Resistance to Chloramphenicol in *Proteus mirabilis* by Expression of a Chromosomal Gene for Chloramphenicol Acetyltransferase

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*Proteus mirabilis* PM13 is a well-characterized chloramphenicol-sensitive isolate which spontaneously gives rise to resistant colonies on solid media containing chloramphenicol (50 μg ml⁻¹) at a plating efficiency of 10⁻⁴ to 10⁻⁵. Such chloramphenicol-resistant colonies exhibit a novel phenotype with respect to chloramphenicol resistance. When a single colony grown on chloramphenicol agar is transferred to liquid medium and grown in the absence of antibiotic for 150 generations, a population of predominantly sensitive cells arises. This mutation-reversion phenomenon has been observed in other *Proteus* species and *Providencia* strains, wherein resistance has been shown to be mediated in each case by the enzyme chloramphenicol acetyltransferase. The *cat* gene responsible for the phenomenon is chromosomal and can be cloned from *P. mirabilis* PM13 with DNA prepared from cells grown in the absence or the presence of chloramphenicol. Recombinant plasmids which confer resistance to chloramphenicol carry an 8.5-kilobase *PstI* fragment irrespective of the source of host DNA. The location of the *cat* gene within the *PstI* fragment was determined by Southern blotting with a *cat* consensus oligonucleotide corresponding to the expected amino acid sequence of the active site region of chloramphenicol acetyltransferase, and the direction of transcription was deduced from homology with the type I *cat* variant.

The mechanisms by which microorganisms express resistance to antibiotics is of clinical and theoretical importance. This report describes a novel phenomenon whereby a population of chloramphenicol-sensitive cells of *Proteus mirabilis* gives rise to resistant cells at a very high frequency (10⁻⁴ to 10⁻⁵ per cell per generation).

Genes specifying the enzyme chloramphenicol acetyltransferase (CAT) occur in many genera of bacteria, wherein they confer resistance to chloramphenicol. The regulation of *cat* gene expression depends in the main on factors which correlate with the taxon of the bacteria in which they reside. The *cat* genes of gram-negative bacteria are commonly found on large R-plasmids and produce CAT constitutively. The most commonly observed variant in this category is the type I polypeptide encoded by *Tn9* and naturally occurring plasmids such as *NR1* (also called *R100* or *R222*) and *R6* or the in vitro plasmid constructs such as pACYC184 and pBR328 (reviewed by Shaw [35]). The nucleotide sequence of the type I *cat* has been determined (1), and the gene has been demonstrated to be subject to catabolite repression mediated by cyclic AMP and the catabolite gene activator protein at the level of transcription (14, 22). Circumstantial evidence suggesting transcriptional activation by the cyclic AMP-catabolite gene activator protein system has not been observed for the type II and type III genes found on non-F plasmids in enteric bacteria (35).

The synthesis of CAT in gram-positive bacteria is induced by chloramphenicol and selected analogs (43), and the regulation appears to be one of posttranscriptional control, which may be regulated by the secondary structure of the CAT mRNA (9, 15).

Any study of chloramphenicol resistance and CAT synthesis in *P. mirabilis* is bound to be complicated by two considerations. The first is the well-documented phenomenon of selective amplification of genes encoding resistance determinants carried by certain R plasmids in *P. mirabilis*. Rownd and Mickel (33) described this phenomenon as transitioning and have shown that the increase in CAT synthesis due to gene amplification is reversible in that growth of transitioned cells in drug-free medium results in loss of resistance determinants with concomitant decrease in resistance. The second consideration has been the knowledge that strains of *P. mirabilis* lacking detectable plasmids may nevertheless harbor *cat* determinants which (i) are expressed poorly, (ii) specify a polypeptide similar to that of the type I *CAT* (45), and (iii) have been described as mutable to high-level expression (37).

The present studies were undertaken to gain a better understanding of the structure and function of the putative chromosomal *cat* gene of *P. mirabilis*, its relationship to analogous genes carried on plasmids in gram-negative and gram-positive bacteria, and the mechanism underlying the high-level expression of CAT in selected strains of *P. mirabilis*.

**MATERIALS AND METHODS**

*Bacteria, plasmids, and phage*. The bacteria, plasmids, and phage used in this report are listed in Tables 1 and 2. All bacteria were grown in Penassay nutrient broth (Difco Laboratories) unless otherwise specifically stated.

**Chromosomal DNA preparations.** *P. mirabilis* genomic DNA was purified by the method of Chow et al. (11), with an additional final cesium chloride buoyant density centrifugation step.

**Plasmid preparations.** Plasmids were prepared by the rapid method of Birboim and Doly (7) with the protocol described by Maniatis et al. (25).

**Conjugation and transformation of *E. coli* and *P. mirabilis*.** Strains were transformed by a modified version (19) of the
VOL. mirabilis P. described by protocol of Kushner tide (5'-CCATCACAGACGGCATGATG-3') fragments with [-y-32P]ATP (39). The groups were freeze-squeeze method of by endonuclease cleaved with and (0.5 U containing plasmid control set were per ml in cultures of chloramphenicol resistance mediated by this study) of CAT antibiotic. Chloramphenicol-sensitive strains may harbor multiple plasmids (43). Induction experiments were carried out as described previously (43) with a range of inducer concentrations from 0.1 to 10 μM of either 3-deoxychloramphenicol or the 3-fluoro analog of chloramphenicol.

Expression of chloramphenicol resistance. Cultures were grown in Penassay broth at 37°C to the mid-exponential phase, and approximately 10^6 cells were spread on Penassay agar plates containing 50 μg of chloramphenicol per ml and incubated at 37°C for 48 h. The resulting colonies containing resistant cells were transferred to plates containing 100 μg of chloramphenicol per ml and thence stepwise to 200 and 500 μg of chloramphenicol per ml.

The fall-off of chloramphenicol resistance in P. mirabilis cultures grown in the absence of selective pressure (as determined by the drop in efficiency of plating on agar containing 50 μg of chloramphenicol per ml and by the concomitant and parallel decrease in CAT levels) was assessed by direct plating experiments and CAT enzyme assays. Studies of the underlying basis of the resistance

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or comment</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAT153</td>
<td>tet Blu, cloning vehicle</td>
<td>(41)</td>
</tr>
<tr>
<td>pCI100</td>
<td>pAT153 with P. mirabilis cat in PstI site</td>
<td>This study</td>
</tr>
<tr>
<td>pCI101</td>
<td>pAT153 with P. mirabilis cat in PstI site</td>
<td>This study</td>
</tr>
<tr>
<td>M13mp9</td>
<td>Cloning vehicle</td>
<td>(42)</td>
</tr>
<tr>
<td>pIC025</td>
<td>M13mp9 with pBR328 cat in Accl site</td>
<td>This study</td>
</tr>
</tbody>
</table>

P. mirabilis

PM13 Wild type, tet` cat' (13) NCTC 3177 and this study
PM2 Wild type, tet` cat' NCTC 3177 and this study
IC100 PM2 containing pCI100 This study
IC101 PM2 containing pCI101 This study
E. coli

S. Kushner via A. R. Hawkins

IC500 Sk3430 containing pCI100 This study
IC501 Sk3430 containing pCI101 This study
JM103 Δlac pro thi rpsL supE endA sbcB hisD2 F' traD36 proA lacI ΔlacZM15 (27)
IC025 JM103 containing pIC025 This study

Proteus mirabilis

10374 + 9559 + 6396 + 6197 + 3177 - 60 -

Proteus vulgaris

10376 - 10015 - 4175 -

Morganella morganii

10375 + 10041 - 7381 + 5845 + 2818 + 2815 + 1707 - 235 - 232 -

Providencia rettgeri

10377 + 8893 C 7481 + 7480 - 7479 - 7477 - 7476 + 7475 +

Providencia species

10318 C 10286 - 8113 - 8056 - 6345 - 2481 -

TABLE 2. Strains of Proteus, Morganella, and Providencia tested for presence of chloramphenicol resistance and CAT synthesis

<table>
<thead>
<tr>
<th>Species</th>
<th>NCTC no.</th>
<th>Selectable CAT synthesis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteus mirabilis</td>
<td>10374</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>9559</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>6396</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>6197</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3177</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>-</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>10376</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>10015</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4175</td>
<td>-</td>
</tr>
<tr>
<td>Morganella morganii</td>
<td>10375</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>10041</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>7381</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5845</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2818</td>
<td>+</td>
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<td></td>
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<tr>
<td></td>
<td>235</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>232</td>
<td>+</td>
</tr>
<tr>
<td>Providencia rettgeri</td>
<td>10377</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>8893</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>7481</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>7480</td>
<td>-</td>
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<td></td>
<td>7477</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>7476</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>7475</td>
<td>+</td>
</tr>
</tbody>
</table>

* + and – indicate the presence and absence, respectively, of selectable chloramphenicol resistance mediated by CAT. Strains marked (+) have the same phenotype as P. mirabilis PM13. C indicates constitutive CAT production. These strains may harbor R plasmids conferring resistance to multiple antibiotics (see Results).
phenotype and estimates of the spontaneous mutation rate were carried out by means of the Luria-Delbruck fluctuation test (24). A cell suspension prepared from a single colony of \textit{P. mirabilis} PM13 was used to inoculate 10 1 ml tubes of Penassay broth as well as a single 10 ml sample. After growth of all cultures to the midexponential phase of growth (approximately 10^7 cells per ml) the large culture was divided into 10 aliquots, and an equal volume (0.1 ml) of each of the 20 samples was plated on Penassay agar plates containing chloramphenicol (50 µg ml^-1). After growth at 37°C for 24 h, the number of colonies on each plate was counted, and the results with samples from independent cultures were compared with the number of resistant cells detected in the larger culture volume (see Results).

\textbf{Definitions.} In this paper we refer to enhancement as the phenomenon whereby there is an increase in the level of \textit{CAT} activity that can be detected by the radiometric method. Although the \textit{CAT} purified from cells grown in the absence of chloramphenicol is referred to as \textit{CAT'}, whereas of \textit{P. mirabilis} cultured in the absence of chloramphenicol is described as \textit{CAT}' (see Results).

\textbf{Sources of materials.} All restriction endonucleases, DNA polymerase I, T4 DNA kinase, and low-melting-point agarose were purchased from BRL Ltd.; T4 DNA ligase and calf-intestinal phosphatase were purchased from Boehringer Mannheim Biochemicals; Fujimix RX X-ray film was from Hannotex UK Ltd.; sheets of nitrocellulose were from Sartorius; agarose was from Miles Laboratories, Inc.; Penassay broth and solid media were from Difco Laboratories; salmon sperm DNA, polyvinyl pyrrolidione, bovine serum albumin, Ficoll 400, and all antibiotics were from Sigma Chemical Co. All other reagents were of analytical grade.

\textbf{RESULTS}

\textbf{Characterization of the \textit{CAT} in \textit{P. mirabilis} PM13.} The \textit{CAT} from strain PM13 was purified to homogeneity both from cells exhibiting the resistant (\textit{CAT}') state and the sensitive (\textit{CAT}'') state, and various properties and kinetic parameters were compared. They were eluted in identical fashion from highly substituted chloramphenicol base-agarose at 1.0 M NaCl in 5 mM chloramphenicol (45) and gave lines of identity with one another by two-dimensional gel diffusion in agar against a goat antiserum to the type I (\textit{Escherichia coli}) variant of \textit{CAT} (45). Both preparations were observed to migrate at the same rate in polyacrylamide gel electrophoresis under nondenaturing conditions (18). The apparent \(K_m\) values for chloramphenicol were 16 and 14 µM for enzyme from \textit{CAT}' cells and \textit{CAT}'' cells, and the values were 73 ± 5 and 82 ± 6 µM for acetyl coenzyme A, respectively. Although the \textit{CAT} purified from cells grown in the absence of chloramphenicol is present at low levels, it is indistinguishable from the \textit{CAT} purified from cells grown in the presence of chloramphenicol (50 µg ml^-1). For cells grown in 0, 50, 100, 200, and 500 µg of chloramphenicol, the \textit{CAT} specific activity was <1, 12, 150, 134, and 118 nmol min^-1 mg^-1. (Extracts from cells grown in the absence of chloramphenicol contained \textit{CAT} which could be detected by the radiometric assay [34] and purified by affinity chromatography [45], but which was below the sensitivity of the spectrophotometric assay [1 nmol min^-1 mg^-1] used in these experiments.) The results support the view that the resistance phenotype is likely to be due to an increase in the amount of enzyme produced rather than to any alteration in the properties of the protein or the synthesis of a second \textit{CAT} variant.

\textbf{Enhancement of \textit{CAT} synthesis in \textit{P. mirabilis} and origin of the resistance phenotype through mutation.} Experiments carried out under conditions which induce \textit{CAT} synthesis in \textit{Staphylococcus aureus} (43) and \textit{Bacillus subtilis} (15) produced no effect on the \textit{CAT} levels in \textit{P. mirabilis} PM13. The incubation of resting cell suspensions with chloramphenicol, 3-deoxychloramphenicol, or 3-fluorochloramphenicol was not accompanied by significant increases in the specific activity of \textit{CAT} (data not shown). Such results, although negative, strongly suggest that induction by chloramphenicol or its analogs is unlikely to play a role in the enhancement of chloramphenicol resistance observed in \textit{Proteus} spp.

The application of the Luria-Delbruck fluctuation test to the efficiency of plating of chloramphenicol-resistant cells revealed a non-Gaussian variation among samples taken from independent cultures as compared with equal samples from a single large culture. Since a mutation to chloramphenicol resistance can occur in any generation before exposure to the selective agent, the large variance observed favors the hypothesis of a mutational event rather than induction of \textit{CAT} by exposure of cells to the antibiotic. In a typical experiment (see Materials and Methods) comparing 10 small cultures with a single 10-fold larger culture volume the results were as follows. Six of the small cultures yielded no chloramphenicol-resistant cells of \textit{P. mirabilis} PM13 in samples containing approximately 10^7 cells per ml, whereas the other four cultures yielded 5, 12, 61, and 267 cells resistant to chloramphenicol from comparable populations. By way of contrast, 10 equivalent samples from the larger culture each yielded from 167 to 233 resistant cells (mean 207 ± 14).

Single step mutants resistant to 50 µg of chloramphenicol per ml after growth for 48 h (see Materials and Methods) were transferred sequentially to plates containing higher concentrations of chloramphenicol (100, 200, and 500 µg per ml) of individual colonies were taken from each plate and grown for transfer to liquid media containing the same concentration of chloramphenicol used in the selection plate, and cells were harvested at the midexponential phase of growth. The specific activity of \textit{CAT} in crude cell extracts was measured by detection of the routine spectrophotometric assay in extracts prepared from bacteria grown in the absence of chloramphenicol (Table 3), whereas the specific activity of \textit{CAT} from cells grown in the presence of chloramphenicol was high and essentially independent of the concentration of antibiotic in the growth medium. The mutation to the \textit{CAT}'' state, (i.e., high efficiency of plating on chloramphenicol agar) seems to be of all-or-nothing nature in that all cells which will grow on 50 µg ml^-1 will also grow on 500 µg ml^-1. Once selected, \textit{CAT}'' cells are maintained in the population so long as there is selective pressure favoring their persistence. Figure 1 epitomizes the results obtained with \textit{P. mirabilis} PM13 (as well as other strains of \textit{Proteus} spp. and related genera) when \textit{CAT}'' cells are grown in the absence of the antibiotic. The exponential decline with time of the efficiency of plating of cells on 50 µg of chloramphenicol per ml and the parallel fall in the specific activity of \textit{CAT} for the population as a whole constitute the phenomenon which is analyzed in greater detail below (see Discussion).

\textit{P. mirabilis} PM13 was tested for enhanced levels of resistance to other antibiotics after selection on 50 µg of...
chloramphenicol per ml but remained uniformly sensitive to ampicillin, kanamycin, nalidixic acid, spectinomycin, and streptomycin (data not shown).

How widespread is the phenomenon? Additional representatives of Proteus spp., Morganella spp., and Providencia spp. were screened for the occurrence of the enhancement phenomenon described above, i.e., the appearance of chloramphenicol-resistant cells arising from a predominantly chloramphenicol-sensitive population at high frequency (approximately $10^{-5}$ per cell per generation). Thirteen of 32 strains from the National Collection of Type Cultures (United Kingdom) showed the phenomenon (Table 2), whereas only two produced CAT at high levels without prior exposure to chloramphenicol. The latter were not studied further because they displayed resistance to multiple antibiotics, suggesting the presence of plasmid-mediated resistance (data not shown).

Absence of a plasmid in P. mirabilis PM13. P. mirabilis PM13 was screened for the presence of plasmids by two methods (7, 20), neither of which yielded evidence of plasmids when the DNA from cell lysates was subjected to agarose gel electrophoresis. Positive controls in such experiments included E. coli and P. mirabilis strains harboring well-characterized plasmids (data not shown). Attempts to transfer chloramphenicol resistance from strain PM13 to suitable E. coli recipients by conjugation were also unsuccessful. Subsequent experiments (see below) were consistent with the view that the cat gene is a chromosomal one in strain PM13.

Cloning of the P. mirabilis cat gene from the CAT+ and CAT− states. Preliminary studies revealed that the CAT from P. mirabilis was likely to be similar to the type I variant in structural and kinetic properties (45). Also, the P. mirabilis CAT bound the dye crystal violet (I. G. Charles, unpublished experiments), a property demonstrated by the type I enzyme (30) but by none of the other CAT variants found in gram-negative bacteria (A. D. Bennett, Ph.D. thesis, University of Leicester, 1984). The likely high degree of homology of the CAT of P. mirabilis with its type I counterpart was exploited in the strategy used to clone the cat gene of P. mirabilis.

Plasmid pBR328 (38) contains a type I cat gene on a 773-base-pair TaqI fragment. To facilitate the isolation of this fragment for hybridization experiments, the entire 773-base-pair fragment was cloned into phage M13 mp9 (42) as outlined in Materials and Methods. One recombinant (pIC025) was isolated as the replicative form, and the entire structural gene for cat was isolated by a double digest with PstI and BamHI.

Identification and isolation of the DNA fragments of the P. mirabilis chromosome carrying the cat gene was achieved by first using the type I cat gene (isolated from pIC025) to probe various restriction endonuclease digests of chromosomal DNA from P. mirabilis. A PstI digest yielded a single band on hybridization (data not shown), indicating that this fragment was likely to contain the entire P. mirabilis cat gene. PstI-digested chromosomal DNA samples (prepared from P. mirabilis in both the CAT+ and CAT− states) were separately ligated with pAT153 which had been cut by PstI and treated with alkaline phosphatase. After transformation, the CAT-containing cells, which were likely to harbor recombinant plasmids (selected on 10 μg of chloramphenicol per ml), were then screened (7) for insert DNA with positive results in each case for an insert of approximately 8.5 kilobases (kb). Plasmids from one each of the resulting transformants (designated pIC100 for the recombinant produced from DNA in the CAT+ state and pIC101 for the recombinant produced from DNA in the CAT− state) were used for large-scale plasmid preparations.

Identification and localization of the cat genes in plasmids pIC100 and pIC101. The synthetic 20-mer cat active site consensus probe was used to locate the cat gene within the
8.5-kb PstI fragment of plasmids pIC100 and pIC101. Hybridization of the $^{32}$P-labeled probe to separated restriction fragments yielded results (Fig. 2) which were interpreted as evidence for the localization of the cat gene on a 500-base-pair fragment located near one end of the insert (Fig. 3). The direction of transcription of the gene was inferred from this hybridization data in relation to the restriction map of the type I cat gene.

Chromosomal organization of the P. mirabilis cat gene. P. mirabilis can amplify cat genes carried on plasmids such as NR1, and the phenomenon of chromosomal cat gene amplification has accounted for increased chloramphenicol resistance in E. coli and B. subtilis (28, 44). The P. mirabilis genome was examined by Southern blotting for selective gene amplification. A ClaI-to-PstI fragment of pIC100, encompassing the entire cat gene, was isolated after electrophoresis through low-melting-point agarose and labeled with $[^{32}P]$dATP by nick translation. P. mirabilis chromosomal DNA samples prepared from late-exponential-phase cultures grown in 0, 50, 100, 200, and 500 μg of chloramphenicol per ml were each digested with PstI and, after electrophoresis, were probed with the labeled fragment. No significant difference in hybridization signal intensity was detected in the restriction fragments arising from DNA prepared from cells that have been grown in chloramphenicol as compared which those that had not (Fig. 4). Southern blotting was also used to detect a possible change in orientation of cat DNA within the chromosome of the CAT$^+$ state compared with the CAT$^-$ state, at least within the 8.5 kb of DNA spanning the cloned fragment. P. mirabilis chromosomal DNA was prepared from late-exponential-phase cultures grown in either no antibiotic or 500 μg of chloramphenicol per ml. Both preparations were separately digested with PstI, HindIII, and EcoRI, subjected to agarose (0.7%) gel electrophoresis, and probed with the entire 8.5-kb $^{32}$P-labeled insert of

**FIG. 2.** Agarose gel (0.7%) showing endonuclease digest pattern of pIC100 after ethidium bromide staining. (a) Lanes after digestion with the following enzymes: A, ClaI; B, ClaI and PstI; C, ClaI, PstI, and PvuII; D, ClaI, PstI, and HindIII; E, ClaI, PstI, and EcoRI; F, ClaI, PstI, and SalI; G, ClaI, PstI, EcoRI, and SalI. (b) Autoradiograph of the DNA shown in part (a) after transfer to nitrocellulose and hybridization with a γ-$^{32}$P-labeled consensus cat active site oligonucleotide (See Fig. 3 and Materials and Methods).
pIC100. Figure 5 shows that the pattern of hybridization produced for each pair of digests is indistinguishable, indicating that no detectable rearrangement has occurred within this 8.5-kb cat-containing fragment.

Expression of the P. mirabilis cat gene in E. coli and in a cat− strain of P. mirabilis. Plasmids pIC100 and pIC101 were both transferred into E. coli and into P. mirabilis PM2, the latter strain having previously been demonstrated to be negative for CAT by a sensitive radiometric assay (34) and to lack homologous cat DNA by Southern blot experiments (data not shown). The resulting transformants were then tested for enhanced expression of cat as observed in strain PM13 (Table 3). Expression of the P. mirabilis cat gene was sufficiently poor in E. coli that the specific activity of the enzyme could not be measured by the spectrophotometric method and required use of the radiometric technique (Fig. 6). The minimum inhibitory concentration for chloramphenicol with E. coli Sk3430 carrying pIC100 or pIC101 is only 10 μg ml−1, whereas the corresponding concentration for strain PM2 with either plasmid is 100 μg ml−1 as compared with 10 μg ml−1 without pIC100 or pIC101. The constitutive expression of the cat gene from strain PM13 in the genetic background of different P. mirabilis strains is addressed in the Discussion.

**DISCUSSION**

The results presented here show that a number of strains of the Proteus-Providence group are capable of expressing high levels of chloramphenicol resistance under appropriate conditions.

The mechanism of the phenomenon appears to involve the selection of mutants in the population. Preexisting mutant cells (which occur spontaneously at a frequency of approximately 10⁻⁷ per cell per generation) are selected by growth on chloramphenicol and are maintained in the population for as long as they are exposed to chloramphenicol selection. Cultures of P. mirabilis in which 100% of the cells are phenotypically resistant to chloramphenicol undergo a population reversion to the chloramphenicol-sensitive state during growth in the absence of the antibiotic, the rate of this process being of the order of 10⁻² per cell per generation. The rate of decrease in the efficiency of plating of cells on antibiotic agar (Fig. 1) is too low to correspond with a model wherein a putative intracellular inducer is progressively diluted by cell division. An alternative explanation, the progressive reduction in the proportion of resistant cells in the population, may be seen to operate by mutation or selection or some combination of both factors. Back-mutation to the sensitive phenotype at a very high rate would yield a concomitant reduction in the efficiency of plating of the population on antibiotic-containing media. Such a model

**TABLE 3. Synthesis of CAT from the cloned cat gene of P. mirabilis PM13 in chloramphenicol-sensitive (cat−) bacteria**

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Plasmid</th>
<th>Sp act of CAT (nmol min⁻¹ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. mirabilis PM2</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>P. mirabilis PM2</td>
<td>pIC100</td>
<td>85</td>
</tr>
<tr>
<td>P. mirabilis PM2</td>
<td>pIC101</td>
<td>85</td>
</tr>
<tr>
<td>E. coli Sk3430</td>
<td>None</td>
<td>&lt;1</td>
</tr>
<tr>
<td>E. coli Sk3430</td>
<td>pIC100</td>
<td>&lt;1</td>
</tr>
<tr>
<td>E. coli Sk3430</td>
<td>pIC101</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

CAT specific activity by the spectrophotometric method in extracts of cells grown in the absence or presence of chloramphenicol (10 μg ml⁻¹). The zero value for P. mirabilis PM2 and for E. coli Sk3430 indicates that no CAT was detected by the more sensitive radiometric method. Although the presence of pIC100 or pIC101 in E. coli promoted CAT synthesis as detected by the latter method (Fig. 6), it was below the level of detection by spectrophotometry. Plasmid-free P. mirabilis PM2 and all three strains of E. coli failed to grow in 10 μg of chloramphenicol per ml.
predicts that in any interval $\Delta t$, a proportion of cells $\mu \Delta t$ (where $\mu$ is the back mutation rate per generation) would revert to sensitivity. If $q$ is the frequency of resistant cells in a population after $t$ generations, then the integrated expression describing the phenomenon is

$$q_t = q_0 e^{-\mu t}$$

(1)

where $q_0$ is the initial frequency of resistant cells in the population and $q_t$ is their frequency after $t$ generations. A plot of $\ln(q_t)$ or $\ln$ (efficiency of plating) against time should yield a straight line, its slope being equal to the back-mutation rate.

A second alternative is selection against resistant cells in the absence of the antibiotic. If $s$ is the selective coefficient against resistant cells, then a proportion of the cells ($s\Delta t$) is selectively eliminated in $\Delta t$ generations, then the change in $q$ in $\Delta t$ generations is

$$q = -s\Delta t q(l - q)$$

(2)

and, when integrated, yields the equation for the proportion of resistant cells remaining after $t$ generations

$$dq_t = \frac{q_0}{(1 - q_0) e^{\mu t} + q_0}$$

(3)

Equation 3 describes a linear plot of $\ln$ (efficiency of plating) against time after an initial slow reduction in the efficiency of plating, the duration of such a lag being dependent upon how close $q_0$ is to 1. The plot becomes linear when nearly half of the cells in the population are sensitive, after which $\ln$ (efficiency of plating) decreases with a slope of $-s$. The compound expression corresponding to equations 1 and 3 in the case wherein both selection and back mutation are involved is

$$q_t = \frac{q_0(\mu + s)}{[\mu + s(1 - q_0)] e^{(\mu + s) t} + sq_0}$$

(4)

The latter reduces to produce equations 1 and 3 as special cases when $s$ and $\mu$, respectively, are zero.

Irrespective of the relative contributions of mutation and selection, as $r$ becomes large, the slope of $\ln$ (efficiency of plating) against $t$ becomes $-(\mu + s)$. The amount of selection, however, determines the duration of the lag before the slope becomes linear.

The shaded region in Fig. 1 describes the graphical limits of predictions from equation 4 when $s + \mu$ is equal to the experimentally derived slope of $-0.044$. A comparison of the possible range of curves calculated from theory for different values of $\mu$ and $s$ with that actually determined experimentally suggests that mutation and selection are likely to be operating (calculations not shown). In addition to such direct evidence bearing on the phenomenon, there are reasons for believing that several conventional explanations are unlikely.

Although there is a superficial resemblance between the induction phenomenon observed for $cat$ expression in gram-positive bacteria and the phenomenon observed with $P. mirabilis$, they differ in important respects. First, nondenuding $P. mirabilis$ cells cannot be induced by chloramphenicol or congeners known to induce $cat$ in staphylococci (e.g., the 3-deoxy and 3-fluoro analogs). Second, the Luria-Delbruck fluctuation test produced marked variation in the frequency with which resistant colonies appeared from cells taken from independent cultures, indicative of a mutation event rather than a response to environmental change. Third, the decay

FIG. 6. Radiometric demonstration of $cat$ activity in crude cell extracts from $E. coli$ Sk3430 harboring pIC100 (lane 1), pIC101 (lane 2), or pBR328 (lane 5). Lanes 3 and 4 contain negative controls which correspond, respectively, to extracts from $E. coli$ Sk3430 without a plasmid and $Methylophilus methylotrophus$, an obligate methylotroph which has been shown previously to lack both $cat$ activity and a so called $cat$ gene detected by hybridization (6). The assay involves ascending chromatography on silica gel in chloroform-methanol (85:15, vol/vol) of the products of acetylation of [14C]chloramphenicol by CAT in the presence of acetyl coenzyme A. The assay is quantitative only when CAT is limiting and both substrates are present in excess (34). In this instance the preponderance of 1,3-diacylchloramphenicol (top arrow) suggests the presence of a large excess of CAT for each of the positive extracts. The middle arrow indicates the relative mobility of 3-acetylchloramphenicol, and the bottom arrow indicates the position of unmodified chloramphenicol.

in the efficiency of plating with time is far too slow to represent the dilution by cell division of a conventional inducer or gene product present only at time zero.

An apparent precedent for the proposed reversible mutation event accounting for increased resistance in $Proteus$ spp. is the so called transitioning phenomenon (reviewed by Rownd [32]). The level of chloramphenicol resistance in such a case is mediated by changes in $cat$ gene dosage via a variation in plasmid copy number, the extent of tandem duplication of plasmid-borne genes, or by a combination of both mechanisms. Two observations argue against transitioning as the explanation for the reversible enhancement of $cat$ expression in $P. mirabilis$. First, in the case of plasmid transitioning all cells appear to express $cat$ constitutively and exhibit a high efficiency of plating on media containing chloramphenicol before an increase in gene dosage occurs. Second, and notwithstanding the limitations of negative experiments, all efforts to detect a plasmid in strain PM13 have been unsuccessful. Furthermore, the possibility of $cat$ amplification by tandem duplication within the genome of $P. mirabilis$ was investigated by Southern hybridization with negative results. No significant difference was observed in the extent of hybridization between the labeled $P. mirabilis$ $cat$ probe and its counterpart in restriction endonuclease digests of total DNA prepared from a population of resistant cells as compared with DNA from cells grown in the absence of chloramphenicol.

Recently it has become clear that gene expression in procaryotes may be modulated by specific and reversible DNA inversions, the best known example being phase variation in $Salmonella$ spp. (36). One of the consequences of such inversions is that the restriction patterns of the DNA
from the alternate states are different. Southern blotting procedures designed to detect rearrangements within cat or its flanking sequences in P. mirabilis PM13 failed to identify any differences when DNA prepared from cells in the CAT\(^+\) state was compared with DNA from CAT\(^-\) cells. There is circumstantial evidence, on the other hand, that the site of action of the mutation may be outside of the cat gene and 5' control regions. The efficiency of plating experiments show that for P. mirabilis in the CAT\(^+\) state virtually 100% of the cells expressing the cat gene at a high rate; thus, the highly transcribed gene must be represented at least once per genome. Furthermore, the Clarke and Carbon equation (12) predicts that not more than 10\(^6\) recombinants need be screened to isolate such a gene in a cloning experiment. In the present study the cat gene was cloned from P. mirabilis DNA isolated both CAT\(^+\) cells (pIC100) and from CAT\(^-\) cells (pIC101). The frequency of isolation of such clones was independent of the origin of the cells and was within the limits of the Clarke-Carbon prediction, and the P. mirabilis DNA inserts from CAT\(^-\) and CAT\(^+\) cells were observed to be identical by restriction endonuclease digestion. Taken together, the results favor the view that the site of the mutation which underlies the enhancement of chloramphenicol resistance and cat expression and their respective levels is likely to be outside the cat gene and its flanking sequences as represented in pIC100 and pIC101. Expression studies employing these plasmids in backgrounds which lack cat (E. coli and P. mirabilis PM2) are consistent with the notion that the control of cat expression in P. mirabilis PM13 may reside outside the cloned segment. Whereas the absence of high-level expression of the P. mirabilis cat in E. coli might be related to the general problem of the anomalous expression which has been observed reciprocally between genes in E. coli and P. mirabilis (3, 4), there are examples of P. mirabilis genes being expressed at normal levels in E. coli backgrounds (5, 16, 17). Furthermore, it is more difficult to account for low-level expression of cat in PM2, since the apparent single copy of the chromosomal cat gene in strain PM13 yields resistance to chloramphenicol concentrations as high as 500 \(\mu\)g \text{ml}^{-1}. The data available are compatible with the hypothesis that a host specific element, present in PM13 but not in PM2, is responsible for promoting the high-level cat expression which is characteristic of the CAT\(^+\) state.

Taken together, the results favor a mechanism wherein a reversible mutation event occurs at high frequency. Such mutations are characteristic of transposition or genetic switch mechanisms (29). Although the data for the P. mirabilis cat system do not support a rearrangement of a DNA segment within an 8.5-kb region which includes the 5' control region, such a switching event could occur at a more distant locus as is the case in phase variation in S. typhimurium. The important predictions of such a model for the CAT system in P. mirabilis would be (i) the absence of a difference between the structural gene for cat in the CAT\(^+\) and CAT\(^-\) states, (ii) evidence of transcriptional control via a trans-acting element (protein or RNA); and (iii) the direct demonstration (by mutation or its isolation and characterization) of a control locus which is external to the P. mirabilis DNA in pIC100 and pIC101, and (iv) that the activity of the putative switch region shows positional dependence.

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