JOURNAL OF BACTERIOLOGY, Feb. 1985, p. 665–672
0021-9193/85/020665-08$02.00/0

Vol. 161, No. 2

Induction of the Chloramphenicol Acetyltransferase Gene *cat-86* Through the Action of the Ribosomal Antibiotic Amicetin: Involvement of a *Bacillus subtilis* Ribosomal Component in *cat* Induction

ELIZABETH J. DUVALL,[1] SKORN MONGKOLSUK,[1] UN JIN KIM,[1] PAUL S. LOVETT,[1]* TINA M. HENKIN,[2]† AND GLENN H. CHAMBLISS[2]

Department of Biological Sciences, University of Maryland Baltimore County, Catonsville, Maryland 21228,[1] and Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706[2]

Received 4 September 1984/Accepted 5 November 1984

The plasmid gene *cat-86* and the *cat* gene resident on pC194 each encode chloramphenicol-inducible chloramphenicol acetyltransferase activity in *Bacillus subtilis*. Chloramphenicol induction has been proposed to result from chloramphenicol binding to ribosomes, which then permits the drug-modified ribosomes to perform events essential to induction. If this proposal were correct, *B. subtilis* mutants containing chloramphenicol-insensitive ribosomes should not permit chloramphenicol induction of either *cat-86* or pC194 *cat*. However, we and others have been unable to isolate chloramphenicol-resistant ribosomal mutants of *B. subtilis* 168. We therefore developed a simple procedure for screening other antibiotics for the potential to induce *cat-86* expression. One antibiotic, amicetin, was found to be an effective inducer of *cat-86* but not of the *cat* gene on pC194. Amicetin and chloramphenicol each interact with the 50S ribosomal subunit, and the mechanism of *cat-86* induction by both drugs may be similar. Amicetin-resistant mutants of *B. subtilis* were readily isolated, and in none of six mutants tested was *cat-86* detectably inducible by amicetin, although the chloramphenicol-inducible phenotype was retained. The *ami-1* mutation which is present in one of these amicetin-resistant mutants was mapped by PBS1 transduction to the "ribosomal gene cluster" adjacent to *cysA*. Additionally, ribosomes from cells harboring the *ami-1* mutation contained an altered BL12a protein, as detected in two-dimensional polyacrylamide gel electrophoresis. Lastly, an in vitro protein-synthesizing system that uses ribosomes from an *ami-1*-containing cell line was more resistant to amicetin than a system that uses ribosomes from an amicetin-sensitive but otherwise isogenic strain. These results indicate that the host mutation, *ami-1*, which effectively abolished the inducibility of *cat-86* by amicetin, altered a ribosomal component.

cat genes specify chloramphenicol acetyltransferase (CAT) and occur in bacteria either as plasmid determinants or in the chromosome (35). CAT mediates the acetylation of chloramphenicol, rendering the drug inactive as an antibiotic (33). *cat* genes therefore confer on host cells resistance to chloramphenicol. Two broad categories of *cat* genes have been identified on the basis of their inducibility by chloramphenicol. Those from several gram-negative bacteria, including the Tn*9* *cat* gene, are expressed in cells at the same level regardless of the presence or absence of chloramphenicol (35). The expression of such genes is therefore constitutive with respect to chloramphenicol. In contrast, the expression of *cat* genes present in two gram-positive bacteria, *Staphylococcus aureus* and *Bacillus pumilus*, is chloramphenicol inducible (19, 40, 42).

A model for the chloramphenicol-inducible regulation of *cat* gene expression is being developed from the study of two nonidentical *cat* genes: *cat-86*, which was cloned from *B. pumilus* in *B. subtilis* on pUB110, and the *cat* gene which is resident on the *S. aureus* plasmid pC194 (6, 10, 15). This model is based initially on the unique nucleotide sequence arrangement that is observed 5′ to the coding sequences for both genes. The 660-base-pair (bp) coding sequence for *cat-86* is immediately preceded by a 40-bp region that consists of two 14-bp inverted-repeat sequences separated by 12 bp (10, 15). This 40-bp region spans the *cat-86* ribosome binding site sequence. Therefore, transcripts of *cat-86*, initiated more than 144 bp upstream from the *cat-86* coding sequence, are predicted to sequester the ribosome binding site in the stem of a stable RNA stem-loop (10). This secondary structure in mRNA could, in theory, block the translation of *cat-86* mRNA by preventing the ribosome binding site from base pairing with 16S rRNA. Furthermore, the RNA stem-loop appears to function as a site of partial termination of transcription in vitro and in vivo (N. Ambulos, S. Mongkolsuk, and P. S. Lovett, submitted for publication). The *cat* coding sequence in pC194 is similarly preceded by a pair of inverted-repeat sequences (10, 19). In this case, the inverted repeats are 12 bp in length and are separated by 13 bp. The inverted repeats span the ribosome binding site sequence and are located between the site of transcription initiation and the *cat* coding sequence. Therefore, transcripts of pC194 *cat* should also sequester the ribosome binding site in an RNA stem-loop. However, this RNA stem-loop appears not to be a significant site of transcription termination in vitro or in vivo (N. Ambulos, unpublished data; see also reference 6).

Two additional observations support the view that the inverted repeats are involved in chloramphenicol regulation of *cat* gene expression. First, constitutive variants of *cat-86* and pC194 *cat* contain deletions that eliminate nearly the entire upstream inverted repeat (2; W. Nicholson, unpublished data). Second, chloramphenicol induction of *cat-86* has been shown to be independent of the promoter that is

* Corresponding author.
† Present address: Department of Microbiology and Molecular Biology, Tufts University School of Medicine, Boston, MA 02111.

TABLE 1. Plasmids

Plasmid[a]	Relevant properties	Reference
pPL708	*cat-86* is activated by a strong promoter within a 284-bp *Eco*RI fragment designated as P4	10, 28
pPL603E	*cat-86* is activated by a moderate promoter within a 203-bp *Eco*RI-*Pst*I fragment designated as P2	11
pPL3*lac*30	Derived from pPL603E; *lacZ* is inserted in frame at codon 30 of *cat-86*	27
pPL3*lac*2	Derived from pPL603E; *lacZ* is inserted in frame at codon 2 of *cat-86*	27
pC194	*cat*-containing plasmid from *S. aureus*	12, 19

[a] The plasmids with the pPL prefix are derivatives of pPL703 (10). pPL703 is a promoter-cloning plasmid constructed by the insertion of the promoterless *cat-86* gene, originally cloned from *B. pumilus*, between the *Bam*HI and *Eco*RI sites of pUB110. The pUB110 portion of pPL703 provides a replicon and a neomycin resistance gene. The four unique restriction sites in pPL703 for the insertion of promoters that activate *cat-86* transcription are within a 21-bp linker located ca. 144 bp upstream from the *cat-86* translation initiation codon. These restriction sites are *Eco*RI, *Bam*HI, *Sal*I, and *Pst*I (see references 10 and 28).

used to activate the gene and is independent of the *cat-86* coding sequence 3′ to codon 2 (27). Thus, the induction of *cat-86* depends only upon transcribed plasmid sequences within a 150-bp region that intervenes between codon 2 and the upstream site of promoter insertion. The inverted repeats are present within this 150-bp region.

If the RNA stem-loops, which are the transcription products of the inverted repeats, control expression of the *cat* genes by blocking translation of mRNA and, in the case of *cat-86*, by acting as partial transcription terminators, chloramphenicol may induce *cat* expression by opening the stem-loop or by blocking the formation of this RNA secondary structure. It has been suggested (6, 10, 11) that the host ribosome or a ribosomal component is modified by binding chloramphenicol and that it is the drug-altered ribosome (or a component thereof) that is active in opening the RNA stem-loop or in blocking its formation. One prediction of this concept is that *B. subtilis* mutants which contain ribosomes that do not bind chloramphenicol or that show reduced or altered binding should not allow the plasmid *cat* genes to be induced by chloramphenicol. However, for reasons that are unclear, chloramphenicol-resistant ribosomal mutants of *B. subtilis* 168 have not been isolated by us or by others (3). Therefore, we have devised a plate test to facilitate the testing of other antibiotics for the potential to induce *cat-86* expression. In the present study, we show that the ribosomally targeted antibiotic amicetin is an effective inducer of *cat-86* in wild-type *B. subtilis*. Amicetin-resistant mutants of *B. subtilis* are readily isolated, and in these mutants *cat-86* can no longer be induced by amicetin. Analysis of one amicetin-resistant mutation, *ami-1*, demonstrates that the mutation alters a ribosomal component.

MATERIALS AND METHODS

Bacteria and plasmids. *B. subtilis* BR151 (*trpC2 metB10 lys-3*) and BGSC1A422 (*leu recE4*) were used throughout. Strain QB944 (*cysA14 purA16 trpC2*) was used for genetic mapping experiments and was obtained from F. Kunst. All are *B. subtilis* 168 derivatives. Transducing phage PBS1 was from the *Bacillus* Genetic Stock Center, Ohio State University. Transductions were performed as previously described (16). The plasmids used are listed in Table 1. Plasmid isolation, transformation, and electrophoresis through 0.7 or 1% agarose gels were performed as previously described (11, 24, 32). The plasmid copy number was estimated as previously described (22, 23). Conditions for cell growth and all growth media were as before (11).

Enzyme assays. CAT was assayed by the colorimetric method (34), and β-galactosidase was measured as described by Miller (26). Protein was determined by the method of Bradford (5). The specific activity of CAT is expressed as micromoles of chloramphenicol acetylated per minute per milligram of protein. The β-Galactosidase activity of cells spread on plates was monitered by incorporating the indicator dye 5-bromo-4-chloro-3-indolyl-β-galactoside into the agar at 50 μg/ml.

Antibiotics. The majority of the antibiotics tested were generously donated by Eli Lilly & Co. and The Upjohn Co. The remainder were purchased from Sigma Chemical Co. Two of the antibiotics that gave a positive result in the plate test have not been, to our knowledge, tested completely for their mode of action. However, the structures of the two antibiotics, A32256 and desmycosin, both from Eli Lilly & Co., suggest possible modes of action. A32256 is of the phenazine class of drugs and gives a positive result in the Ames test (N. Allen, personal communication). Hence, we assume that the compound interacts with DNA. Desmycosin is structurally related to tylosin (N. Allen, personal communication). Hence, the mode of action is probably similar to that of the macrolide antibiotics, which are known to interact with the 50S ribosomal subunit. Amicetin donated by The UpJohn Co. was lot number 10754. This lot assayed as 89.5% active amicetin several years ago (J. Coats, personal communication). Drug-resistant mutants were obtained by plating BR151 cells previously exposed to nitrosoguanidine (25) onto tryptose blood agar base (Difco Laboratories) containing 10 μg of amicetin per ml.

Induction of *cat* gene expression. To test the inducing activity of antibiotics, we grew plasmid-containing BR151 or 1A422 cells to the middle of the exponential growth phase in Penassay broth (Difco) and split the culture. To one-half of the culture we added a subinhibitory level of the antibiotic being tested. Incubation at 37°C was continued for 1 or 2 h, and the culture was sampled periodically to assay for CAT activity.

Ribosomal resistance to amicetin. Cells were grown in nutrient sporulation medium (31) to the mid-exponential growth phase (absorbance at 570 nm, 0.5) and were harvested and washed as previously described (7). Ribosomes were prepared and tested for resistance to amicetin during in vitro translation of mRNA isolated from BR151 cells infected with phage SPO1; initiation factors and a high-speed supernatant were from strain BR151 (7). The electrophoretic migration of ribosomal proteins was examined with one-dimensional sodium dodecyl sulfate-polyacrylamide gradient gels (1) and two-dimensional polyacrylamide gels (20).

RESULTS

Plate test. To rapidly test many antibiotics for their potential to induce the expression of *cat-86*, we took advantage of the observation that the expression of heterologous genes becomes chloramphenicol inducible in *B. subtilis* when the coding sequences for the heterologous genes are fused in frame to the 5′ end of the *cat-86* coding sequence (27, 41). Plasmid pPL3*lac*30 is suited to this type of study because it

contains the *Escherichia coli lacZ* gene fused in frame to codon 30 of *cat-86* (27). Therefore, the expression of β-galactosidase activity specified by pPL3*lac*30 is chloramphenicol inducible in *B. subtilis* (27). The replicon and a neomycin resistance determinant of pPL3*lac*30 are from pUB110, and the promoter that is used to transcriptionally activate the *cat-86–lacZ* fusion is the natural promoter for *cat-66*, a gene closely related to *cat-86* (11, 27).

B. subtilis BGSC1A422 (*recE4 leuB6*) harboring pPL3*lac*30 forms a beige lawn after 24 h of growth on tryptose blood agar base containing neomycin sulfate (10 μg/ml) and the β-galactosidase indicator dye 5-bromo-4-chloro-3-indolyl-β-galactoside (50 μg/ml). However, when chloramphenicol powder is spotted onto a freshly spread lawn and the plate is incubated for 24 h, the lawn contains a zone of no cell growth at the site of drug application surrounded by a diffuse blue halo. The halo results from the diffusion of chloramphenicol from the spot of application and induction of the *cat-86–lacZ* fusion by drug concentrations below that required for bacteriostasis. We used this assay to test various antibiotics for their ability to enhance β-galactosidase activity. The positive result obtained with several of the drugs (Table 2) differed somewhat from that obtained with chloramphenicol in that the blue zone was closer to the periphery of the killing zone. This could be due to a reduced diffusion of the drugs relative to chloramphenicol or may indicate that the drug concentration needed for induction is closer to that required for bacteriostasis than was seen with chloramphenicol.

Several of the drugs which enhanced β-galactosidase activity are known to interact with the 50S ribosomal subunit (Table 2). This result seems consistent with the idea that induction is not directly mediated by the drug but by a drug-induced modification of the ribosome. Most tested antibiotics did not enhance β-galactosidase activity; these antibiotics were: A204 (Eli Lilly & Co.), A21978C (Eli Lilly & Co.), A2315A (Eli Lilly & Co.), A26771B (Eli Lilly & Co.), A33853 (Eli Lilly & Co.), A39183 (Eli Lilly & Co.), ampicillin, anthelmycin, apramycin, asperlin, berninamycin, bluensomycin, celesticetin, cephalosporin, clindamycin (Cleocin; The Upjohn Co.) decoyinine, echinocandin B, endomycin, erythromycin, farvenulin, fusidic acid, geldanamycin, gentamycin, lipiarmycin, lipoxamycin, marincamycin, netropsin, pactamycin, plicacetin, polymyxin B, psicofuranine, rutamycin, scopafungin, sinefungin, spectinomycin, steffimycin, stendomycin, streptolydigin, streptomycin, streptovitacin A, streptozocin, tetracycline, tobramycin, tirandamycin, tubercidin, and tylosin. Certain antibiotics that are known to interact with the 50S ribosomal subunit, such as erythromycin and tylosin, did not enhance β-galactosidase activity. Therefore, perhaps inducing drugs must cause a relatively specific ribosomal alteration. None of the antibiotics tested which are known to bind specifically to the 30S subunit enhanced β-galactosidase activity. However, we found that several drugs which are known not to interact with the procaryotic ribosome did elicit a positive response in the plate test. Mitomycin, novobiocin, and nalidixic acid each enhanced β-galactosidase activity and each interacts with DNA in a direct or indirect manner. We suspect that these drugs do not mimic chloramphenicol induction but rather may enhance transcription or alter the structure or the copy number of the plasmid. β-Galactosidase activity was also enhanced by two cell wall synthesis inhibitors, vancomycin and amphomycin. The structure and mechanism of action of these drugs are so dissimilar from those of chloramphenicol that we feel it likely that enhancement by these drugs is the result of mechanisms dissimilar from that responsible for induction by chloramphenicol.

TABLE 2. Antibiotics that enhanced β-galactosidase activity in the plate test

Antibiotic	Mode of action	Source or reference
A-32256	Unknown; possibly affects DNA	N. Allen, personal communication
Amicetin	Protein synthesis inhibitor; interferes with chloramphenicol binding to ribosomes	8, 39
Amphomycin	Affects cell wall biosynthesis	37
Desmycosin	Structurally related to tylosin, a macrolide antibiotic	N. Allen, personal communication
Lincomycin	Macrolide antibiotic	9, 38, 39
Mitomycin	Affects DNA	9, 38
Nalidixic acid	Affects DNA	9, 38
Novobiocin	Affects DNA	9, 38
Sparsomycin	Protein synthesis inhibitor	39
Vancomycin	Affects cell wall biosynthesis	38

The advantages of the plate test for identifying potential inducers of *cat-86* include its simplicity and the fact that such a test permits a drug to form a diffusion gradient. Therefore, enhanced gene expression can be obtained at the optimal concentration of the particular drug being tested. The major weakness of the test seems to be lack of specificity. Our interpretation of the results suggests that agents which probably do not mimic the mode of action of chloramphenicol can enhance β-galactosidase activity. A second weakness we have uncovered is that some of the ribosomal antibiotics, such as lincomycin and sparsomycin, which consistently gave a positive result in the plate test, were very poor inducers of *cat-86* in broth cultures of *B. subtilis*. For example, subinhibitory levels of lincomycin (0.5 to 5 μg/ml) gave no more than a twofold induction of *cat-86* during 2 h of growth of BR151 cells harboring either of two promoter-containing derivatives of pPL703, pPL603E or pPL708. Thus far we have not been able to predict, on the basis of the plate test, the effectiveness of a drug in inducing *cat-86* in broth cultures of strain BR151.

Amicetin induction of *cat-86*. Among the ribosomally targeted antibiotics which enhanced β-galactosidase activity, the biological activity of amicetin was examined further because the drug was found to be an effective inducer of *cat-86* and because amicetin-resistant mutants of *B. subtilis* were readily isolated.

The MICs of amicetin for strain BR151 and for plasmid-containing derivatives were determined by incubation of the cells for 24 h in Penassay broth containing various concentrations of the drug. An MIC of amicetin of 4 μg/ml was obtained for BR151 and for derivatives containing either pC194 or a plasmid that harbors *cat-86* and is activated by a strong promoter, pPL708 (Table 1). Therefore, neither pC194 nor pPL708 conferred detectable resistance to amicetin. Subinhibitory levels of amicetin (0.2 to 1 μg/ml) were found to enhance CAT levels in BR151(pPL708) ca. 3- to 4-fold during 1 h of incubation, and incubation of BR151(pPL708) with 0.5 μg of amicetin per ml for 2 h resulted in a 6- to 10-fold increase in CAT levels (Fig. 1 and Table 3). By contrast, BR151(pC194) incubated for up to 3 h with 0.5 or 1 μg of amicetin per ml exhibited no increase in the specific activity of CAT. Hence, pC194 *cat* appeared not to be

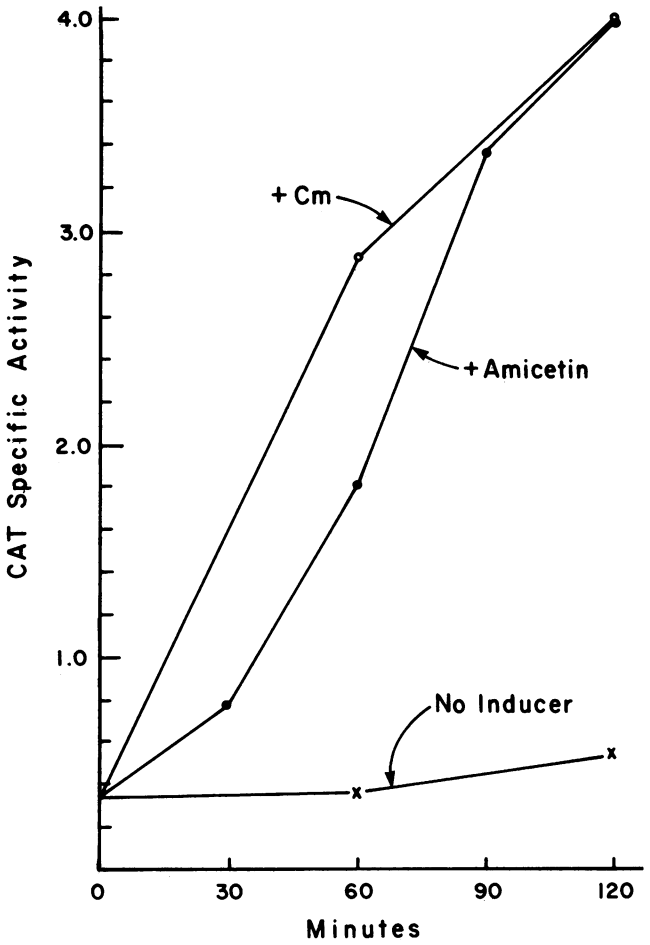

FIG. 1. Induction of *cat-86* in *B. subtilis* by chloramphenicol (Cm) and amicetin. Strain BR151(pPL708) was grown to the mid-log phase in Penassay broth, and the cells were distributed into three flasks. Chloramphenicol and amicetin were added to two of the flasks at 2 and 0.5 μg/ml, respectively. Incubation was continued at 37°C. Samples were periodically withdrawn and assayed for CAT activity and total protein.

amicetin inducible, whereas *cat-86* appeared to be amicetin inducible.

The possibility that amicetin enhancement of *cat-86* expression was not due to induction but to an increase in the copy number of plasmid pPL708 or to the duplication of regions within the plasmid was considered. The copy number of pPL708 in BR151 cells grown for 2 h in Spizizen minimal medium containing no drug or 0.5 μg of amicetin per ml was 27 (±5%). Thus, the enhancement of CAT levels by amicetin was not paralleled by an increase in the plasmid copy number. In addition, pPL708 DNA isolated from BR151 (pPL708) grown for 2 h with 0.5 μg of amicetin per ml comigrated during agarose gel electrophoresis with pPL708 isolated from cells grown without the drug. Thus, it seems highly unlikely that the *cat-86* gene in pPL708 is amplified when host cells are grown with amicetin. Lastly, the possibility that amicetin caused a general enhancement of the expression of genes located on a pUB110 vector was tested. For this test, we used two promoter-deleted versions of the pC194 *cat* gene, each of which is inserted into a pUB110-based replicon (2). In both cases, the deletions that remove the *cat* promoter also remove all or the majority of the upstream inverted-repeat sequence for the pC194 *cat* gene (2, 4). Therefore, when these *cat* genes are activated by a promoter, the expression is constitutive (2). We found that the CAT levels specified by both of these constitutively expressed *cat* genes was not enhanced by the growth of host cells with 0.5 μg of amicetin per ml for 2 h (data not shown). Therefore, amicetin does not cause a general enhancement of the expression of any gene that is joined to a pUB110 replicon. These data indicate that the enhanced expression of *cat-86* by amicetin is likely due to specific induction.

Amicetin-resistant mutants of *B. subtilis*. Amicetin is a 4-aminohexose nucleoside antibiotic that has been shown to interfere with the binding of chloramphenicol to ribosomes of *B. stearothermophilus* (8, 39). As the primary site of chloramphenicol binding is the 50S ribosomal subunit, it is inferred that the primary site of binding of amicetin is also the 50S ribosomal subunit. If the mechanism through which each drug induces *cat-86* expression requires that the drug interacts with a ribosomal component, we would anticipate that chromosomal mutations which alter the site of ribosomal binding for chloramphenicol or amicetin would eliminate or reduce the ability of the respective drug to induce *cat-86*. Such mutations should be readily isolated by the selection of drug-resistant mutants. However, the occurrence of chloramphenicol-resistant mutants among untreated or mutagenized BR151 cells is very infrequent, and the few chloramphenicol-resistant mutants which have been reported do not appear to result from drug-insensitive ribosomes; rather, it has been suggested that such mutants may be defective in chloramphenicol uptake (3). It is therfore conceivable that ribosomal changes which result in an alteration of chloramphenicol binding are lethal in BR151. In striking contrast, BR151 mutants resistant to 10 μg of amicetin per ml were easily isolated from BR151 cells mutagenized with nitrosoguanidine. In one experiment, more than 300 mutants resistant to 10 μg of amicetin per ml were isolated, whereas the same mutagenized cell population yielded no mutants resistant to 10 μg of chloramphenicol per ml. Six independently isolated amicetin-resistant mutants gave comparable results in the induction studies. Experimental results obtained with one mutant are shown, as the corresponding mutation was further analyzed.

A mutation conferring amicetin resistance was transferred from the original mutagenized host to nonmutagenized BR151 cells by transformation. A resulting amicetin-resistant transformant, BR151 *ami-1*, was capable of growth in Penassay broth containing 20 μg of amicetin per ml, but the transformant was as sensitive to chloramphenicol as the amicetin-sensitive parent. To test the inducibility of *cat-86* in the mutant, we transformed plasmid pPL708 into BR151 *ami-1*. In BR151 *ami-1*(pPL708), CAT activity was induced by chloramphenicol but not by amicetin, whereas the CAT

TABLE 3. Lack of amicetin induction of *cat-86* in an amicetin-resistant mutant of *B. subtilis*[a]

Strain	CAT sp act under indicated incubation conditions (2 h)		
	No inducer	Amicetin (0.5 μg/ml)	Chloramphenicol (2 μg/ml)
BR151	0.41	3.1	2.5
BR151 *ami-1*	0.36	0.44	2.2

[a] BR151 and BR151 *ami-1*, each harboring pPL708, were grown to the mid-log phase at 37°C in penassay broth. The individual cultures were split into three flasks, and chloramphenicol and amicetin were added to two of the flasks. Incubation was continued for 2 h, at which time the cells were harvested and assayed for CAT activity.

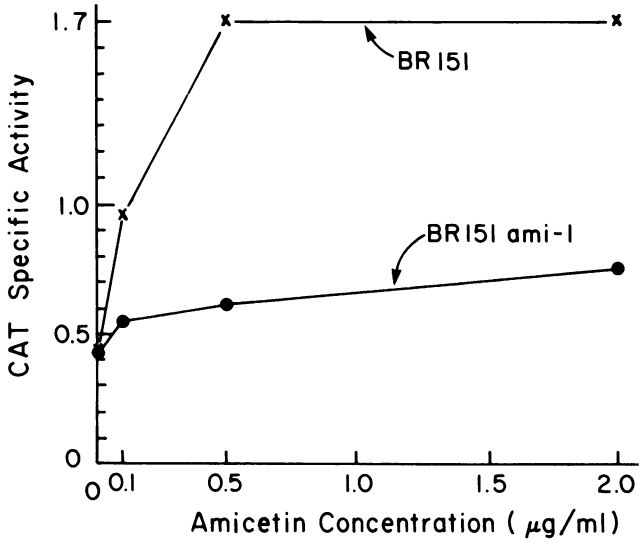

FIG. 2. Amicetin induction of CAT activity in strains BR151 and BR151 *ami-1*, each containing pPL708. Cultures of BR151(pPL708) and BR151 *ami-1*(pPL708) were grown to the mid-log phase in Pennassay broth. Each culture was distributed into five flasks, and amicetin was added to four of the flasks at concentrations of 0.1, 0.5, 1, and 2 μg/ml. The flasks were shaken at 37°C for 1 h, and the cells were harvested and assayed for CAT activity and total protein.

activity specified by pPL708 in wild-type (amicetin-sensitive) BR151 was induced by both amicetin and chloramphenicol (Table 3). As shown in Fig. 2, we tested the inducibility of *cat-86* in BR151 *ami-1*(pPL708) by using amicetin levels ranging from 0.1 to 2 μg/ml and found no significant increase in CAT specific activity after 1 h of exposure to the inducer. However, in this experiment (Fig. 2) and in two other similar experiments, incubation of BR151 *ami-1*(pPL708) with 2 μg of amicetin per ml resulted in CAT levels slightly higher than those seen after incubation with 0.5 μg of the drug per ml. These results and those of in vitro protein synthesis studies (see below) indicate that the amicetin target in BR151 *ami-1* retains a low level of sensitivity to the antibiotic.

Resistance to ribosomal antibiotics is commonly the result of mutations that alter the structure of a component of a ribosome. However, it is also possible that a mutation such as *ami-1* could confer amicetin resistance by reducing the uptake of the drug. Therefore, we attempted to correlate the *ami-1* mutation with alterations in the function and structure of the host ribosomes. The amicetin sensitivity of in vitro protein-synthesizing systems was tested with ribosomes from strains BR151 and BR151 *ami-1*. Ribosomes from BR151 *ami-1* were consistently more resistant to amicetin than were ribosomes from BR151 (Fig. 3). Although amicetin inhibited translational activity in both systems, the activity of BR151 ribosomes was more strongly inhibited by lower concentrations of the drug than was the activity of BR151 *ami-1* ribosomes. For example, at 2.5 μg amicetin per ml, the activity of BR151 ribosomes was reduced to 29% of the activity obtained in the absence of the drug, whereas BR151 *ami-1* ribosomes exhibited 60% activity. Ribosomes from BR151 *ami-1* exhibited no cross-resistance to chloramphenicol in vitro (data not shown), in agreement with the absence of chloramphenicol resistance in vivo in BR151 *ami-1*. It therefore appears that the *ami-1* mutation specifically affects ribosomal sensitivity to amicetin.

The electrophoretic migration pattern of strain BR151 *ami-1* ribosomal proteins was also examined. A minor difference in the electrophoretic pattern in one-dimensional sodium dodecyl sulfate-polyacrylamide gradient gels was observed, with the BR151 *ami-1*-associated form of one protein migrating more slowly than the wild-type form. However, the identities of protein bands in this gel system are not known. In two-dimensional polyacrylamide gels of the basic ribosomal protein, protein BL12a (nomenclature of Osawa et al. [30]) from BR151 *ami-1* was observed to migrate slightly more rapidly toward the cathode in the first dimension (i.e., it was more basic than the wild-type form) (Fig. 4). Interestingly, an alteration in a 50S protein identified as protein BL12 (30) has been reported to be associated with chloramphenicol resistance in a different strain of *B. subtilis* than that used in the present experiments (29). The determinant for this protein was mapped to the major ribosomal protein gene cluster near *cysA* in *B. subtilis* (29, 30).

Genetic mapping of the *ami-1* mutation. As the majority of *B. subtilis* ribosomal protein genes, including the gene for protein BL12, are located in a single region of the chromo-

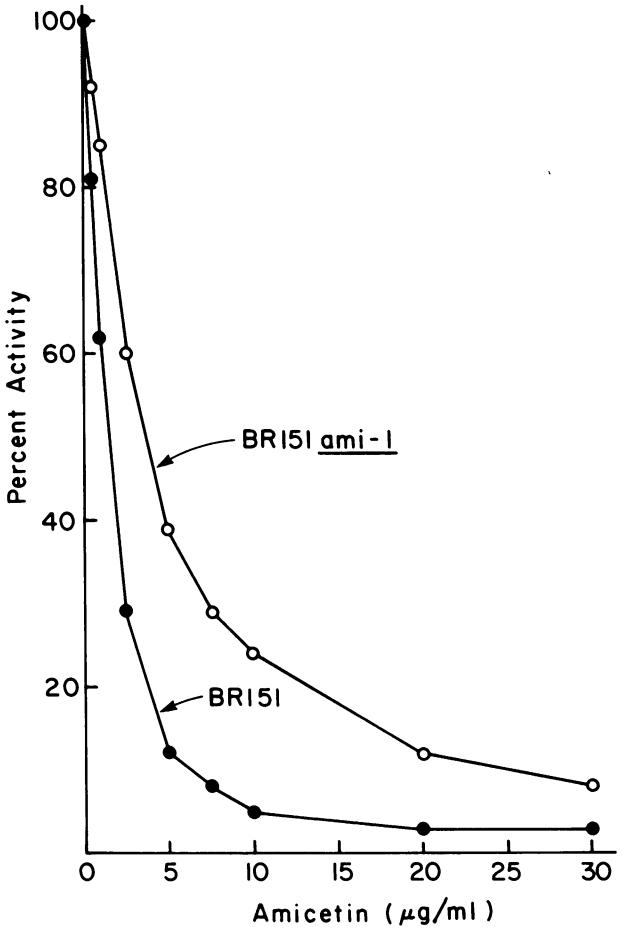

FIG. 3. In vitro amicetin resistance. The incorporation of [^{14}C]phenylalanine (14 cpm/pmol) into material insoluble in hot trichloroacetic acid was measured with phage SPO1 RNA as the template. The percent activity is expressed relative to the activity in the absence of the drug, which was 567 pmol incorporated for strain BR151 ribosomes and 616 pmol incorporated for strain BR151 ami-1 ribosomes. Stimulation by the template addition was 16-fold.

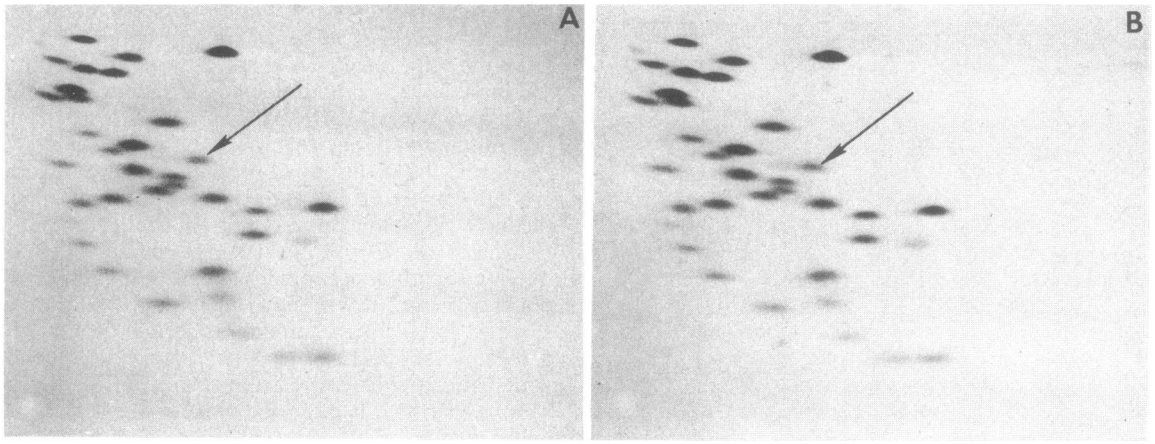

FIG. 4. Two-dimensional polyacrylamide gel electrophoresis of basic 70S ribosomal proteins. (A) Strain BR151; (B) strain BR151 *ami-1*. The arrows point to the BL12a protein, which is identified in accordance with the nomenclature of Osawa et al. (30).

some adjacent to the *cysA* gene (36), the map location of the *ami-1* mutation was determined. The *ami-1* mutation was 90% linked to *cysA*, as determined by PBS1 transduction, and three-factor analyses indicated that the marker order is *purA cysA ami-1* (Table 4). These results place the *ami-1* mutation in the major cluster of ribosomal genes and further support the definition of *ami-1* as a mutation in a ribosomal gene.

DISCUSSION

The plate test we have devised takes advantage of the observation that in-frame fusion of *lacZ* to *cat-86* renders β-galactosidase activity inducible by chloramphenicol. By testing a wide spectrum of antibiotics for their ability to enhance the β-galactosidase activity specified by a *cat-86–lacZ* fusion gene, we identified two classes of enhancing antibiotics. One class includes antibiotics which interact with the 50S ribosomal subunit, and the other class includes drugs that are known not to interact with ribosomes. The ability of the 50S ribosomal antibiotics to enhance β-galactosidase activity lends support to the previous suggestion that chloramphenicol induction of *cat* genes from *B. pumilus* and *S. aureus* is due to a chloramphenicol-induced modification of the 50S ribosomal subunit. We suspect that β-galactosidase enhancement by drugs such as amicetin, lincomycin, and sparsomycin is the result of an induction mechanism analogous to chloramphenicol induction. In contrast, the enhancement of β-galactosidase activity by antibiotics that do not interact with ribosomes is thought not to be analogous to chloramphenicol induction.

TABLE 4. PBS1 transduction mapping of *ami-1*

Selected marker	Recombinant class[a]			No. of recombinants[b]
	purA	*cysA*	*ami-1*	
Pur$^+$	1	1	1	15
	1	0	1	1
	1	1	0	5
	1	0	0	184
Cys$^+$	1	1	1	28
	1	1	0	5
	0	1	1	154
	0	1	0	17

[a] 1 and 0 indicate donor and recipient markers, respectively. The donor strain was BR151 *ami-1*; the recipient strain was QB944 (*purA16 cysA14 trpC2*) obtained from F. Kunst.

[b] Cotransduction of *ami-1* with *purA*: 16 of 204 (8%); cotransduction of *cysA* with *purA*: 20 of 204 (10%); cotransduction of *ami-1* with *cysA*: 183 of 204 (90%); and cotransduction of *purA* with *cysA*: 33 of 204 (16%).

Several of the ribosomal antibiotics which should be capable of inducing *cat-86* on the basis of the plate test were tested for their ability to induce CAT activity in BR151 cells harboring pPL708. These experiments were performed in a manner comparable to that described in the legend to Fig. 1. Subinhibitory levels of lincomycin and sparsomycin gave only a twofold induction during 2 h of incubation or strongly positive induction was detected in one trial but only a two-fold induction was obtained in subsequent trials. Our interpretation of these data is that such drugs qualitatively induce *cat-86*, but only weakly. In contrast to the weak inducing activity of lincomycin and sparsomycin, the antibiotic amicetin has been shown to be an effective inducer of *cat-86*, but the drug did not detectably induce the expression of the *cat* determinant on plasmid pC194. Amicetin induction of *cat-86* expression is not dependent on the specific promoter that is used to activate the gene. The *cat-86* gene in pPL708 is activated by a promoter different from the one that activates the *cat-86–lacZ* fusion in pPL3*lac*30. Additionally, amicetin induces the expression of the *cat-86–lacZ* fusion gene in pPL3*lac*2, in which *lacZ* replaces all *cat-86* coding sequences 3′ to codon 2 of *cat-86* (27). Hence, sequences essential for amicetin induction appear to reside in the 144-bp region that intervenes between the promoter and the beginning of the *cat-86* coding region. This is the same region that has been shown to contain sequences essential to chloramphenicol induction of *cat-86* (10, 27).

Our model for chloramphenicol induction of *cat-86* (see above) proposes that induction depends on an interaction between the inducing antibiotic and its ribosomal target. Therefore, host mutations that block or perturb the ribosome-drug interaction should diminish or eliminate the ability of a drug to induce *cat-86*. This hypothesis has not been experimentally tested for chloramphenicol because mutations in *B. subtilis* 168 that alter the ability of ribosomes to bind chloramphenicol have not been identified. However, amicetin is an alternative ribosomally targeted antibiotic that induces *cat-86*, and each of six amicetin-resistant mutants of *B. subtilis* 168 tested were found to be incapable of allowing *cat-86* to be induced by amicetin. One of the amicetin-resistant mutations, *ami-1*, caused both structural and functional

alterations in *B. subtilis* ribosomes, and the mutation mapped to the region of the *B. subtilis* genetic map known to contain ribosomal genes. Therefore, amicetin induction, and by inference possibly chloramphenicol induction, involves a component of host ribosomes. How drug-modified ribosomes might induce *cat* genes has been speculated on in several studies (6, 11). The common feature of both models is the proposal that the drug-modified ribosomes block the formation of the RNA secondary structure that would sequester the *cat* ribosome binding site. Precisely how this is accomplished remains to be experimentally demonstrated.

It is becoming apparent that aspects of the inducible regulation of *cat-86* and pC194 *cat* bear some resemblance to the regulation proposed for the erythromycin-inducible *erm* genes present in several gram-positive microorganisms (13, 14, 17, 18). *erm* encodes an enzyme that methylates 23S rRNA, thereby conferring erythromycin resistance. The gene is induced as the result of erythromycin binding to ribosomes (14), and induction appears to result from a ribosome-mediated conformational change in the *erm* mRNA, resulting in the destabilization of the secondary structure that sequesters the ribosome binding site for *erm*. Although significant aspects of the regulation of the two classes of genes, *cat* and *erm*, may be shown to differ, the apparent pattern of regulation involving changes in the RNA secondary structure may well predict a mode of regulation for other inducible determinants which confer resistance to ribosomal antibiotics in gram-positive bacteria.

ACKNOWLEDGMENTS

P.S.L. thanks Norris Allen of Eli Lilly & Co. and John Coats of The Upjohn Co. for valuable information and discussion and for making it possible to obtain most of the antibiotics used in this study.

This investigation was supported by grant PCM-8202701 from the National Science Foundation and Public Health Service grants AI-21350 and GM-32199 from the National Institutes of Health.

LITERATURE CITED

1. **Adoutte-Panvier, A., J. E. Davies, L. R. Gritz, and B. J. Littlewood.** 1980. Studies of ribosomal proteins of yeast species and their hybrids. Gel electrophoresis and immunochemical cross-reactions. Mol. Gen. Genet. **179:**273–282.
2. **Ambulos, N. P., J. H. Chow, S. Mongkolsuk, L. H. Preis, W. R. Vollmar II, and P. S. Lovett.** 1984. Constitutive variants of the pC194 *cat* gene exhibit DNA alterations in the vicinity of the ribosome binding site sequence. Gene **28:**171–176.
3. **Anderson, L. M., T. M. Henkin, G. H. Chambliss, and K. F. Bott.** 1984. New chloramphenicol resistance locus in *Bacillus subtilis*. J. Bacteriol. **158:**386–388.
4. **Band, L., D. G. Yansura, and D. Henner.** 1983. Construction of a vector for cloning promoters in *Bacillus subtilis*. Gene **26:**313–315.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Byeon, W.-H., and B. Weisblum.** 1984. Post-transcriptional regulation of chloramphenicol acetyltransferase. J. Bacteriol. **158:**543–550.
7. **Chambliss, G. H., T. M. Henkin, and J. M. Leventhal.** 1983. Bacterial *in vitro* protein synthesizing systems. Methods Enzymol. **101:**598–605.
8. **Chang, F. N., C. Jiddhikol, and B. Weisblum.** 1969. Subunit localization of antibiotic inhibitors of protein synthesis. Biochem. Biophys. Acta **186:**396–398.
9. **Corcoran, J. W., and F. E. Hahn (ed.).** 1974. Mechanism of action of antimicrobial and antitumor agents. Springer-Verlag, New York.
10. **Duvall, E. J., D. M. Williams, P. S. Lovett, C. Rudolph, N. Vasantha, and M. Guyer.** 1983. Chloramphenicol-inducible gene expression in *Bacillus subtilis*. Gene **24:**171–177.
11. **Duvall, E. J., D. M. Williams, S. Mongkolsuk, and P. S. Lovett.** 1984. Regulatory regions that control expression of two chloramphenicol-inducible *cat* genes cloned in *Bacillus subtilis*. J. Bacteriol. **158:**784–790.
12. **Ehrlich, S. D.** 1977. Replication and expression of plasmids from *Staphylococcus aureus* in *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. **74:**1680–1682.
13. **Gryczan, T., M. Israeli-Reches, M. DelBue, and D. Dubnau.** 1984. DNA sequence and regulation of *ermD*, a macrolide-lincosamide-streptogramin B resistance element from *Bacillus licheniformis*. Mol. Gen. Genet. **194:**349–356.
14. **Gryczan, T., M. Israeli-Reches, and D. Dubnau.** 1984. Induction of macrolide-lincosamide-streptogramin B resistance requires ribosomes able to bind inducer. Mol. Gen. Genet. **194:**357–361.
15. **Harwood, C. R., D. M. Williams, and P. S. Lovett.** 1983. Nucleotide sequence of a *Bacillus pumilus* gene specifying chloramphenicol acetyltransferase. Gene **24:**163–169.
16. **Henkin, T. M., and G. H. Chambliss.** 1984. Genetic mapping of a mutation causing an alteration in *Bacillus subtilis* ribosomal protein S4. Mol. Gen. Genet. **193:**364–369.
17. **Horinouchi, S., W.-H. Byeon, and B. Weisblum.** 1983. A complex attenuator regulates inducible resistance to macrolides, lincosamides, and streptogramin type B antibiotics in *Streptococcus sanguis*. J. Bacteriol. **154:**1252–1262.
18. **Horinouchi, S., and B. Weisblum.** 1980. Posttranscriptional modification of mRNA conformation: mechanism that regulates erythromycin-induced resistance. Proc. Natl. Acad. Sci. U.S.A. **77:**7079–7083.
19. **Horinouchi, S., and B. Weisblum.** 1982. Nucleotide sequence and functional map of pC194, a plasmid that specifies inducible chloramphenicol resistance. J. Bacteriol. **150:**815–825.
20. **Howard, G. A., and R. R. Traut.** 1973. Separation and radioautography of microgram quantities of ribosomal proteins by two dimensional polyacrylamide gel electrophoresis. FEBS Lett. **29:**177–180.
21. **Jamet, D., and C. Anagnostopoulos.** 1969. Étude d'une mutation tres faiblement transformable au locus de la threonine desaminase de *Bacillus subtilis*. Mol. Gen. Genet. **105:**225–242.
22. **Keggins, K. M., P. S. Lovett, and E. J. Duvall.** 1978. Molecular cloning of genetically active fragments of *Bacillus* DNA in *Bacillus subtilis* and properties of the vector plasmid pUB110. Proc. Natl. Acad. Sci. U.S.A. **75:**1423–1427.
23. **Lovett, P. S.** 1973. Plasmid in *Bacillus pumilus* and the enhanced sporulation of plasmid-negative variants. J. Bacteriol. **115:**291–298.
24. **Lovett, P. S., and K. M. Keggins.** 1979. *Bacillus subtilis* as a host for molecular cloning. Methods Enzymol. **68:**342–357.
25. **Lovett, P. S., and F. E. Young.** 1970. Genetic analysis in *Bacillus pumilus* by PBS1-mediated transduction. J. Bacteriol. **101:**603–608.
26. **Miller, J.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Mongkolsuk, S., N. P. Ambulos, Jr., and P. S. Lovett.** 1984. Chloramphenicol-inducible gene expression in *Bacillus subtilis* is independent of the chloramphenicol acetyltransferase structural gene and its promoter. J. Bacteriol. **160:**1–8.
28. **Mongkolsuk, S., Y.-W. Chiang, R. B. Reynolds, and P. S. Lovett.** 1983. Restriction fragments that exert promoter activity during postexponential growth of *Bacillus subtilis*. J. Bacteriol. **155:**1399–1406.
29. **Osawa, S., and R. Takata.** 1973. Chloramphenicol resistant mutants of *Bacillus subtilis*. Mol. Gen. Genet. **127:**163–173.
30. **Osawa, S., A. Tokui, and H. Saito.** 1978. Mapping by interspecies transformation experiments of several ribosomal protein genes near the replication origin of *Bacillus subtilis* chromosome. Mol. Gen. Genet. **164:**113–129.
31. **Schaeffer, P., J. Millet, and J. P. Aubert.** 1965. Catabolic repression of bacterial sporulation. Proc. Natl. Acad. Sci. U.S.A. **54:**704–711.
32. **Schoner, R. G., D. M. Williams, and P. S. Lovett.** 1983. Enhanced expression of mouse dihydrofolate reductase in *Ba-*

cillus subtilis. Gene **22**:47–57.
33. **Shaw, W. V.** 1967. The enzymatic acetylation of chloramphenicol by extracts of R factor resistant *Escherichia coli*. J. Biol. Chem. **242**:687–693.
34. **Shaw, W. V.** 1975. Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. Methods Enzymol. **43**: 737–755.
35. **Shaw, W. V.** 1983. Chloramphenicol acetyltransferase: enzymology and molecular biology. Crit. Rev. Biochem. **14**:1–43.
36. **Smith, I.** 1982. The translational apparatus of *Bacillus subtilis*, p. 111–145. *In* D. Dubnau (ed.), The molecular biology of the bacilli, vol. 1. Academic Press, Inc., New York.
37. **Tanaka, H., R. Oiwa, S. Matsukura, and S. Omura.** 1979. Amphomycin inhibits phospho-N-acetylmuramylpentapeptide translocase in peptidoglycan synthesis of *Bacillus*. Biochem. Biophys. Res. Commun. **86**:902–908.
38. **Vazquez, D.** 1979. Inhibitors of protein biosynthesis. Mol. Biol. Biochem. Biophys. **30**:108–112.
39. **Weissbach, A., and S. Pestka (ed.).** 1977. Molecular mechanism of protein biosynthesis. Academic Press, Inc., New York.
40. **Williams, D. M., E. J. Duvall, and P. S. Lovett.** 1981. Cloning restriction fragments that promote expression of a gene in *Bacillus subtilis*. J. Bacteriol. **146**:1162–1165.
41. **Williams, D. M., R. G. Schoner, E. J. Duvall, L. H. Preis, and P. S. Lovett.** 1981. Expression of *Escherichia coli trp* genes and the mouse dihydrofolate reductase gene cloned in *Bacillus subtilis*. Gene **16**:199–206.
42. **Wilson, C. R., S. E. Skinner, and W. V. Shaw.** 1981. Analysis of two chloramphenicol resistance plasmids from *Staphylococcus aureus*: insertional inactivation of Cm resistance, mapping of restriction sites and construction of cloning vehicles. Plasmid **5**:245–258.