Inhibition by Antibacterial Compounds of the Hypersensitive Reaction Induced by *Pseudomonas pisi* in Tobacco

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ABSTRACT

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Screening of 50 selected antibacterial compounds for inhibition of the hypersensitive reaction (HR) induced by *Pseudomonas pisi* in tobacco identified 19 experimentally useful compounds. None of the materials active against bacterial cell wall synthesis inhibited the HR, but some membrane-active compounds and some of those that inhibit bacterial RNA synthesis or protein synthesis were effective. Bacterial mutants resistant to specific compounds induced the HR in the presence of these compounds, indicating that their effects were on the bacteria rather than on the plants. Apparent selective inhibition of bacterial plasmid replication by acridine compounds reduced the ability of *P. pisi* to induce the HR in tobacco. Inhibition of the bacteria-induced HR by chloramphenicol was reversible while that caused by streptomycin above 64 μg/ml was essentially irreversible. The use of twofold serial dilutions of bacterial suspensions increased the sensitivity of the HR evaluation.

Additional key word: antibiotics.

The use of differentially active antibiotics (those active against bacteria, but not plants) was suggested by Klement and Goodman (22) for use in the study of the bacteria-induced hypersensitive reaction (HR) in plants. An earlier study by Katznelson and Sutton (19) reported the in vitro growth inhibition of phytopathogenic bacteria by antibiotics and quaternary ammonium compounds, but they did not investigate the HR. Sequeira (37) used rifampicin, an RNA polymerase inhibitor, to investigate the induction time of the HR induced by *Pseudomonas solanacearum* B1 in tobacco. Meadows (26) and Meadows and Stall (27) reported the use of many antibiotics to study the induction time of the HR induced by *Xanthomonas vesicatoria* in a cultivar of *Capsicum annuum* resistant to bacterial leaf spot They found that antibiotics that inhibited bacterial respiration also inhibited the HR, as did those with modes of action reported to be due to inhibition of protein synthesis or disruption of membranes.

Sasser (36) found that streptomycin and chloramphenicol inhibited the HR by an effect on *Pseudomonas pisi* rather than by an effect on the plant. The bacteria were found to be respiring at a normal rate for 8 hr following in vitro treatment with either antibiotic, thus the antibiotics were presumed to be inhibiting the HR by specific inhibition of bacterial protein synthesis.

Numerous antibiotics and antibacterial compounds (11,15) are available that are active against bacterial cell wall synthesis, membrane integrity, nucleic acid synthesis, or protein synthesis. In the studies reported here, the effects of 50 of these on the HR induced by *P. pisi* in tobacco were investigated. A parallel study of compounds active in plants is currently underway and will be reported separately.

MATERIALS AND METHODS

Chemicals. Acriflavine; actinomycin-D(dactinomycin); amethopterin (methotrexate); 5-aminouracil; 5-aminouridine; ampicillin; 8-azaguanine; 6-azauridine; bacitracin (bacitracin-A); 5-bromodeoxyuridine; carbenicillin; carzinophillin-A; cetrimide(CTAB); chloramphenicol; chloroquine; cordycepin (grade III); D-cycloserine; cytosine arabinoside hydrochloride; distamycin-A(grade II); erythromycin; ethidium bromide; 5-fluorodeoxyuridine; 5-fluorouracil; gramicidin-D; gramicidin-S; kanamycin sulfate; kasugamycin hemi-sulfate; 6-mercaptopurine (purinethiol); mitomycin-C; nalidixic acid; neomycin sulfate; novobiocin; oleandomycin phosphate (grade II); phenethanol (benzeneethanol); polymyxin-B sulfate; proflavine; puromycin dihydrochloride; rifampicin; showdomycin; sodium dodecyl sulfate (SDS); streptomycin sulfate; tetracycline hydrochloride; trimethoprim; and tubericidin were obtained from Sigma Chemical Co., St. Louis, MO 63178. Tyrocidine and tyrothricin (960 μg/mg, a mixture of gramicidin and tyrocidine) were obtained from United States Biochemical Corp., Cleveland, OH 44128. Streptovaricin complex (a mixture of eight macrolide antibiotics) and streptolydigin (stereoisomer of 4-[6-(1,4-dimethylspiro[2,9-dioxabicyclo[3.3.1]non-6-ene-8,2'-oxiran]3-yl)-4-methyl-1-oxo-2,4-heptadienyl]-2,5-dihydro-3-hydroxy-*N*,α-dimethyl-5-oxo-1-(tetrahydro-5-hydroxy-6-methyl-2H-pyran-2-yl)-1H-pyrrole-2-acetamide) were kindly given by the Upjohn Co., Kalamazoo, MI 49003.

Plants. Tobacco (*Nicotiana tabacam* L. 'Burley 2') plants were grown in the greenhouse until six leaves were fully expanded. For 3 days prior to experimentation, the plants were placed in a controlled environment with a 12-hr photoperiod, 25 ± 1 C, 31 ± 5% relative humidity, and light from high-intensity-discharge metal halide lamps at 980 ± 60 μeinsteins/m^2/sec (25,800 lux) as measured by quantum sensor. Inoculations were made by the hypodermic syringe method of Klement (20). The HR consistently occurred 7–8 hr postinoculation and all visual observations of confluent necrosis were made 10 hr postinoculation.

Bacteria. *Pseudomonas syringae* van Hall pv. *pisi*, isolate D10176 was obtained from R. N. Goodman, Department of Plant Pathology, University of Missouri. Inocula were grown at 27 ± 0.5 C for 24 hr on nutrient agar slants and were harvested by rinsing from the surface with 0.05 M phosphate buffer, pH 6.8. The cells were washed once and adjusted turbidimetrically to ~5×10^7 cells per milliliter in the buffer, and held at 25 C until use.

For the determination of inhibition of bacterial growth by the antibacterial compounds (minimal inhibitory concentration = MIC) in vitro, the bacteria were introduced at ~10^3 cells per milliliter into nutrient broth containing the appropriate

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concentration of an antibacterial compound and incubated on a shaker at 25 ± 1 C for 48 hr. Results were recorded at 24 and 48 hr postinoculation. The medium in the presumptive plasmid elimination experiment was nutrient broth made pH 7.2 by addition of pH 7.2 potassium phosphate buffer to 0.05 M, and of NaOH.

In all experiments involving serial dilutions of antibacterial compounds, twofold dilutions from 1,024 to 1 μg/ml were used, and were added immediately prior to injection. Where serial dilutions of the bacteria were used, they were also twofold dilutions from ~5×10^7 to ~10^5 cells per milliliter. Plant injections were two spots ~2 cm in diameter in the tobacco leaf constituting one replicate. A separate leaf was used for each treatment and the treatments and replicates were randomized throughout. Repeat experiments were performed on fresh plants at weekly intervals. Except where noted, treatments were replicated four times and experiments were repeated three times.

In order to determine whether the HR-inhibiting compounds were having an effect on the bacteria or on the plant, bacterial mutants resistant to 11 of the compounds were obtained. No attempt was made to obtain mutants resistant to compounds inhibiting cell wall synthesis, DNA replication, nucleotide metabolism, or nucleotide interconversion due to the relative inactivity of these compounds in inhibition of the HR.

To obtain resistant mutants of *P. pisi,* culture plates containing 20 ml of nutrient agar (with 200 μg of the compound per milliliter filter-sterilized and added after autoclaving of the media) were slanted during pouring of the media. Following cooling, the plates were placed flat and the sloping surfaces of the media were overlaid with nutrient agar containing 2×10^7 cells of *P. pisi* per milliliter. Resistance to at least 16 times the MIC of the antibiotic was obtained in the first attempt on plates containing chloramphenicol, mitomycin-C, rifampicin, streptomycin, neomycin, kanamycin, streptovaricin, or tetracycline. Repeated transfers to higher concentrations were necessary with bacteria on plates containing cetrimide, polymyxin-B, or tubericidin. Mutants resistant to kanamycin, neomycin, and kasugamycin were found to be unstable and the mutants had to be maintained on the respective antibiotic media to avoid reversion to antibiotic sensitivity. Antibiotic-resistant mutants thus obtained were grown for 24 hr on slants of nutrient agar containing 100 μg of the corresponding antibiotic per milliliter. The bacteria were rinsed from the surface of the slants with distilled water, washed once, and turbidimetrically adjusted to 10^8 cells per milliliter. A solution of the corresponding antibiotic was added to the suspension of each bacterial mutant so that the final dilution contained 5×10^7 bacterial cells per milliliter and four times the concentration of the antibiotic previously required to block the HR. The resulting suspension/solutions were incubated for 30 min on a shaker and injected into tobacco leaves, which subsequently were observed for HR induction.

To compare the relative reversibility of the chloramphenicol and stremptomycin inhibition of bacterially-induced HR, 10^8 cells of *P. pisi* per milliliter were incubated for 30 min with 0, 4, 16, or 64 μg/ml of either streptomycin or chloramphenicol. Following incubation, the cells were centrifuged from the suspension (10,000 *g* for 10 min), the antibiotic solutions were decanted, and the bacteria were resuspended in water to ~10^8 cells per milliliter (by turbidimetric measurement). Twofold serial dilutions were performed and these were injected into tobacco leaves and evaluated for ability to induce the HR. The four replications of this experiment were performed at 30-min intervals to facilitate the necessary operations.

RESULTS AND DISCUSSION

Ampicillin was 16 times as inhibitory to the growth of *P. pisi* in vitro as was carbenicillin (Table 1, 16 versus 256 μg/ml). Despite the broad-spectrum activity of carbenicillin against Gram-negative organisms, it is generally less active than ampicillin (33). Although Hoitink (18) reported that D-cycloserine-induced bacterial sphaeroplasts of *Pseudomonas tabaci* did not cause the HR, the

TABLE 1. The effect of 50 antibacterial compounds on the *Pseudomonas pisi*-induced hypersensitive reaction (HR) induced in tobacco, phytotoxicity levels, and in vitro bacterial growth inhibitory levels

Antibiotic	Effective concentration (μg/ml)			
	in vitro MIC[a]	Phyto-toxic level	HRIC[b]	30-min HRIC[c]
Cell wall synthesis				
ampicillin	16[d]	>1,024	>1,024	>1,024
bacitracin	>1,024	>1,024	>1,024	>1,024
carbenicillin	256	>1,024	>1,024	>1,024
D-cycloserine	32	>1,024	>1,024	>1,024
Membrane integrity				
cetrimide	16	128	8	<1
colistin methane sulfonate	2	512	256	4
gramicidin-D	>1,024	1,024	1,024	1,024
polymyxin-B	<1	256	8	<1
sodium dodecyl sulfate	>1,024	>1,024	>1,024	>1,024
tyrocidine	>1,024	64	†[e]	32
tyrothrycin	>1,024	512	†	128
gramicidin-S	>1,024	128	64	16
Inhibit DNA replication				
nalidixic acid	8	512	†	†
phenethanol	>1,024	>1,024	>1,024	>1,024
Nucleotide metabolism				
5-aminouracil	>1,024	>1,024	>1,024	>1,024
5-aminouridine	>1,024	>1,024	>1,024	>1,024
6-azauridine	>1,024	>1,024	>1,024	>1,024
amethopterin	>1,024	>1,024	>1,024	>1,024
trimethoprim	>1,024	>1,024	>1,024	>1,024
5-fluorodeoxyuridine	>1,024	>1,024	>1,024	>1,024
Necleotide interconversion				
5-fluorouracil	16	>1,024	>1,024	>1,024
6-mercaptopurine	>1,024	>1,024	>1,024	>1,024
6-mercaptopurine riboside	>1,024	>1,024	>1,024	>1,024
showdomycin	>1,024	>1,024	>1,024	>1,024
Nucleotide use				
cytosine arabinoside	>1,024	>1,024	1,024	256
Incorporated into RNA and DNA				
8-azaguanine	>1,024	>1,024	>1,024	>1,024
5-bromodeoxyuridine	>1,024	>1,024	>1,024	>1,024
cordycepin	>1,024	>1,024	>1,024	>1,024
tubericidin	4	>1,024	256	4
Cause DNA cross linking				
carzinophyllin-A	>1,024	>1,024	>1,024	>1,024
mitomycin-C	<1	>1,024	64	4
DNA template intercalation				
actinomycin-D	64	128	†	†
chloroquine	>1,024	>1,024	>1,024	>1,024
distamycin-A	>1,024	>1,024	256	32
ethidium bromide	16	512	256	8
proflavine	16	128	32	4
acriflavine	8	64	32	2
Inhibit RNA polymerase				
rifampicin	4	>1,024	8	<1
streptovaricin	32	256	64	32
streptolydigin	512	>2,024	1,024	512
Protein synthesis				
chloramphenicol	<1	>1,024	8	8
erythromycin	256	>1,024	>1,024	1,024
kanamycin	<1	>1,024	32	<1
kasugamycin	8	>1,024	64	32
neomycin	<1	>1,024	8	4
novobiocin	64	>1,024	>1,024	>1,024
oleandomycin	512	>1,024	>1,024	>1,024
puromycin	128	512	>1,024	>1,024
streptomycin	<1	>1,024	128	2
tetracycline HCl	<1	1,024	4	<1

[a] Abbreviations, MIC = minimal growth inhibitory concentration and HRIC = minimal inhibitory concentration that prevented HR.
[b] Compound and bacteria mixed immediately prior to injection.
[c] Compound and bacteria mixed with shaking for 30 min prior to injection.
[d] Compounds used in twofold serial dilution from 1,024 to 1 μg/ml. *Pseudomonas pisi* used at 5×10^7 cells per milliliter.
[e] † = Phytotoxic levels as low or lower than level required to prevent the HR.

resulting abnormal physiological state of the bacteria may have been the reason for this. Sphaeroplasts were not used in the work reported here. Klement (21) and Stall and Cook (41) have postulated that bacterial and plant cell "contact" is necessary for HR induction, which suggests a possible role for the bacterial cell wall in the causation of the HR.

Polymyxin B, colistin methane sulfonate, gramicidin-S, and cetrimide were very effective inhibitors of the HR. Membrane-active materials produced by *Bacillus polymyxa* or *B. cereus* include polymyxin, gramicidin-S, gramicidin-D, tyrothrycin, and tyrocidin (17). In my experiments, tyrocidin and tyrothrycin caused phytotoxicity at levels too close to those needed to inhibit the HR to be of value in bacteria-plant interaction studies. Polymyxin and cetrimide are cationic surface-active materials that have been shown to cause release from bacterial cells of phosphate, amino acids, purines, and pyrimidines, indicating general damage to the cellular membrane (31,32,35). With polymyxin treatment of *Pseudomonas aeruginosa*, a linear relationship between bactericidal activity and release of purines and pyrimidines has been reported (30). Although it may be merely coincidental, it is interesting to note that polymyxin-B at 25 μg/ml caused "blebs" on *E. coli* (23) that appear strikingly similar to those observed on *P. phaseolicola* inducing the HR in a resistant bean cultivar (39).

Although nalidixic acid at a low concentration (8 μg/ml) inhibited bacterial growth, neither it nor phenethanol caused an inhibition of the HR (phenethanol typically is used at 0.25% to inhibit DNA synthesis [1,12,24]). The fact that nalidixic acid causes rapid selective inhibition of DNA synthesis (14,47, and Fig. 1) with only minor effects on RNA or protein synthesis suggests that bacterial DNA synthesis is not necessary for induction of the HR. This finding appears to be contrary to Klement's suggestion (21) that bacterial multiplication is necessary for HR induction.

The effective inhibition of the HR by mitomycin-C, seems to counter the above suggestion of lack of involvement of DNA synthesis, in that the rapid bactericidal effect of mitomycin coincides with the inhibition of DNA synthesis while RNA and protein synthesis continue (34). Reich et al (34), however, also reported breakdown of the cellular DNA which subsequently (3,43) was suggested to be due to repair mechanism excision. Such DNA degradation would make it unsuitable as a template for RNA synthesis. The relative inactivity of carzinophyllin-A in preventing either the growth of the bacteria or the HR induced by the bacteria, may be due to lack of uptake by the bacteria.

P. pisi was remarkably resistant to the nucleotide analogs; only tubericidin was active at a low concentration in inhibiting both growth and the HR. Tubericidin is an adenine analog which is incorporated into both RNA and DNA and seems to owe its activity largely to changes in RNA structure following incorporation (42).

The lack of inhibition of growth of *P. pisi* due to exposure to distamycin is in sharp contrast to the pronounced inhibition of the bacteria-induced HR. Distamycin-A is an oligopeptide antibiotic which binds to the DNA template (6), thus inhibiting both DNA polymerase and RNA polymerase. Chloroquine is an antimalarial drug that inhibits nucleic acid synthesis in some bacteria (7), but it was relatively inactive against *P. pisi.* Actinomycin-D was phytotoxic to tobacco at concentrations equal to, or lower than those required to block the bacterially-induced HR. Thus, while it may have research value due to its action on the eukaryote, it was not of use in the current study.

Ethidium bromide, proflavine, and acriflavine bind to DNA and inhibit both DNA replication and DNA-dependent RNA synthesis (9). At low concentrations, these three compounds may selectively interfere with closed circular duplex DNAs and thus have been used to cause elimination of plasmids from bacteria (2,13). Because of this partially selective action on plasmids, the acridines and ethidium bromide were investigated further to ascertain their usefulness in study of presumptive plasmid determinants related to *P. pisi*-induction of the HR (Table 2). Following growth in sublethal levels of acriflavine, proflavine, or ethidium bromide the bacteria were found to be less active in inducing the HR in tobacco leaves than were untreated bacteria (Table 2).

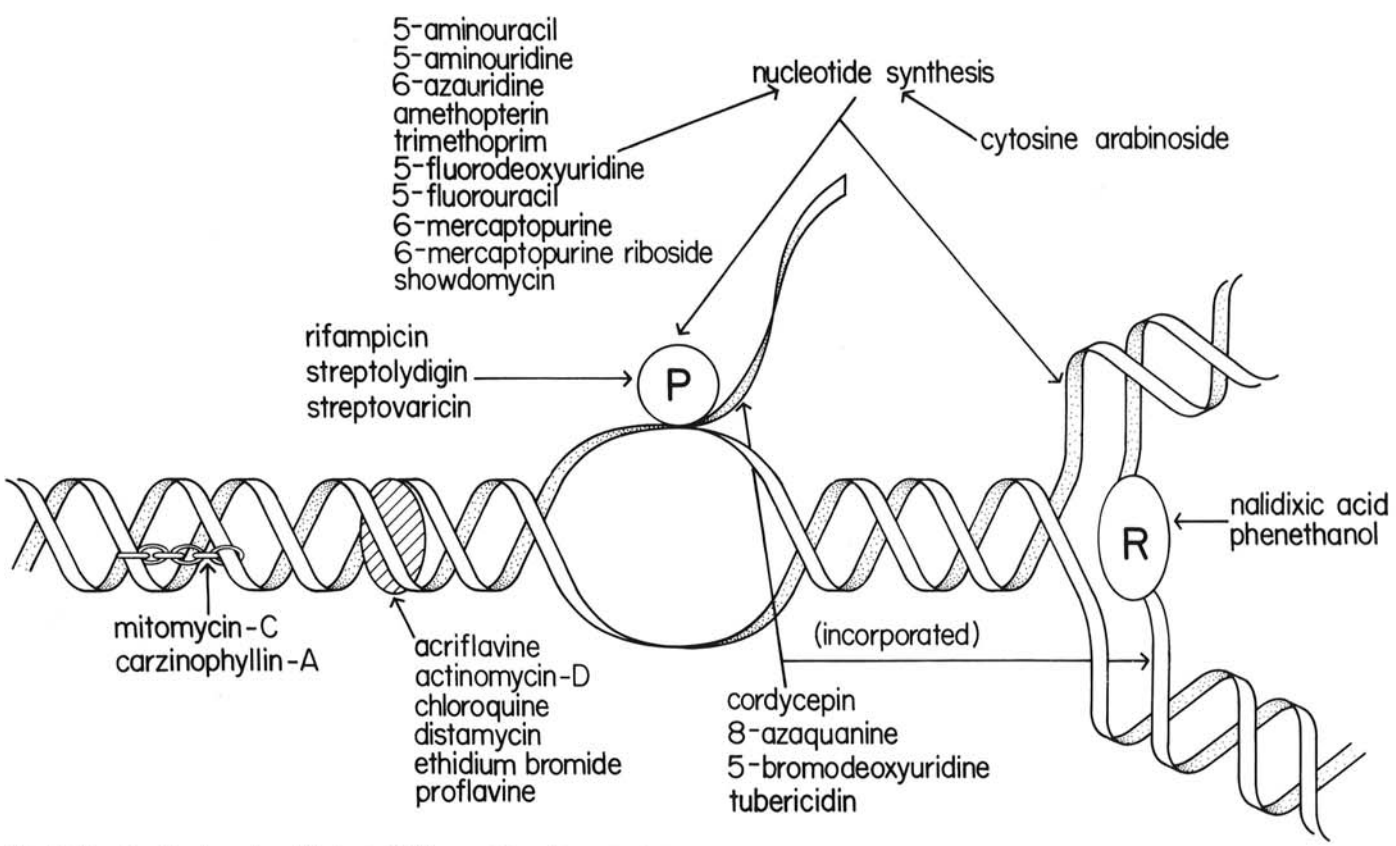

Fig. 1. The site of action of antibiotics inhibiting nucleic acid synthesis. P represents RNA polymerase and R represents DNA replicase. Mitomycin and carzinophyllin-A cause cross-linking of the DNA and the group headed by acriflavine inhibits synthesis by being intercalated into DNA.

The pronounced inhibition of the HR by rifampicin (Table 1), coupled with its known specificity for inhibition of RNA polymerase (16,40), is evidence for the necessity of bacterial RNA synthesis in induction of the HR, and is in agreement with the results of Sequeira (37). Streptovaricin appears to have an identical mode of action (28) to rifampicin, but it was not nearly as effective in inhibiting the HR. Streptolydigin binds to bacterial RNA polymerase (5) and inhibits the RNA chain-polymerization reaction, but binds much more weakly than rifampicin. This weak binding allows for the reversibility of streptolydigin action through dilution (38) and may make the compound useful in research despite the high concentrations required.

Tetracycline HCl was very active against *P. pisi* both in growth inhibition and in inhibiting the HR. The results of other studies (*unpublished*) indicate that chlorotetracycline (10) is even more active than tetracycline HCl against this bacterium. Erythromycin (4), novobiocin, oleandomycin, and puromycin (29) were somewhat inhibitory to bacterial growth in vitro, but did not inhibit the HR under the experimental conditions used here.

Kanamycin and neomycin (8,46) are similar to streptomycin in inhibition of ribosome function and in that their action is irreversible. When no preincubation of the antibiotic and bacteria was allowed, both neomycin and kanamycin inhibited the HR at lower levels than did streptomycin. This suggests that they are taken up more rapidly than is streptomycin and thus may be useful in plant/bacteria studies. Streptomycin has been used to inhibit the bacterially-induced HR (22,26,27,36) and does so despite the fact that the bacteria continue to show a linear increase in respiration rate (26,36) for at least 6 hr following addition of the antibiotic. However, streptomycin is bactericidal in that it binds irreversibly to the ribosomes of bacteria (25,45), while chloramphenicol has been found to be a reversible inhibitor of protein synthesis (45). The reversible nature of the chloramphenicol inhibition of the HR (Table 3) suggests that it may be more useful than streptomycin in the study of bacteria/plant interactions. Meadows and Stall (27) found chloramphenicol to be the most rapid-acting antimicrobial agent of the 24 compounds tested for inhibition of the HR induced by *Xanthomonas vesicatoria* in a resistant pepper cultivar. They concluded that chloramphenicol was superior to streptomycin for studies involving pepper and *X. vesicatoria.*

In most of the research on the bacteria-induced HR, evaluation is typically done by determining presence or absence of confluent necrosis. Although Turner and Novacky (44) have reported a staining and microscopic procedure for determination of death of individual plant cells, it requires considerably more time and labor than visual evaluation. A simple procedure to gain increased information about HR causation is to use twofold serial dilutions of the bacterial inoculum when comparing treatments. This allows differences in treatments to be detected that often would be missed in experiments with a single level of inoculum. Thus, in the chloramphenicol/streptomycin reversibility experiment (Table 3) partial inhibition of the bacterial potential for causing the HR can be detected. If the dilutions are performed in large spot-plates using a preset repeating pipet with disposable nonwetting sampler tips, the process is simple and rapid.

Bacterial mutants were selected for individual resistance for 11 of the compounds (Table 1) that were effective in inhibition of the HR. The HR resulted in every case following injection of the bacteria and antibiotic mixture into tobacco leaves. The inhibition of the bacterially-induced HR by antibiotics is therefore due to their action on *P. pisi* and not on the plant.

Bacterial division in the plant is apparently not required for induction of the HR, as some compounds inhibiting bacterial DNA replication did not inhibit the HR, nor was the HR inhibited by compounds which prevent bacterial cell wall synthesis. It should be noted, however, that bacterial fission was not measured in these experiments. The pronounced inhibition of the HR by several compounds that prevent formation of bacterial RNA or protein agrees with earlier reports about some of these materials (22,27,37) and with the finding that bacterial protein synthesis is necessary for induction of the HR (36). Continued bacterial respiration and bacterial membrane integrity appear to also be necessary for the induction of the HR.

TABLE 2. Cell concentrations of *Pseudomonas pisi* required to induce the hypersensitive reaction in tobacco following growth of the bacteria in the presence of compounds which selectively interfere with closed circular duplex DNAs

Antibacterial compound[a]	Concentration (μg/ml)	Bacteria to induce HR (no./ml)
None	...	2.5×10^{6}[b]
Ampicillin[c]	8	2.5×10^{6}
Proflavine	8	10^{7}
Acriflavine	4	2.5×10^{7}
Ethidium bromide	8	10^{8}

[a] Following logarithmic growth for 16 hr in nutrient broth (pH 7.2) containing the antibacterial compound, the bacteria were harvested, washed once and resuspended in water to $\sim 10^{8}$ cells per milliliter and in a twofold serial dilution.

[b] Numbers are the means of four replications. The results were taken 12 hr postinoculation and were identical for all replications of a selected treatment. Bacterial populations did not differ among the antibacterial compound treatments after 12 hr in vivo when 1 cm^2 leaf disk samples were taken from the leaves injected with 1.25×10^{6} cells per milliliter.

[c] Cell-wall specific antibiotic as an additional control treatment.

TABLE 3. Comparative reversibility of the effect of chloramphenicol and of streptomycin on inhibition of the hypersensitive reaction (HR) caused in tobacco by *Pseudomonas pisi*

Antibiotic (μg/ml)	Number of bacteria required to cause HR[a]	
	Chloramphenicol	Streptomycin
0	3.1×10^{6}	3.1×10^{6}
4	6.2×10^{6}	5.0×10^{7}
16	1.2×10^{7}	...[b]
64	1.2×10^{7}	...

[a] Bacteria were suspended in the antibiotic solution for 30 min, centrifuged out at 5,000 *g*, resuspended and twofold serially diluted in water prior to injection. Numbers are means of four replications. The results were identical for all replications of a selected treatment.

[b] No HR after exposure to inoculum containing 10^{8} cells per milliliter.

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