Polygenes and Modifier Genes for Tetracycline and Penicillin Resistance in *Neisseria gonorrhoeae*

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The genetic basis for spontaneous resistance to tetracycline (Tet) and penicillin (Pen) in *Neisseria gonorrhoeae* was investigated. *Tet* and *pen* are polygenes which confer small but distinct levels of resistance to Tet and Pen, respectively. *Mtr* is a multiple-drug resistance polygene which increases resistance to Tet and Pen (as well as to other unrelated antibiotics). *Tem* is a modifier gene affecting resistance to Tet and Pen. *Pem* is a modifier gene for Pen resistance.

The following gene combinations code for resistance to five antibiotics: *tet*, *mtr* and *tem* for Tet; *pen*, *mtr*, *pem* and *tem* for Pen; *tet*, *tem* and *mtr* for doxycycline; *pen* and *pem* for ampicillin; *pen*, *pem* and *mtr* for nafcillin.

INTRODUCTION

Transformation experiments with *Neisseria gonorrhoeae* demonstrate that different levels of resistance to penicillin (Pen) and tetracycline (Tet) are due to individual genes and a combination of genes (Maier *et al.*, 1977; Maier *et al.*, 1975*a, b*; Sarubbi *et al.*, 1974; Sparling *et al.*, 1975). The *pen* gene or *pen A* and another, *mtr* or *ery-2*, code for different low levels of resistance to Pen (Sparling, 1977; Sparling *et al.*, 1975). In combination, these genes have additive effects. Another gene, *pen B*, together with the two Pen resistance genes results in a further increase in resistance to penicillin (Sparling *et al.*, 1975). The combination of these three genes still does not account for the level of resistance to Pen which is exhibited by the multiple-drug-resistant DNA donor strain. With regard to Tet resistance, the *tet* gene codes for a low level of resistance (Maier *et al.*, 1975*a, b*; Sparling *et al.*, 1975). This gene together with *pen B* and *mtr* account for the full donor level resistance to Tet (Maier *et al.*, 1975*b*; Sparling *et al.*, 1975).

We also have detected a gene, *tem*, which enhances Tet and Pen resistance, but, unlike *pen B*, it has no phenotypic expression of its own. *Tem* is by definition a modifier gene (Bryan, 1961). Another modifier gene, *pem*, in concert with *pen*, *mtr* and *tem* accounts for the full donor level of resistance to Pen. *Tet*, *mtr* and *pen* are polygenes (Bryan, 1961). The *tem* and *pem* genes in combination with appropriate polygenes affect resistance to doxycycline, ampicillin and nafcillin.

METHODS

Media, chemicals and conditions for cultivation of gonococci were as previously described (Maier *et al.*, 1975*a*).

Bacterial strains. The multiple-drug-resistant strain *Neisseria gonorrhoeae* 24392, the drug-susceptible strain *N. gonorrhoeae* 49191 (Maier *et al.*, 1975*b*) and their derivatives are listed in Table 1. Both 24392 and 49191 are clinical isolates.

Table 1. *Gonococcal strains*

Strain designation	Antibiotic resistance genotype*	Source
24392	<i>tem str tet cml ery mtr pen pem</i>	Clinical isolate
49191	<i>tem⁺ str⁺ tet⁺ cml⁺ ery⁺ mtr⁺ pen⁺ pem⁺</i>	Clinical isolate
Genotype and phenotype ($\mu\text{g ml}^{-1}$) relevant to Tet and Pen		Transformation† (Donor → recipient)
7P16	<i>pen</i> (Pen 0.05)	24392 → 49191 §
4B10	<i>mtr</i> (Tet 0.2; Pen 0.025)	24392 → 49191 §
6T20	<i>tet</i> (Tet 0.3)	24392 → 49191 §
MP81	<i>mtr pen</i> (Tet 0.2; Pen 0.1)	4B10 → 7P16 §
MT93	<i>mtr tet</i> (Tet 0.6; Pen 0.025)	4B10 → 6T20 §
TM14‡	<i>tem tet</i> (Tet 0.6)	24392 → 49191
TM59‡	<i>tem tet mtr</i> (Tet 1.0; Pen 0.025)	4B10 → TM14
PM50	<i>pen pem</i> (Pen 0.1)	24392 → 49191
PM51	<i>pen pem mtr</i> (Pen 0.2)	PM50 → 4B10
PM54	<i>pem pen mtr tem</i> (Tet 0.2; Pen 0.4)	TM14 → PM51
TP32‡	<i>pen tem tet</i> (Tet 0.6; Pen 0.1)	7P16 → TM14

* + indicates wild-type or susceptibility to antibiotics. The following genes code for resistance to antibiotics ($\mu\text{g ml}^{-1}$): *str* 1000; *tet* 0.3; *cml* 1.0; *ery* 0.25; *pen* 0.05; *mtr*, a multiple-drug resistance gene for Tet 0.2, Cml 0.5; Ery 1.0, Pen 0.025; *pem*, modifier for Pen; *tem*, modifier for Tet and Pen.

† The genes were added sequentially except in the case of *pen pem* which are closely linked and were cotransferred.

‡ TM14, TM59 and TP32 also possess the *str*, *cml* and *ery* (low level) resistance genes of linkage group I which do not affect Tet or Pen resistance (Maier *et al.*, 1975*b*).

§ Maier *et al.* (1975*a*).

Isogenic crosses. Strain 24392 was the parent for all the genes listed. These genes were individually transformed into strain 49191. The genetic basis for antibiotic resistance presented is based on isogenic crosses between strains of 49191.

Extraction of DNA. For standard transformation procedures, DNA was extracted by a phenol method (Young & Wilson, 1974). For rapid transformation procedures, a crude DNA preparation was used (Wharton & Zubrzycki, 1976).

Standard transformation. The standard transformation procedure previously described (Maier *et al.*, 1975*a*) was modified by using phenol-extracted DNA and 2 mM-CaCl₂ in the transformation mix. These modifications allowed detection of greater linkage between genes (Warner *et al.*, 1975).

Rapid transformation. The rapid transformation procedure (Zubrzycki & Robinson, 1978) involved spotting crude DNA preparations on to a lawn previously spread with a competent recipient, using either a standard inoculating loop or a 25-pronged replicator. The use of the latter allows the delivery of duplicate preparations of DNA and broth controls on to a variety of antibiotic-containing plates.

Phenotypic lag. For selecting antibiotic-resistant transformants, a 4 to 6 h lag time was allowed before the agar with micro-colonies was layered on to antibiotic-containing media (Maier *et al.*, 1975*a*).

The Tet and Pen resistance genotypes. The genes for Tet and Pen resistance were identified by the levels of resistance conferred (Maier *et al.*, 1975*a*). The genotypes and phenotypes with regard to Tet and Pen resistance are shown in Table 1.

Minimal inhibitory concentrations (m.i.c.). The procedure for determining the m.i.c. values of antibiotics involved a multipronged replicator system which allows delivery of many cell suspensions on to plates containing different concentrations of antibiotics (Maier *et al.*, 1975*a*). To ensure reproducibility and proper evaluation of results, the following strains with known antibiograms were used: 24392, 49191, 7P16, 4B10, 6T20, MP81, MT93. M.i.c. values for Tet were determined by testing at concentrations of ($\mu\text{g ml}^{-1}$): 0.15, 0.2, 0.25, 0.3, 0.5, 1.0 and 1.5. Those for Pen were: 0.01, 0.015, 0.02, 0.025, 0.03, 0.05, 0.1, 0.12, 0.15, 0.2, 0.4 and 0.8.

RESULTS

Tem, a modifier locus affecting tetracycline resistance

Previous work using sodium dodecyl sulphate (SDS)-prepared DNA established the genotypes and phenotypes of three classes of Tet-resistant transformants (Maier *et al.*,

Fig. 1. Results of an experiment in which DNA from four strains was spotted with a loop on to agar plates previously spread with a suspension of gonococci and then layered, after a 5 h phenotypic lag, on to agar containing different concentrations of Tet ($\mu\text{g ml}^{-1}$) as follows: plate A, 49191, 0.3; plate B, 49191, 0.6; plate C, 6T20, 0.6; plate D, TM14, 1.0. On plates A, B and C the DNA spotted (from top to bottom) was extracted from strains TM14, 4B10, MT93 and 24392. On plate D the DNA came from MT93, 4B10, 6T20 and 24392.

1975*b*). Typical strains (4B10, 6T20 and MT93) are listed in Table 1. Using phenol-extracted DNA, identical strains were isolated, but in addition, a fourth class, represented by TM14 (Table 1), was observed.

Transformants MT93 and TM14 (Table 1) exhibited the same level of resistance to Tet, but differed in the presence or absence of the *mtr* gene (characterized by resistance to erythromycin at $1 \mu\text{g ml}^{-1}$). It was deduced that a third locus, called *tem*, was involved in Tet resistance.

In order to establish the role of *tem* in Tet resistance, rapid transformation experiments were conducted. The results from part of one such experiment are shown in Fig. 1. Plate A shows that the $0.3 \mu\text{g ml}^{-1}$ level of resistance is conferred by *tet* ($0.3 \mu\text{g ml}^{-1}$) from TM14 or MT93 but not *mtr* ($0.2 \mu\text{g ml}^{-1}$) from 4B10. The absence of growth spots on plate B shows that the $0.6 \mu\text{g ml}^{-1}$ level of resistance requires two or more genes which cannot be transferred simultaneously into 49191 by this technique. Plate C shows that the $0.6 \mu\text{g ml}^{-1}$ level of resistance can be attained if the recipient 6T20, which contains *tet* ($0.3 \mu\text{g ml}^{-1}$), receives *tem* (no phenotype) from TM14 or *mtr* ($0.2 \mu\text{g ml}^{-1}$) from 4B10. The 6T20 DNA is a negative control. Plate D shows that an even higher level of resistance ($1 \mu\text{g ml}^{-1}$) can be attained if the recipient TM14, which contains *tet* ($0.3 \mu\text{g ml}^{-1}$) and *tem* (no phenotype), receives *mtr* ($0.2 \mu\text{g ml}^{-1}$) from MT93 or 4B10. The *tet-tem* combination (6T20-TM14) does not confer this level of resistance. DNA from 24392 containing all the Tet genes was used as a control. Analyses of these results is an example of how the existence of a *tem* locus can be detected by using strains with known genotypes as recipient and DNA donors in a variety of genetic crosses. The results of a complete experiment using a multipronged replicator are shown in Table 2. The use of 24392 DNA, containing the *tet*, *mtr* and *tem*

Table 2. Association of different genotypes with the various phenotypic classes of tetracycline-resistant strains obtained from transformation experiments

Bacterial lawn* [gene(s) and level of resistance to Tet ($\mu\text{g ml}^{-1}$)]	Selection plate with Tet ($\mu\text{g ml}^{-1}$)	Growth in area in which DNA was spotted†					
		Source of DNA					
		6T20	4B10	MT93	TM14	24392	49191
6T20 (<i>tet</i> 0.3)	0.3	+	+	+	+	+	+
	0.6	—	+	+	+	+	—
	0.8	—	—	—	—	—	—
4B10 (<i>mtr</i> 0.2)	0.3	+	—	+	+	+	—
	0.6	+	—	+	+	+	—
	0.8	—	—	—	—	—	—
MT93 (<i>mtr tet</i> 0.6)	0.8	—	—	—	+	+	—
	1.0	—	—	—	+	+	—
	1.5	—	—	—	—	—	—
TM14 (<i>tet tem</i> 0.6)	0.8	—	+	+	—	+	—
	1.0	—	+	+	—	+	—
	1.5	—	—	—	—	—	—
49191 (sensitive wild-type 0.1)	0.2	+	+	+	+	+	—
	0.3	+	—	+	+	+	—
	0.6	—	—	—	—	—	—

* A lawn of 24392 grew on all Tet plates except 1.5 $\mu\text{g ml}^{-1}$.

†+, Growth; —, no growth. Growth means that the transformants were resistant to Tet at the concentration in the plate, as indicated.

genes, shows that no more than one Tet resistance gene can be transformed by this rapid technique. DNA from 49191 is the negative control. In all cases DNA from one strain spotted on a lawn of that same strain did not increase its level of resistance to Tet. This means that a 'double dose effect' does not occur. The *tet* in 6T20, MT93 and TM14 DNA preparations has additive effects with the *mtr* in 4B10, increasing its level of resistance from 0.3 to 0.6 $\mu\text{g ml}^{-1}$. The same effect occurs using *mtr* DNA (4B10 or MT93) in combination with *tet* (6T20). The *tet* + *tem* in TM14 DNA has additive effects with the *tet* in 6T20 and with the *mtr* + *tet* combination in MT93, increasing the Tet resistance of these strains to the levels shown in Table 2. 'Double dose effects' do not occur; therefore, these results are due to the *tet* + *tem* and *tet* + *mtr* + *tem* combinations, respectively. The latter genotype with the same phenotypic result can be constructed by adding *mtr* from 4B10 to *tet* + *tem* in TM14. Such a strain, TM59 (Table 1), exhibits the same level of resistance to Tet as does strain 24392. It should be reported that results similar to those in Table 2 were obtained with many other isolates (Warner, 1978).

Although the effects of *tem* on Tet resistance are obvious, the *tem* gene has not been isolated alone in any strain. Many attempts to do so after rapid or standard transformation experiments have been unsuccessful (Warner, 1978). The most likely explanation is that *tem* has no phenotypic expression of its own.

The results obtained and conclusions reached as a result of the rapid transformation experiments were confirmed by standard transformation experiments. In fact, the strains used to obtain the results shown in Table 2 were also obtained from standard transformation experiments.

Linkage of the *tet* and *tem* loci

The nature of the rapid spotting technique using a crude DNA preparation and small amounts of DNA per spot does not permit the transfer of more than one Tet resistance gene at a time. However, in standard transformation experiments using phenol-extracted DNA from TM14, transformants resistant to Tet at the 0.6 $\mu\text{g ml}^{-1}$ level occurred at a frequency (10^{-3}) associated with a single transformation event (Maier *et al.*, 1975a). This

Table 3. Association of different genotypes with the various phenotypic classes of penicillin-resistant strains obtained from transformation experiments

Bacterial lawn [gene(s) and level of resistance to Pen ($\mu\text{g ml}^{-1}$)]	Selection plate with Pen ($\mu\text{g ml}^{-1}$)	Growth in area in which DNA was spotted*			
		4B10 (<i>mtr</i>)	7P16 (<i>pen</i>)	PM50 (<i>pen pem</i>)	MP81 (<i>mtr pen</i>)
Type A (<i>pen</i> 0.05)	0.1	+	—	+	+
	0.2	—	—	—	—
	0.4	—	—	—	—
Type B (<i>pen</i> + <i>pem</i> 0.1)	0.1	+	—	+	+
	0.2	+	—	—	+
	0.4	—	—	—	—

* +, Growth; —, no growth.

level of resistance in these strains is due to two genes, *tet* and *tem*. Their presence in these transformants was confirmed by rapid transformation tests. The close linkage of the *tet* and *tem* loci was confirmed by routine DNA dilution experiments (Goodgal, 1961). DNA from strain TM14 was used to transform strain 49191. Below the saturating concentration of $1.0 \mu\text{g DNA ml}^{-1}$, the number of Tet-resistant transformants at the $0.6 \mu\text{g ml}^{-1}$ level decreased in linear proportion to the decreasing DNA concentration (Warner, 1978). Therefore, these Tet-resistant transformants have received only one piece of DNA which carries the two loci *tet* and *tem*.

Pem, a modifier locus affecting penicillin resistance

As a result of transformation experiments using SDS-extracted DNA, three phenotypic classes of Pen-resistant transformants were observed by Maier *et al.* (1975*b*). The representative strains 4B10, 7P16 and MP81 are listed in Table 1. Identical transformants were also obtained when phenol-extracted DNA was used. In addition, other classes of transformants were obtained. The class of transformants represented by strain PM50 was of considerable interest. With regard to Pen resistance, MP81 and PM50 differ only in the presence or absence of *mtr* (characterized by resistance to erythromycin at $1 \mu\text{g ml}^{-1}$). It was deduced that another locus, labelled *pem*, was involved in Pen resistance.

From standard transformation experiments using PM50 DNA, only two phenotypic classes of Pen-resistant transformants were isolated. Type A was resistant to $0.05 \mu\text{g ml}^{-1}$ and presumed to contain *pen*, and type B was resistant to $0.1 \mu\text{g ml}^{-1}$ and presumed to contain *pen* and *pem*. These two types were used in rapid transformation experiments. Because *mtr* alone confers a low level of Pen resistance, the effect of this gene on the type A and type B transformants was also determined. The results (Table 3) indicate that a third locus, *pem*, does account for an increase in the level of resistance to Pen. Attempts to isolate *pem* in strains obtained from rapid and standard transformation experiments have been unsuccessful (Warner, 1978). Similar to *tem*, the *pem* locus has no phenotypic expression of its own other than enhancing the resistance expressed by *pen* and *mtr*.

The results of these rapid and subsequently standard transformation experiments prove that the combination of *pen*, *pem* and *mtr* codes for resistance to Pen at $0.2 \mu\text{g ml}^{-1}$. However, this gene combination does not produce the full level of resistance to Pen of $0.4 \mu\text{g ml}^{-1}$ observed in strain 24392. It was deduced that at least one other gene was involved in Pen resistance.

The tem locus affects penicillin resistance

Rapid transformation experiments showed that DNA from TM14 in combination with *pen*, *pem* and *mtr* in PM51 resulted in transformants, such as PM54 (Table 1), which exhibit the full level of resistance to Pen as observed for strain 24392. TM14 contains *tem*

Table 4. *Role of the tem and pem loci in cross-resistance of 49191 transformants to other antibiotics**

Strain	Minimal inhibitory concn ($\mu\text{g ml}^{-1}$)		
	Ampicillin	Nafcillin	Doxycycline
49191	0.06	0.6	0.25
24392	0.25	15	2.0
6T20 (<i>tet</i>)	0.06	0.6	1.0
4B10 (<i>mtr</i>)	0.06	1.25	0.5
7P16 (<i>pen</i>)	0.15	2.5	0.25
MP81 (<i>mtr pen</i>)	0.15	10	0.5
MT93 (<i>mtr tet</i>)	0.06	1.25	1.5
PM50 (<i>pem pen</i>)	0.25	10	0.25
PM51 (<i>mtr pem pen</i>)	0.25	15	0.5
TM14 (<i>tet tem</i>)	0.06	0.6	1.0
TM59 (<i>mtr tet tem</i>)	0.06	1.25	2.0

* Strains 49191 and 24392 are the drug-susceptible and drug-resistant strains, respectively.

and *tet*, but *tet* does not confer resistance to Pen (Maier *et al.*, 1975*a, b*; Warner, 1978). Since only one gene can be transferred, it had to be *tem* which increased resistance to Pen. These results were confirmed by standard transformation experiments.

Linkage of the pen and pem loci

DNA from PM50 (*pen pem*) was used to transform Pen resistance genes into 49191. The *pen* and *pem* markers cotransfer 90% of the time at a frequency of 10^{-3} which is associated with a single transformational event (Maier *et al.*, 1975*a*). A DNA dilution curve also confirmed that transformants resistant to Pen at $0.1 \mu\text{g ml}^{-1}$ received only one piece of DNA, which carries the two genes *pen* and *pem* (Warner, 1978). Therefore, the *pem* locus is linked to *pen*.

Cross-resistance of transformants to doxycycline, ampicillin and nafcillin

Transformants which carry a single antibiotic resistance locus or a specific two-gene combination exhibit cross-resistance to other antibiotics and antibacterial agents (Maier *et al.*, 1975*b*). However, the gene combinations previously reported did not account for the full level of resistance of 24392 to a semi-synthetic tetracycline (doxycycline), a broad spectrum penicillin (ampicillin) and a β -lactamase-resistant penicillin (nafcillin). M.i.c. analyses of many transformants demonstrated that the *tem* and *pem* loci were necessary for the full level of resistance to these antibiotics. As shown in Table 4, the following gene combinations code for cross-resistance to the three antibiotics: doxycycline, *tet tem mtr*; ampicillin, *pen pem*; nafcillin, *pen pem mtr*. In addition, the current studies confirm the phenotypic effects of the individual genes *tet*, *mtr* and *pen* for these three antibiotics (Maier *et al.*, 1975*b*).

DISCUSSION

Genetic analyses of two clinical isolates and a laboratory-induced mutant of *N. gonorrhoeae* has led to the identification of chromosomal loci responsible for resistance to a variety of antibacterial agents (Maier *et al.*, 1975*b*; Sparling, 1977). However, the genetic basis for resistance to Tet, Pen and related antibiotics was not completely established.

It was reported that transformants with a variety of phenotypic patterns were observed in mixed-culture experiments (Warner *et al.*, 1975). These phenotypes were not observed from standard transformation experiments previously reported (Maier *et al.*, 1975*a, b*). The results of mixed-culture experiments suggested the possible genotypes which could

account for the donor (24392) level of resistance to Tet and Pen. However, it was difficult to determine the genes involved because of the plethora of genetic exchanges which occur in mixed cultures. The genetic basis for resistance could only be established from controlled experimental situations.

An essential approach for resolving the interplay of genotypes controlling Tet and Pen resistance was the use of strains containing individual genes or combinations of genes coding for distinct levels of resistance to antibiotics. For example, the phenotypic effects of the *tet*+*tem* combination could not be distinguished from that of *tet*+*mtr* without using strains known to contain only *tet* or *mtr* (see Table 1). The selection and use of strains containing only *mtr* or *pen* allowed us to establish three different genotypes which result in the same phenotype (see Table 1).

An important technical approach was the use of the rapid transformation procedure. This allowed us to test the effects of various combinations of genotypes before confirming the results by standard transformation experiments. With the rapid transformation and m.i.c. procedures about 2500 transformants were tested during the various stages of the experiments described. Therefore, the conclusions presented are not based merely on the results obtained with a few strains. Most of the work on the genetic basis for antibiotic resistance of *N. gonorrhoeae* conducted in our laboratory and that of Dr P. F. Sparling are in agreement (Maier *et al.*, 1977; Maier *et al.*, 1975*b*; Sparling, 1977). Even the *pen* B and *tem* loci are functionally similar. However, there are important differences.

From studies involving *pen* B, it was concluded that *ery*-2, now designated *mtr* (Sparling, 1977), is a modifier gene for *pen* B. This gene combination results in an increased resistance to Tet and Pen. However, all recipients in these studies of the *pen* B gene contained *mtr* (*ery*-2) and other antibiotic resistance genes. Therefore, it is not clear whether *mtr* (*ery*-2) or *pen* B is a modifier. In these studies it was found that *mtr* (*ery*-2) confers a low level of resistance to Pen and a slight, almost indistinguishable, level of resistance to Tet. It may be that the concentrations of Tet used in the m.i.c. test did not allow the investigators to associate a distinct level of Tet resistance with *mtr* (*ery*-2). In our current and previous studies (Maier *et al.*, 1975*b*) *mtr* has been shown to confer a distinct level of resistance to Tet and Pen. As concluded herein, the *tem* gene is a modifier gene (Bryan, 1961) enhancing resistance to Tet and Pen conferred by *mtr*, *tet* or *pen*, but having no other detectable phenotypic expression of its own. With regard to Tet and Pen, *tet*, *mtr* and *pen* are polygenes which make a small but distinct contribution to the phenotype of an organism (Bryan, 1961).

It may be that *tem* and *pen* B are different genes. By three-factor crosses *pen* B was found to be linked to the *spc* (spectinomycin) and *cml* (chloramphenicol) loci (Sparling *et al.*, 1975). The fact that *tem* is cotransferred with *tet* suggests that it is distal to the *spc*-*cml* region. Only a comparative study of the *pen* B and *tem* genes in a common recipient will determine whether they are different genes with functional similarities.

No gene similar to, nor a functional equivalent of, the *pem* gene has been reported. Like the *tem* gene, it is a modifier for gonococcus. These two modifier genes in combination with *mtr* and *pen* account for the full level of donor resistance to Pen. It should be noted that neither strain 24392 nor 49191 produces β -lactamase (Warner, 1978) as determined by an iodometric test (Catlin, 1975). This, plus the fact that 24392 and all transformants tested are stably resistant to Pen, Tet and the other antibiotics referred to, is consistent with the proposition that these genes are chromosomally located. However, one cannot eliminate the possibility that antibiotic resistance genes of *N. gonorrhoeae* are plasmid determined (Sparling, 1977).

Reyn (1961) reported that gonococci which are resistant to one antibacterial agent tend to be resistant to others. The genetic basis for this cross-resistance can be ascertained from previous publications (Maier *et al.*, 1975*b*; Sparling, 1977). The modifier genes *tem* and *pem* can be added to the list of genes which in combination with other genes account for cross-resistance to doxycycline, ampicillin and nafcillin.

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