Organization of Two Sulfonamide Resistance Genes on Plasmids of Gram-Negative Bacteria

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The organization of two widely distributed sulfonamide resistance genes has been studied. The type I gene was linked to other resistance genes, like streptomycin resistance in R100 and trimethoprim resistance in R388 and other recently isolated plasmids from Sri Lanka. In R388, the sulfonamide resistance gene was transcribed from a promoter of its own, but in all other studied plasmids the linked genes were transcribed from a common promoter. This was especially established with a clone derived from plasmid R6-5, in which transposon mutagenesis showed that expression of sulfonamide resistance was completely dependent on the linked streptomycin resistance gene. The type II sulfonamide resistance gene was independently transcribed and found on two kinds of small resistance plasmids and also on large plasmids isolated from clinical material.

Plasmid-borne sulfonamide resistance in Escherichia coli is due to the production of a sulfonamide-resistant variant of the enzyme dihydropteroate synthase, the normal target for sulfonamide resistance (19). During enzymatic characterization of plasmid-coded sulfathiazole-resistant dihydropteroate synthases, a tentative characterization of two kinds of plasmid-coded enzymes was made. Enzymes of the two kinds differed from each other mainly in their heat lability in crude extracts and differed from the chromosomal enzyme both in stability and in susceptibility to sulfathiazole inhibition. Of five clinical isolates, two (pGS01 and pGS02) were classified as producers of labile enzyme activity (type I), and the three others (pGS03, pGS04, and pGS05) produced stable enzyme activity (type II) (13).

The type I enzyme is also specified by several earlier characterized plasmids. In a previous paper, restriction enzyme mapping and DNA-DNA hybridizations established the identity of the sulfonamide resistance in the clinical isolates pGS01 and pGS02 with the previously described plasmids R1, R100, R6, and R388 (14).

There exists a large population of small, nonconjugative plasmids carrying linked sulfonamide and streptomycin resistance. Two well-characterized plasmids of this group are RSFI010 (5) and pBP1 (16). van Treeck et al. showed that the resistance genes of these two plasmids are homologous, although the rest of the plasmid DNAs differ substantially (16). Heffron et al. (6) showed by Tn5 insertions that in RSFI010 the two resistance genes form an operon with the sulfonamide resistance (sal) gene proximal to the promoter.

In this paper the organization of the two types of sulfonamide resistance genes is described. First, in plasmid R6-5 the linked sulfonamide and streptomycin resistance genes were shown to be expressed as an operon with the streptomycin resistance gene as the first in the operon. Second, linkage between sulfonamide and trimethoprim resistance genes was investigated. In the previously known plasmid R388 and in some newly isolated plasmids from clinical material, genes encoding resistance to sulfonamides and trimethoprim were linked in an arrangement similar to that found in R6-5. Third, genes encoding type II sulfonamide-resistant dihydropteroate synthase, which previously have been found only on small plasmids, were also found to be incorporated into large plasmids isolated from clinical material.

MATERIALS AND METHODS

Bacterial strains. For most purposes the E. coli K-12 strain C600 (F' thi thr leu lac tonA supE) (1) was used. The E. coli K-12 strain J53 (F' pro met) (3) was used for transposon mutagenesis, and the E. coli K-12 strain JM83 (ara Δlac-pro strA thi Δ80dlacZ ΔM15) (17) was used as a host for plasmids pUC8 and pUC9 and their derivatives.

Plasmids. The plasmids used are listed in Table 1. Plasmid DNA preparations, restriction enzyme digestions, and agarose gel electrophoresis were carried out as described before (14). Ligation of DNA was performed in a buffer containing 66 mM Tris chloride (pH 7.5), 0.1 mM EDTA, 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP, and 0.1 mg of bovine serum albumin per ml. T4 DNA ligase was purchased from Boehringer GmbH, Mannheim, Federal Republic of Germany. Transformation was performed as described by Dagert and Ehrlich (4).

Tn5 insertions in plasmid pGS105. Transposition mutagenesis was done essentially as described by Heffron et al. (6). pGS105 was introduced into strain J53 by transformation, and bacteriophage λ::Tn5 was added. Plasmids with inserted Tn5 were isolated by transformation of E. coli C600.

DNA-DNA hybridization. DNA was transferred from agarose gels to nitrocellulose paper (Millipore Corp., Bedford, Mass.) by the method of Southern (11). Hybridization and autoradiography were performed as described earlier (14).

Preparation of ³²P-labeled DNA probes. Specific DNA fragments were recovered by cutting out corresponding bands from an agarose gel. The gel slice was subjected to electrophoresis in a 0.7% agarose gel cast in a tube (0.9 by 8 cm). DNA was recovered in a dialysis tube tied to the bottom of the electrophoresis tube, precipitated by the addition of ethanol, and dissolved in 10 mM Tris chloride (pH 7.5) containing 1 mM EDTA. Isolated DNA fragments were labeled with α-³²P]TP (600 Ci/mmol; New England Nuclear Corp., Boston, Mass.) by the method of Maniatis et al. (9).

RESULTS

Construction of hybrid plasmids for determination of the expression of type I dihydropteroate synthase activity. Earlier, when type I sulfonamide resistance determinants were
TABLE 1. Plasmids

| Designation | Size (kb) | Resistance markers* | Reference or derivation |
|-------------|-----------|----------------------|-------------------------|
| pGS03B      | 6.0       | Su Sm                | 14                      |
| pJM55B      | 6.0       | Su Sm                | 10                      |
| pJM4B       | 13.0      | Ap Sm Su             | 10                      |
| pGS04       | 100       | Sm Su                | 13                      |
| pGS05       | 100       | Sm Su                | 13                      |
| R388        | 32        | Su Tp                | 18                      |
| pLMO20      |           | Ap Hg Km Su Tc Tp    | Description in text     |
| pLMO27      |           | Cl Hg Tp Tc          | Description in text     |
| pUC8        | 2.7       | Ap                   | 17                      |
| pUC9        | 2.7       | Ap                   | 17                      |
| pBR322      | 4.4       | Ap Tc                | 2                       |
| pFC012      | 9.5       | Sm Su                | 15                      |
| pGS105      | 7.9       | Ap Sm Sm             | 3.5-kb BamHI fragment from pFC012 inserted in pBR322 |
| pGS105a     | 6.2       | Ap Sm                | 1.7-kb HindIII fragment deleted from pGS105 |
| pGS106      | 7.9       | Ap Sm Sm             | Like pGS105, but inverted orientation of insertion |
| pGS106a     | 5.3       | Ap                   | 2.6-kb HindIII fragment deleted from pGS106 |
| pGS107      | 7.4       | Ap Sm                | 3.0-kb EcoRI-HindIII fragment from pFC012 inserted in pBR322 |
| pGS108      | 6.7       | Ap Su                | 2.3-kb EcoRI-HindIII fragment from pFC012 inserted in pBR322 |
| pGS72       | 6.1       | Ap Su                | 14                      |
| pGS73       | 6.1       | Ap                   | Like pGS72, but insertion in inverted orientation |
| pGS74       | 7.9       | Ap Su Tp             | Two BamHI fragments from R388 inserted in pBR322 |
| pGS74a      | 5.8       | Ap Su                | 2.1-kb EcoRI fragment deleted from pGS74 |
| pGS41       | 5.5       | Ap Su Tc             | 14                      |
| pGS51       | 5.5       | Ap Su Tc             | 14                      |

* Abbreviations: Ap, ampicillin resistance; Cl, chloramphenicol resistance; Hg, mercury resistance; Km, kanamycin resistance; Sm, streptomycin resistance; Su, sulfonamide resistance; Tc, tetracycline resistance; Tp, trimethoprim resistance.

cloned on HindIII fragments, only one of the orientations in pBR322 was possible for the expression of the sulfonamide resistance (14). More hybrid plasmids covering the region outside the HindIII site used in cloning and including the streptomycin gene known to be located close to the sulfonamide resistance gene were constructed. A 3.5-kilobase (kb) BamHI fragment contained within the EcoRI fragment from R6-5 cloned in pFC012 (15) and expressing both sulfonamide and streptomycin resistance was cloned in the BamHI site of pBR322 in both possible orientations (pGS105 and pGS106; Fig. 1). By further subcloning, the two plasmids pGS105a and pGS106a were constructed. The 2.2-kb fragment containing the streptomycin resistance gene as it is present in plasmid pGS105a gave expression of streptomycin resistance. However, the 1.3-kb fragment carrying the sulfonamide resistance gene as in plasmid pGS106a did not give any detectable sulfonamide resistance. In pGS106a no vector promoter is available for the expression of the cloned fragment (12).

In another set of constructions (pGS107 and pGS108; Fig. 2), pFC012 was digested with EcoRI and HindIII, which separates the sulfonamide and streptomycin resistance genes on two fragments. These two fragments were inserted between the EcoRI and HindIII sites in pBR322. The streptomycin resistance gene in pGS107 was expressed as well in this plasmid as in the earlier constructs. In pGS108, the HindIII-EcoRI fragment is oriented in such a way that of the sul and aadA genes are from published data (2, 8) and my own observations. The resistance traits expressed by each plasmid are indicated by the inscriptions in the circles. Restriction endonuclease cleavage sites: B, BglII; Ba, BamHI; E, EcoRI; H, HindIII; P, PstI; S, SacI. Resistance markers: Ap, ampicillin; Sm, streptomycin; Su, sulfonamides; Tc, tetracycline. Heavy lines indicate pBR322 sequences.
transcription starting from a weak promoter in pBR322 (12) can read into the fragment. It gave rise to a low level of resistance, as expected from the similarity to the situation in pGS11 (14).

Transposon Tn5 insertions in the streptomycin resistance determinant. The results with plasmid constructions indicated that the streptomycin resistance is independently transcribed from its own promoter, but that the sulfonamide resistance is dependent on a promoter located outside the HindIII–EcoRI fragment cloned in pGS108. To determine directly whether the sulfonamide resistance is dependent on transcription of the streptomycin resistance gene, the plasmid pGS105 was used as a receptor for Tn5 insertions. Invariably, clones that were selected as susceptible to streptomycin were also susceptible to sulfonamides; also some clones that were streptomycin resistant and sulfonamide susceptible were found.

The positions of insertion for several of the streptomycin susceptible clones were identical or very similar (Fig. 3). Two insertions that gave streptomycin-resistant and sulfonamide-susceptible clones were located on the 2.2-kb HindIII–BamHI fragment where the streptomycin resistance gene is located. This insertion then puts a limit to the extension of the streptomycin resistance gene. Both classes of insertion were located well outside the coding region for the sulfonamide resistance gene.

Linkage of sulfonamide and trimethoprim resistance determinants. A different context in which a sulfonamide resistance determinant is connected to trimethoprim resistance is found in the plasmid R388. The map of this plasmid shows the two resistances closely linked (18). The cloning of sulfonamide resistance from R388 on a 1.7-kb BamHI fragment was described in a previous paper (14), where the homology between sul genes from R388 and R100 also was shown. The hybrid plasmid pGS72 expressed sulfonamide resistance well, probably resulting from the fact that the tet promoter of pBR322 could be utilized for transcription. To further characterize the transcription properties of the clone pGS72, the BamHI fragment was cleaved out and reinserted to obtain fragments in both orientations. The plasmid pGS73, which carries the same 1.7-kb BamHI fragment as pGS72 but in the inverted orientation, does not express...
The sulfonamide resistance (Fig. 4). Furthermore, the plasmid pGS74 was derived by cloning DNA from a partial BamHI digest of R388 so that both trimethoprim and sulfonamide resistance were conferred by the plasmid (Fig. 4). By restriction mapping, it was confirmed that the two BamHI fragments were oriented in the same way as in the plasmid R388. The plasmid pGS74 was then cleaved with EcoRI and ligated to yield plasmid pGS74a, in which the trimethoprim resistance gene is deleted. With this construction, no pBR322 promoter reading into the cloned fragment is available (12). Still, sulfonamide resistance is conferred by plasmid pGS74a, a fact which strongly suggests that a promoter is present on the cloned fragment and located between the EcoRI and the BamHI sites.

Recently, other plasmids carrying linked sulfonamide and trimethoprim resistance have been isolated from samples of fecal gram-negative bacteria collected in Sri Lanka (L. Sundström, T. Vinayagamoorthy, and O. Sköld, Antimicrob. Agents Chemother., in press). Among the plasmids a group of six conferring transmissible resistance to trimethoprim was selected for further study. Some of these plasmids were analyzed for the expression of the sulfonamide resistance gene. Common to all these six plasmids was a 3.5-kb BamHI fragment that carried both sulfonamide and trimethoprim resistance genes. The plasmid pLMO20 carries such a 3.5-kb BamHI fragment which was cloned in pBR322 and expressed both resistances. There are differences between R388 and pLMO20 in the region concerned (Fig. 5). Note that the BamHI and EcoRI sites in R388 have counterparts in pLMO20 and that pLMO20 has a third HindIII site in addition to the two that are similar to the ones found in R388. This is noteworthy because the data from constructions of pGS73 and pGS74a suggest a promoter location between the BamHI site and the EcoRI site in R388. By partial digestion with HindIII, the trimethoprim resistance gene of pLMO20 was deleted to each of the three HindIII sites in the intercistronic region. Even with the shortest deletion, expression of sulfonamide resistance was lost.

Another interesting plasmid found in the material mentioned above was called pLMO27. It contains a 3.5-kb BamHI fragment conferring trimethoprim resistance but not sulfonamide resistance. However, the restriction map indicated that a sulfonamide resistance gene should be present. For instance (Fig. 5), pLMO27 contained a 1.3-kb BamHI-HindIII fragment that corresponded well with fragments from several plasmids conferring sulfonamide resistance (14). To investigate whether the lack of resistance is caused by a defective gene or lack of transcription, the 1.3-kb BamHI-HindIII fragment from pLMO27 was excised and cloned in the plasmids pUC8 and pUC9, in which the cloned fragment is under the control of the lac promoter-operator system (17). After transformation of E. coli JM83, in which lac gene expression is constitutive, pUC9 carrying the fragment expressed sulfonamide resistance, whereas with pUC8 as vector plasmid no expression was detected. In pUC9, the orientation of the inserted fragment is such that transcription would run from the HindIII site toward the BamHI site, which is also the expected order of transcription for the sul gene. The reverse orientation is created with pUC8 as the vector; this would therefore not be expected to express the sulfonamide resistance.

**Restriction maps of plasmids encoding dihydropteroate synthase of type II.** Plasmids encoding type II dihydropteroate synthase are of two kinds. The plasmid pGS03B (14) is a 6-kb, nonconjugative plasmid, which by restriction enzyme digestion was shown to be identical to the plasmid pBP1 described by van Treec et al. (16) (data not shown). Both pGS04 and pGS05 (13), on the other hand, are 100-kb conjugative plasmids, differing only in that pGS05, in addition to conferring sulfonamide resistance, gives resistance to streptomycin. The sulfonamide resistance genes from both pGS04 and pGS05 have been cloned as 1.1-kb EcoRI fragments in pBR322 (14). A restriction map was made on the inserted EcoRI fragments and compared with maps of pGS03B and another small plasmid, pJM4B (10), which was isolated in Denmark and is related to the plasmid RSF1010 (Fig. 6). A region of similarity, extending from the left EcoRI site, is apparent from the presence of cleavage sites for PstI, BglII, HincII, and Aval in the same positions in both pGS04 and pGS05 and in the small plasmids. At the right end of the region depicted in Fig. 6, a difference can be noted in that a PstI site is present in pGS04, pGS05, and pJM4B but not in pGS03B. Outside the PstI site the sequences seem to differ; the next EcoRI site in pGS04 and pGS05 has no counterpart in either pJM4B or pGS03B. The restriction map similarity between the different plasmids is thus confined to a small region, just enough to code for the dihydropteroate synthase.

**Interrelations between small plasmids and larger ones transferring sulfonamide resistance by conjugation.** The sulfonamide resistance genes of the small plasmids were compared with those of the earlier-described transferable plasmids (14). The plasmid pJM35B (10), which according to restriction enzyme analysis was identical to pGS03B, was
labeled with $^{32}$P by nick translation and used as a probe in hybridization with cloned restriction enzyme fragments carrying sulfonamide resistance from the large plasmids. No hybridization was observed between pJMSSB and sulfonamide resistance genes from the plasmids R1, R100, R6, pGS01, pGS02, R388, or R22259. However, pJMSSB hybridized to sulfonamide resistance genes from pGS04 and pGS05 as well as to the other small plasmids, pGS03B and pJM4B (data not shown).

The homology between pJMSSB and the larger plasmids pGS04 and pGS05 was investigated further. The small plasmid was digested with EcoRI and PvuII. The resulting two fragments, 4.2 and 1.8 kb, were isolated and $^{32}$P labeled by nick translation. The large plasmids pGS04 and pGS05 were both digested with EcoRI, and the fragments were transferred to nitrocellulose paper after separation on an agarose gel (Fig. 7). The 4.2-kb fragments from pJMSSB hybridized to two fragments in each digest of the larger plasmids. The smallest of the two hybridizing fragments corresponded in size (1.1 kb) to the fragments cloned in the recombinant plasmids pGS41 and pGS51 (14). This fragment should thus contain the sulfonamide resistance gene. That hybridization occurred with another fragment found in both pGS04 and pGS05 indicated that the homology extends beyond the sulfonamide resistance gene.

The 1.8-kb fragment gave hybridization with one fragment from pGS04 and two fragments from pGS05. The larger fragment from pGS05 showing hybridization could contain the streptomycin resistance gene, since this resistance marker is present in pGS05 but not in pGS04. The hybridization observed with another fragment found both in pGS04 and pGS05 supported the idea that the homology between the small plasmids and the large plasmids extends beyond the resistance genes.

**DISCUSSION**

It has previously been shown that there exist two types of drug-resistant, plasmid-coded dihydropteroate synthase activities (13). The genetic organization of the two resistance determinants has been studied before (14), and the results presented in this paper reflect the different environments in which the two gene types have been set while maintaining functional integrity. The type I dihydropteroate synthase gene is found on the plasmids R1, R6, and R100 among others and is located close to a streptomycin resistance gene coding for an adenylylating enzyme. In other plasmids (R388, pLM020, pLM027), a type I gene was linked to trimethoprim resistance genes. The type II dihydropteroate synthase gene is found on small plasmids such as RFS1010 and pBP1, where the sulfonamide resistance gene is linked to another streptomycin resistance gene, which codes for a phosphorylating enzyme; the gene has now also been found on large, conjugative plasmids.

In this paper the organization of the sulfonamide and streptomycin resistance genes in plasmid R6-5 has been studied. I conclude that the streptomycin resistance gene is expressed independently but that the sulfonamide resistance gene does not have a promoter of its own. In all constructed plasmids where the two resistance genes are separated the streptomycin gene is always expressed independently of the vector, as for example in pGS103a and in pGS107. The sulfonamide resistance gene on the other hand is only expressed when a vector promoter can be used for transcription. Compare the plasmids pGS106a, in which no vector promoter is available, and pGS108, in which a pBR322 promoter is available and sulfonamide resistance is expressed, although at a low level. The results of transposon inactivation where insertions into the streptomycin resistance gene in pGS105 invariably lead to extinction also of sulfonamide resistance point more directly to an operon organization and common transcription for the two resistance genes.

Kratz et al. (8) have pointed out the large degree of rearrangements in the vicinity of the sulfonamide resistance gene in Tn21 and related transposons. My results show that the DNA sequences where the rearrangements take place are important for the expression of the sulfonamide resistance gene. In R388, which harbors a sulfonamide resistance gene homologous to the one in R100 (14), it was possible to tentatively locate a promoter that might be responsible for the expression of the resistance gene. This promoter must be located at least 400 base pairs from the coding sequence for the dihydropteroate synthase, since we can define the limit for the structural gene at a HindIII site and that for the promoter close to a BamHI site 400 base pairs upstream toward the trimethoprim resistance gene. In all other plasmids included in this study the restriction map differs from R388 in the region where this promoter is supposed to be located. The two HindIII sites at the starting point for the coding sequence are conserved, so the divergence must start between the coding start and the promoter. The operon structure in the streptomycin and sulfonamide resistance plasmid pGS105 can be interpreted as a consequence of insertion of the streptomycin resistance gene in a previous sequence in such a way as to separate the promoter and the coding sequence of dihydropteroate synthase. The same type of interpretation could explain the results with pLM020. In both of these cases, however, sulfonamide resistance is still expressed, presumably because of readthrough of the mRNA of the preceding gene. In another case, pLM027, the observed lack of sulfonamide resistance could be caused by insertion of the dihydrofolate reductase gene in such a way as to destroy totally the transcription of the sulfonamide resistance gene.
As an alternative explanation, the distance between the promoters and the structural genes in the different plasmids studied here can be a consequence of the transfer of the type I resistance gene from a bacterium with a different type of transcription control so that its original promoter sequence is not recognized by the RNA polymerase of E. coli. For expression to occur it is necessary for the gene to be placed under the control of an E. coli promoter.

The type II gene has its own promoter and is mainly found on small plasmids. Such small plasmids are evidently widely spread and stably maintained in bacterial populations, although their role in bacteria apart from antibiotic resistance is unknown. It has been argued that the small size of the plasmids makes them a small burden for the host cell, so the selection pressure against the plasmid is not great even in the absence of antibiotic treatment (7, 16). Now we see also the presence of the type II gene on large, conjugative plasmids, which further increases the ability of the resistance gene to be spread effectively among gram-negative bacteria. There is no evidence for the presence of a transposable element that is responsible for the translocation of the resistance gene to a large plasmid. Possibly a small plasmid has been integrated into a larger one and is thereafter maintained as a stable cointegrate, perhaps after loss of some plasmid DNA. The hybridization results presented in this paper favor the idea of integration of a small plasmid into a preexisting conjugative plasmid.

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