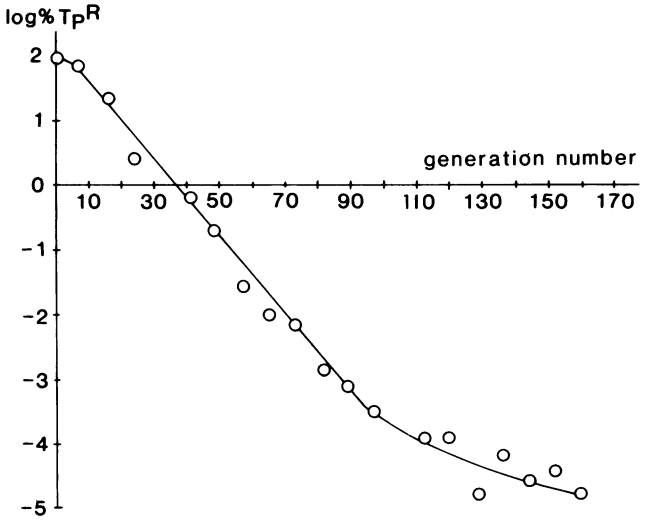

FIG. 2. Instability of trimethoprim resistance trait in hybrid plasmid pJF76. The logarithmic values along the ordinate denote percent trimethoprim-resistant bacteria in a culture of HB101(pJF76) growing in the absence of trimethoprim in a rich medium containing tetracycline (25 μg/ml) for the number of generations marked on the abscissa.

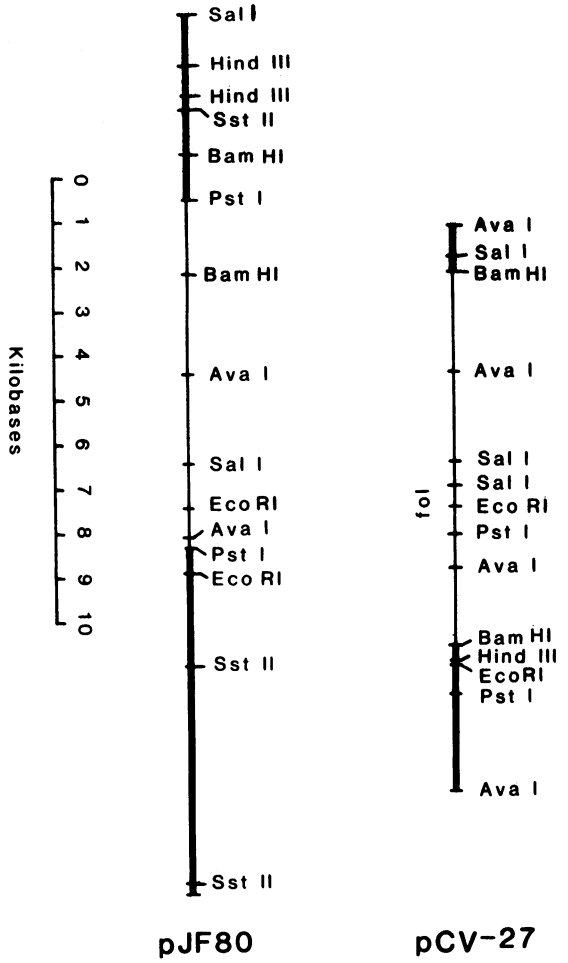

FIG. 3. Restriction enzyme digestion map of hybrid plasmid pJF80, which carries the 7.8-kb trimethoprim resistance fragment from strain 1810 on vector pCT398. Thick lines represent vector DNA, which in the case of pCV27 (data from Smith and Calvo [21]), shown for comparison, is pBR322.

washed five times with 2× SCC and then incubated for 50 min at 20°C with 20 μg of RNase A per ml followed by 10 min at 37°C (13). The filters were finally washed five times with 2× SSC, dried, and assayed for radioactivity.

Preparation of bacterial extracts and assay of dihydrofolate reductase activity. Bacteria were grown in 40 ml of M9 medium appropriately supplemented with the required amino acids. Trimethoprim was added to 5 μg/ml for bacteria carrying plasmid pCV27, whereas a concentration of 100 μg/ml was used for strain 1810 and also for strains harboring recombinant plasmids carrying the resistance trait from 1810. Cells were harvested by centrifugation during exponential growth, washed three times with buffer A (0.05 M Tris-hydrochloride [pH 7.2], 0.05 M KCl, 10 mM dithiothreitol, 1 mM disodium EDTA), and finally suspended in 1 ml of buffer A. The cells were kept chilled on ice and disintegrated by sonication twice for 20 s at an amplitude of 18 μm in an MSE Ultrasonic desintegrator MK2. Cell debris was removed by centrifugation at 25,000 × *g* for 10 min.

Determination of dihydrofolate reductase activity was carried out by the method of Warner and Lewis (30). The assay was performed at 30°C in a Zeiss PM6 spectrophotometer equipped with a thermostat and a Servogor 5 recorder. Enzyme activity was followed for at least 5 min. Unspecific oxidation of NADPH, i.e., decrease in absorbance at 340 nm in the absence of dihydrofolate, was subtracted. Enzyme activity was measured as decrease in absorbance at 340 nm for 5 min and is expressed as nanomoles of NADPH oxidized per minute per milligram of protein. As an approximate measure of protein content, absorption at 280 nm was used.

Chemicals. Folic acid, NADPH, RNase A, DNAse I (electrophoretically purified), lysozyme, and Triton X-100 were bought from Sigma Chemical Co., St. Louis, Mo. Dihydrofolic acid was prepared by the method of Blakley (2). Trimethoprim lactate was a gift from the Wellcome Research laboratories, England. Cesium chloride was purchased from JMC, Roystone, Great Britain, and ethidium bromide was from Calbiochem, San Diego, Calif. Restriction endonucleases and T4 DNA ligase were from New England Biolabs, Beverly, Mass. [α-^{32}P]TTP (600 Ci/mmol) and [2-^{14}C]uridine (50 Ci/mol) were purchased from the Radiochemical Centre, Amersham, England. Agarose (electrophoresis grade) was from Bio-Rad Laboratories, Richmond, Calif. Ampicillin was from Astra Läkemedel AB, Södertälje, Sweden. Tetracycline was from Lederle Laboratories, Pearl River, N.Y. Chloramphenicol was from Parke-Davis, Ponty Pool, Great Britain.

RESULTS

Cloning of the chromosomal trimethoprim resistance trait from strain 1810 on a small vector plasmid. To investigate the molecular mechanism behind the overproduction of dihydrofolate reductase causing trimethoprim resistance in strain 1810, the resistance trait was cloned on a small vector as follows. The genetic region responsible for trimethoprim resistance was transferred into a genetically well known background, strain C600, by transduction with P1CMc1r100 (16). Transductants were isolated on minimal plates containing 100 μg of trimethoprim per ml. One transductant, OS01, showing the same high resistance to trimethoprim and almost the same high enzyme activity as the parental strain 1810 (Table 2) was selected. An F′ factor, KLF4 (10), containing *thr*, *fol*, and *leu* was introduced into OS01 by conjugation with strain AB2463(KLF4) (1) with selection for Thr and Leu prototrophy. In the resulting KLF4-carrying strain recombination could take place between the trimethoprim resistance-mediating chromosomal *fol* gene of the transductant and the *fol* gene of the F′ factor. The resistance-carrying F′ factor was transferred to strain SN01 (*recA*) (14) by conjugation with selection for rifampin and trimethoprim resistance. This recombinant strain, called SN01(pJF01), had the same high enzyme activity and the same high minimal inhibitory concentration value toward trimethoprim as OS01 (Table 2). Plasmid DNA from strain SN01(pJF01) was isolated, treated with restriction enzyme *Pst*I, and ligated to plasmid pBR322 DNA that had also been cleaved with *Pst*I. After transformation into strain HB101 (4), isolates expressing trimethoprim resistance were selected on plates containing 200 μg of trimethoprim per ml. One such clone, HB101(pJF76), after plasmid isolation and redigestion with *Pst*I gave rise to a 4.4-kb fragment corresponding to plasmid pBR322 and a 7.8-kb insert. The level of enzyme activity expressed by strain HB101(pJF76) was about 9 times higher than that of strain OS01 (Table 2).

Intracellular instability of hybrid plasmid pJF76. Hybrid plasmid pJF76, after purification by cesium chloride gradient centrifugation and retransformation, was always found to coexist with a smaller plasmid, which was seen at electrophoretic analysis of transformants. The smaller plasmid was

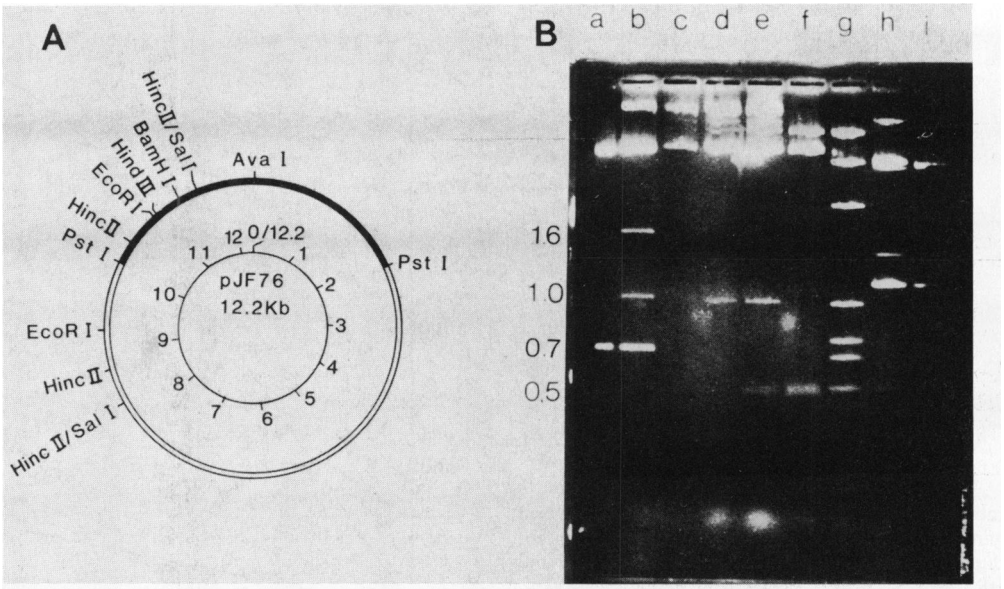

FIG. 4. Characterization of the 7.8-kb DNA fragment carrying trimethoprim resistance from strain 1810. (A) Restriction enzyme digestion map of hybrid plasmid pJF76 carrying the 7.8-kb resistance fragment from strain 1810 linked to vector pBR322. The vector part of the molecule is represented by the black line, and the resistance carrying fragment is represented by the open line. (B) Patterns of restriction fragments from different hybrid plasmids. Lanes: a, pBR322 digested with *Eco*RI and *Sal*I; b, pJF76 digested with *Eco*RI and *Sal*I; c, pCV27 digested with *Eco*RI followed by partial *Sal*I digestion for 3 min; d, pCV27 digested with *Eco*RI followed by partial *Sal*I digestion for 8 min; e, pCV27 digested with *Eco*RI followed by partial *Sal*I digestion for 20 min; f, pCV27 digested with *Eco*RI and *Sal*I; g, pCV27 digested with *Hinc*II; h, pJF76 digested with *Hinc*II; i, pBR322 digested with *Hinc*II.

of the same size as the vector plasmid pBR322, but did not express ampicillin resistance. Several attempts were made to separate the hybrid from the vector-like plasmid, by isolating hybrid DNA from the electrophoresis gel and using it for the retransformation of strain HB101. The hybrid pJF76, however, always seemed to segregate and form the small plasmid intracellularly. This is demonstrated in Fig. 1, where four successive electrophoretic isolations of pJF76 DNA and ensuing transformations consistently gave the same pattern of segregation. By restriction enzyme digestion analysis the smaller plasmid seemed to be almost identical with pBR322. However, after *Msp*I and *Hha*I digestions fragments of 110 and 337 base pairs, respectively, were missing in comparison with pBR322. It should be noted that at segregation of the fragment from the hybrid plasmid the original *Pst*I site was always regenerated in spite of the loss of small fragments.

The stability of hybrid plasmid pJF76 was furthermore measured directly, by growing HB101(pJF76) in a rich medium containing tetracycline (25 μg/ml). The trimethoprim resistance trait disappeared quickly in the absence of trimethoprim (Fig. 2), as judged by plating on minimal plates containing 25 μg of the drug per ml. After about 37 generations of growth, only about 1% of tetracycline resistant bacteria also showed trimethoprim resistance. Plasmid analysis of cell samples withdrawn after 7 and 25 generations, respectively, showed a weak electrophoretic band of pJF76 after 7 generations. This band was, however, undetectable at the later time point.

The instability of hybrid plasmid pJF76 could be interpreted to mean that the overproduction of dihydrofolate reductase by the gene dose from the small hybrid plasmid would lead to unmanagable levels of the enzyme in the cell, which then under the selection pressure of trimethoprim (200 μg/ml) has to lower the gene dose by the observed segregation.

Expression of trimethoprim resistance from the 7.8-kb fragment cloned in low-copy-number plasmids. The 7.8-kb chromosomal fragment from strain 1810 was subcloned on pCT398, which is a derivative of RK2 with a size of 12.0 kb (28). In addition to the published restriction sites, pCT398 also contains one *Pst*I site (C. M. Thomas, personal communications). Plasmid pCT398 was opened at its *Pst*I locus and ligated to the 7.8-kb *Pst*I fragment derived from pJF76. After transformation of strain HB101 and analysis of trimethoprim-resistant clones a plasmid with the size of 19.8 kb was isolated, which after *Pst*I digestion was cleaved into two fragments of 7.8 and 12.0 kb, respectively. This hybrid plasmid was denoted pJF80, and it mediates high resistance to trimethoprim and effects a dihydrofolate reductase activity in strain HB101 that is about 10 times higher than that of strain OS01 (Table 2).

The trimethoprim resistance-carrying fragment was also cloned to the low-copy-number vector pBEU1, which is a derivative of plasmid R1 (29). Plasmid pBEU1 has nine *Pst*I sites (B. E. Uhlin, personal communication); after partial digestion with this enzyme and ligation to the 7.8-kb *Pst*I fragment derived from pJF76, two kinds of hybrid plasmids were observed in strain HB101 transformed to trimethoprim resistance. They were denoted pJF60 and pJF70, respectively. Double digestion of these with *Eco*RI and *Sal*I showed that fragments were inserted in different *Pst*I sites of pBEU1 and also in different directions. From Table 2 it can be seen that the two hybrid plasmids mediated high resistance to trimethoprim and that they both expressed dihydrofolate reductase activity to the same level. The latter observation suggests that the dihydrofolate reductase gene is expressed from its own promoter in the hybrid plasmids and thus independently of vector promoters.

Restriction enzyme digestion map of the 7.8-kb resistance fragment from strain 1810. A detailed restriction endonuclease digestion map was constructed of the 7.8-kb chromosomal DNA fragment expressing the trimethoprim resistance of strain 1810 (Fig. 3). This map is compared with that of the

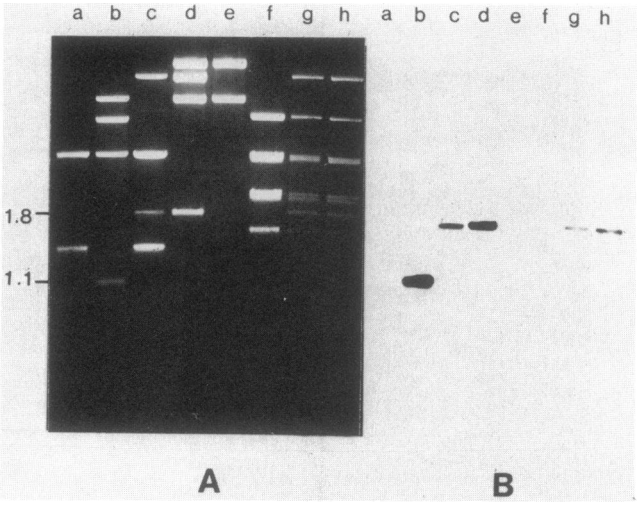

FIG. 5. Restriction enzyme digestion patterns of different hybrid plasmids. (A) Agarose gel electrophoresis of DNA from different hybrid plasmids digested by a combination of the enzymes *Pst*I and *Sal*I. Lanes: a, pBR322; b, pCV27; c, pJF76; d, pJF80; e, pCT398; f, pBUE1; g, pJF60; h, pJF70. (B) Autoradiogram after transfer of fragments from the gel onto nitrocellulose paper and subsequent hybridization to a 1.1-kb ^{32}P-labeled *Pst*I-*Sal*I-generated fragment containing the entire *fol* gene of pCV27.

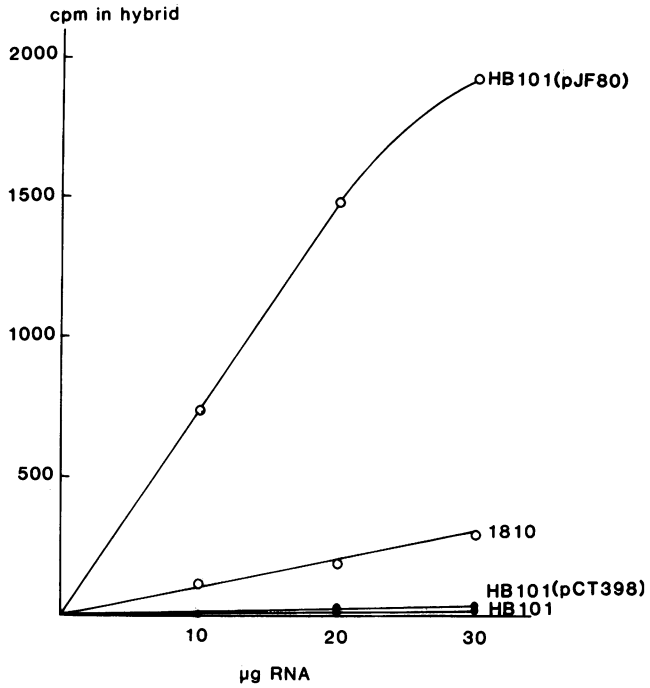

FIG. 6. Titration of *fol* mRNA from strains containing different hybrid plasmids. Determination of mRNA was by hybridization of RNA pulse-labeled with [^{14}C]uridine to a 1.1-kb *Pst*I-*Sal*I fragment of pCV27 containing *fol*. Different amounts of RNA were incubated with 5 μg of the DNA fragment bound to nitrocellulose filters. Filter-bound radioactivity was determined and plotted as a function of added amounts of RNA. The specific activities for the different RNA preparations were as follows: strain 1810, 6,060 cpm/μg; strain HB101(pJF80), 2,440 cpm/μg; strain HB101(pCT398), 2,900 cpm/μg; strain HB101, 2,700 cpm/μg. The counts per minute along the ordinate were standardized to the specific activity given for strain 1810.

fol gene region of the *E. coli* K-12 chromosome (21). The latter map was obtained from analysis of the 8.3-kb *Bam*HI fragment of the hybrid plasmid pCV27 (21). The *fol* gene of *E. coli* K-12 was localized within a 1.1-kb stretch of DNA between a *Sal*I site and a *Pst*I site as indicated in Fig. 3 (21). The two maps show extensive similarities and could be aligned without difficulty, e.g., by the *Eco*RI site, which is situated within the structural gene for dihydrofolate reductase of *E. coli* K-12 (21). Above this site on the map, apparently identical *Sal*I, *Ava*I, and *Bam*HI sites, respectively, are found in the two DNA sequences. About 0.5 kb above the common *Eco*RI site in pCV27, however, there is a *Sal*I site that is missing in the 7.8-kb *fol* fragment of pJF80. This is also demonstrated in Fig. 4, where the double digestion of pCV27 with *Eco*RI and *Sal*I resulted in an 0.5-kb fragment (Fig. 4, lane f), which is, however, absent after the corresponding digestion of pJF76 containing the 7.8-kb fragment (Fig. 4, lane b). The corresponding 1-kb *Eco*RI-*Sal*I fragment was clearly seen after digestion of pJF76 (Fig. 4, lane b), but was absent after digestion of pCV27 (Fig. 4, lane f). Complete digestion of pCV27 with *Eco*RI followed by partial *Sal*I digestion for 3, 8, and 20 min resulted in the gradual appearance of the 0.5-kb fragment (Fig. 4, lanes d, c and e, respectively). The latter, small fragment was also seen after digestion of pCV27 with *Hin*cII, which cuts like *Sal*I, but is unspecified regarding pyrimidines and purines centrally in the recognized hexanucleotide. Interestingly, *Hin*cII also cuts pJF76 in the same way, which then indicates that the disappearance of the *Sal*I site is due at the most to two transitions in this area of the DNA sequence. The *Sal*I and *Hin*cII loci of pJF76 are demonstrated in the circular map of Fig. 4A.

About 650 base pairs below the mentioned *Eco*RI site, there is an *Ava*I site in the 7.8-kb fragment of pJF80 (Fig. 3), which is missing in the corresponding fragment of pCV27. Furthermore, below this *Ava*I site in pJF80 there is a *Pst*I site, which could correspond to a *Pst*I site in pCV27, but it is about 300 base pairs more distant from the *Eco*RI site than the one in pCV27. This could be interpreted as an insert in the DNA just below the *fol* gene.

Cross-hybridization between the *fol* gene from *E. coli* K-12 and the corresponding genetic area from the trimethoprim-resistant strain 1810. The 1.1-kb *Sal*I-*Pst*I fragment of pCV27 containing the entire *fol* gene of *E. coli* K-12 (21) (Fig. 3) was used as a probe in hybridization experiments. Different hybrid plasmids containing the 7.8-kb fragment from the trimethoprim-resistant strain 1810 were doubly digested with the enzymes *Sal*I and *Pst*I. After fragment separation by agarose gel electrophoresis, DNA was transferred to nitrocellulose paper, and hybridization to the ^{32}P-labeled probe was performed as described by Swedberg and Sköld (26). Strong hybridization took place to the 1.8 kb *Sal*I-*Pst*I fragment (Fig. 3) derived from the different clones containing the 7.8-kb resistance fragment from 1810 (Fig. 5). This indicates the intimate relationship between the *fol* gene area of *E. coli* K-12 and the domain of trimethoprim resistance in strain 1810.

Determination of *fol* mRNA levels in strains overproducing dihydrofolate reductase. Levels of *fol* mRNA in strains 1810, HB101(pJF80), HB101(pCT398), and HB101 were determined by hybridization techniques. The 1.1-kb fragment of plasmid pCV27 containing the entire *fol* gene of *E. coli* K-12 (21) was isolated after cleavage of the plasmid with restriction endonucleases *Pst*I and *Sal*I and bound to nitrocellulose filters. The different bacterial strains were pulse-labeled with

[^{14}C]uridine, and RNA was isolated and hybridized to the DNA on the filters. The values obtained for bound radioactivity indicate that the relative rate of *fol* mRNA production in strain 1810 is 15 to 20 times higher than in HB101, which contains a normally regulated K-12 *fol* gene. The relative rate of *fol* mRNA production in strain HB101(pJF80) is approximately 150 times higher than that in strain HB101 and 8 times higher than that in strain 1810 (Fig. 6).

DISCUSSION

Organisms with chromosomally linked but derepressed levels of dihydrofolate reductase readily arise in the laboratory in response to trimethoprim treatment (18). In spite of the ubiquitous use of the drug for about 10 years and close observation, such organisms did not seem to occur clinically, however. This was explained either as an interference with other bacterial functions if the tetrahydrofolate biosynthetic pathway were deregulated or by the suspicion that the increased levels of dihydrofolate reductase would not be sufficient under clinical conditions (9). Here a clinical isolate of *E. coli* was studied which was resistant to more than 1,000 μg of trimethoprim per ml and showed an enzyme activity that was several hundred-fold increased over that in a strain of *E. coli* K-12 (Table 2). In other cases of drug resistance due to large increases in chromosomal enzyme levels, extra gene copies seemed to be required, as shown by Schimke et al. (17) for mammalian cells and by Edlund et al. (6) for *E. coli* β-lactamase. The high trimethoprim resistance and the intense overproduction of dihydrofolate reductase in the clinical strain described here was stable during growth for more than 150 generations in the absence of drug (data not shown), which speaks somewhat against multiple gene copies as an explanation of increased enzyme synthesis. The increased levels of dihydrofolate reductase obtainable in the laboratory by deregulation mutations amount to about 30-fold. Since strain 1810 had clinically developed an overproduction that was severalfold larger, we thought it interesting to investigate the mechanism of this changed regulation.

First, the cloned resistance gene from the isolate hybridized to a 1.1-kb DNA fragment containing the complete dihydrofolate reductase gene *fol* of *E. coli* K-12, which indicates the close similarity between the overproduced dihydrofolate reductase and the corresponding enzyme in *E. coli* K-12.

A comparison of the restriction enzyme digestion maps for the resistance gene of the isolate and the *fol* gene of *E. coli* K-12, also showed a close similarity between the two genetic areas. Differences are discernible, however. One is the disappearance of an *Sal*I locus at the beginning of the dihydrofolate reductase gene of the resistant isolate. This locus, however, kept its sensitivty to *Hin*cII, which indicates a difference to the *E. coli* K-12 gene of at the most two transitional changes. This observation could be compared with results obtained by Smith and Calvo (22) and Smith et al. (23), who constructed recombinant plasmids containing the *fol* gene of several trimethoprim-resistant mutants of *E. coli* K-12. These authors found that some of the mutants overproduced dihydrofolate reductase about 15-fold and that a *Sal*I site, corresponding to the one mentioned above at the beginning of the *fol* gene, was missing when compared with the wild type. At this site in these mutants, *Hin*cII sensitivity was retained, however. Sequence analysis by Smith and Calvo (22) and Smith et al. (23) showed that a transition from C to T in the −35 region of the *fol* promoter resulted in loss of the *Sal* site. This transition then results in a promoter up mutation leading to an about 15-fold increase in *fol* mRNA formation (20). This increase corresponds to that found for the trimethoprim-resistant isolate described here.

The determination of mRNA described in Fig. 6 shows an about 15-fold higher production of *fol* mRNA in the isolate, strain 1810, than in *E. coli* K-12. It thus seems as if the resistant strain had evolved part of its resistance by this promoter up mutation, which is not sufficient, however, to explain the several hundred-fold increase in enzyme activity observed. Other clues to the mechanism of overproduction might be found in other changes in the restriction enzyme digestion map. It was observed for example, that at the end of the *fol* gene there seemed to be an insert of about 300 base pairs the significance of which cannot be assessed for the time being. It could be noted that the dihydrofolate reductase from the resistant strain was earlier shown by methotrexate titration to have a turnover number roughly equal to that of the *E. coli* K-12 enzyme (B. Tennhammar-Ekman, Ph.D. thesis).

Cloning of the trimethoprim resistance gene on plasmid vectors resulted in expression of the dihydrofolate reductase gene in proportion to the expected copy number, with the exception of the unstable plasmid pJF76. Strain HB101(pCV27) expressed a dihydrofolate reductase activity which was 25-fold higher than that of strain HB101 (Table 2). This is in accordance with a measured copy number of 28 plasmids per chromosome (B. Tennhammar-Ekman, Ph.D. thesis). Although a direct measure of copy number for pJF76 was not performed here, the amount of plasmid DNA from strain HB101(pJF76) was found to be roughly the same as that from strain HB101(pCV27). From the data in Table 2 we then concluded that the dihydrofolate reductase activity formed in strain HB101(pJF76) was lower than expected. This could be interpreted as a negative interference with cell growth of enzyme production enhanced from both deregulation and high gene dose. An alternative interpretation would be that an intensive transcription from the inserted fragment interferes with the replication of the vector plasmid (25).

In conclusion, the remarkable overproduction of dihydrofolate reductase mediating trimethoprim resistance in the clinical isolate could be explained partly by a promoter up mutation and partly by a still unknown change in translation.

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LITERATURE CITED

1. **Bachmann, B. J.** 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. **36**:525–557.
2. **Blakley, R. L.** 1960. Crystalline dihydropteroyl-glutamic acid. Nature (London) **188**:231–232.
3. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles III: a multipurpose cloning system. Gene **2**:95–113.
4. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
5. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. A manual for genetic engineering: advanced bacterial genetics. Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.
6. **Edlund, T., T. Grundström, and S. Normark.** 1979. Isolation and characterization of DNA repetitions carrying the chromosomal beta-lactamase gene of *Escherichia coli* K-12. Mol. Gen. Genet. **173**:115–125.
7. **Hansen, J. B., and R. H. Olsen.** 1978. Isolation of large bacterial

plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. J. Bacteriol. **135**:227–238.
8. **Hershfield, V., H. W. Boyer, C. Yanofsky, M. A. Lovett, and D. R. Helinski.** 1974. Plasmid Col EI as a molecular vehicle for cloning and amplification of DNA. Proc. Natl. Acad. Sci. U.S.A. **71**:3455–3459.
9. **Koch, A. L.** 1981. Evolution of antibiotic resistance gene function. Microbiol. Rev. **45**:355–378.
10. **Low, B.** 1968. Formation of merodiploids in matings with a class of *rec*$^-$ recipient strains of *Escherichia coli* K12. Proc. Natl. Acad. Sci. U.S.A. **60**:160–167.
11. **Mandel, M., and A. Higa.** 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. **53**:159–162.
12. **Maniatis, T., A. Jeffrey, and D. G. Kleid.** 1975. Nucleotide sequence of the rightward operator of phage λ. Proc. Natl. Acad. Sci. U.S.A. **72**:1184–1188.
13. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.
14. **Normark, S., and L. G. Burman.** 1977. Resistance of *Escherichia coli* to penicillins: fine structure mapping and dominance of chromosomal beta-lactamase mutations. J. Bacteriol. **132**:1–7.
15. **Pattishall, K. H., J. Acar, J. J. Burchall, F. W. Goldstein, and R. J. Harvey.** 1977. Two distinct types of trimethoprim-resistant dihydrofolate reductase specified by R-plasmids of different compatibility groups. J. Biol. Chem. **252**:2319–2323.
16. **Rosner, J.** 1972. Formation, induction and curing of bacteriophage P1 lysogens. Virology **48**:679–684.
17. **Schimke, R. T., F. W. Alt, R. E. Kellems, R. J. Kaufman, and J. R. Bertino.** 1977. Amplification of dihydrofolate reductase genes in methotrexate-resistant cultured mouse cells. Cold Spring Harbor Symp. Quant. Biol. **42**:649–657.
18. **Sheldon, R., and S. Brenner.** 1976. Regulatory mutants of dihydrofolate reductase in *Escherichia coli* K-12. Mol. Gen. Genet. **147**:91–97.
19. **Sköld, O., and A. Widh.** 1974. A new dihydrofolate reductase with low trimethoprim sensitivity induced by an R-factor mediating high resistance to trimethoprim. J. Biol. Chem. **249**:4324–4325.
20. **Smith, D. R., and J. M. Calvo.** 1979. Regulation of dihydrofolate reductase synthesis in *Escherichia coli*. Mol. Gen. Genet. **175**:31–38.
21. **Smith, D. R., and J. M. Calvo.** 1980. Nucleotide sequence of the *E. coli* gene coding for dihydrofolate reductase. Nucleic Acids Res. **8**:2255–2274.
22. **Smith, D. R., and J. M. Calvo.** 1982. Nucleotide sequence of dihydrofolate reductase genes from trimethoprim-resistant mutants of *Escherichia coli*: evidence that dihydrofolate reductase interacts with another essential gene product. Mol. Gen. Genet. **187**:72–78.
23. **Smith, D. R., J. I. Rood, P. I. Bird, M. K. Sneddon, J. M. Calvo, and J. F. Morrison.** 1982. Amplification and modification of dihydrofolate reductase in *Escherichia coli*. Nucleotide sequence of *fol* genes from mutationally altered plasmids. J. Biol. Chem. **257**:9043–9048.
24. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
25. **Stueber, D., and H. Bujard.** 1982. Transcription from efficient promoters can interfere with plasmid replication and diminish expression of plasmid specified genes. EMBO J. **1**:1399–1404.
26. **Swedberg, G., and O. Sköld.** 1983. Plasmid-borne sulfonamide resistance determinants studied by restriction enzyme analysis. J. Bacteriol. **153**:1228–1237.
27. **Tennhammar-Ekman, B., and O. Sköld.** 1979. Trimethoprim resistance plasmids of different origin encode different drug-resistant dihydrofolate reductases. Plasmid **2**:334–346.
28. **Thomas, C. M., A. A. K. Hussain, and C. A. Smith.** 1982. Maintenance of broad host range plasmid RK2 replicons in *Pseudomonas aeruginosa*. Nature (London) **298**:674–676.
29. **Uhlin, B. E., and A. J. Clark.** 1981. Overproduction of the *Escherichia coli recA* protein without stimulation of its proteolytic activity. J. Bacteriol. **148**:386–390.
30. **Warner, H. R., and N. Lewis.** 1966. The synthesis of deoxycytidylate deaminase and dihydrofolate reductase and its control in *Escherichia coli* infected with bacteriophage T4 and T4 amber mutants. Virology **29**:172–175.