Use of a Streptomycin-Dependent Escherichia coli Strain for the Quantitative Determination of Streptomycin Residues in Fruit

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The Federal Food and Drug Administration has established a finite tolerance of 0.25 ppm of streptomycin for pome fruits. This edict has prevented the use of streptomycin for control of fire blight of pears in California, since there are no California data on streptomycin residues. A project, therefore, was initiated to obtain information on whether or not streptomycin exists in pear fruit above the tolerance level when adopting standard spray programs.

Streptomycin has been detected in pear tissue at concentrations of 0.25 ppm using the antibiotic assay technique (2). However, we were not satisfied with the accuracy and sensitivity of the technique. Therefore, a bioassay method was developed for detecting streptomycin residues in plants with a streptomycin-dependent bacterium as the assay vehicle.

Basal and assay media.—The basal medium contained nutrient broth (Difco), 8 g; glucose, 10 g; streptomycin sulfate (78.1% streptomycin base), 100 mg; and distilled water, 1 liter. The assay medium was similar, except that it excluded streptomycin sulfate and included Ionagar II (Colab), 10 g; and 50 ml of 1 M phosphate buffer pH 6.8. The buffer was added after autoclaving the medium to avoid precipitation.

Organism.—The organism used in this study was *Escherichia coli* strain JC425, a spontaneous streptomycin-dependent mutant of JC355 isolated from strain K12 by John Clarke, Molecular Biology Department, University of California, Berkeley. This strain requires 0.5 ppm streptomycin for growth; 4-8 ppm is optimal. It tolerates a streptomycin concentration of 800 ppm. A more sensitive strain capable of growth with 0.15 ppm streptomycin was selected by plating the parent culture in the assay medium, pipetting 10 ppm streptomycin sulfate into a well of a cylinder, and selecting colonies farthest from the streptomycin source. This cylinder antibiotic assay technique (1) was periodically used to insure maximum sensitivity of the strain.

Preparation of the organism.—The organism was prepared for the bioassay by streaking a loopful of cells on the basal medium containing 2% agar. The high concentration of antibiotic stimulates growth and also discourages back mutation. A single colony was selected and examined for purity by plating on the assay medium. The growth of colonies indicates the presence of back mutants not requiring streptomycin. The inspected organism then was grown in the basal medium for 24 hr at 28 C. The culture was centrifuged at 7,000 *g* for 5 min, resuspended in the basal medium, and incubated on a rotary shaker for 20 hr at 28 C to exhaust endogenous streptomycin (4).

Assay standardization.—One ml of a cell suspension (80-150 Klett reading, green filter) was added to 1 liter of melted assay medium (46 C). Ten ml of the medium were dispensed/petri dish. The dishes were dried with the lids on for 10 hr at 28 C. Glass assay cylinders, ID-6 mm, OD-8 mm × 10 mm in length, were sterilized with ethanol and placed on the assay plate. The sensitivity of the organism to streptomycin was determined by pipetting into the assay cylinders 0.2 ml of 0.1 to 10 ppm streptomycin sulfate solutions. After incubation at 28 C for 72 hr, the diam of the growth rings around the cylinders were recorded.

Streptomycin sulfate was detected in concentrations as low as 0.15 ppm; the sizes of the growth rings about the cylinders accurately reflected the quantity of antibiotic placed in the rings (Fig. 1).

Extraction of streptomycin from pear tissues.—One M phosphate buffer, pH 6.8, was used to extract streptomycin from pear fruit. The efficiency of the buffer for extraction was evaluated by cutting 25-g cross sections of tissue through the centers of pear fruit and blending with a Waring Blendor for 30 sec. An amendment of 8 mg streptomycin sulfate was added to each section (0.32 ppm). This mixture was blended with an equal amount (w/v) of buffer for 30 sec, incubated 10 min, and strained through a fine-mesh, nylon cloth. The filtrate was then centrifuged at 10,000 *g* for 15 min. Two-tenths cc of supernatant was assayed, and the plates were examined after 72-hr incubation at 28 C.

The average diam of the bacterial growth ring around

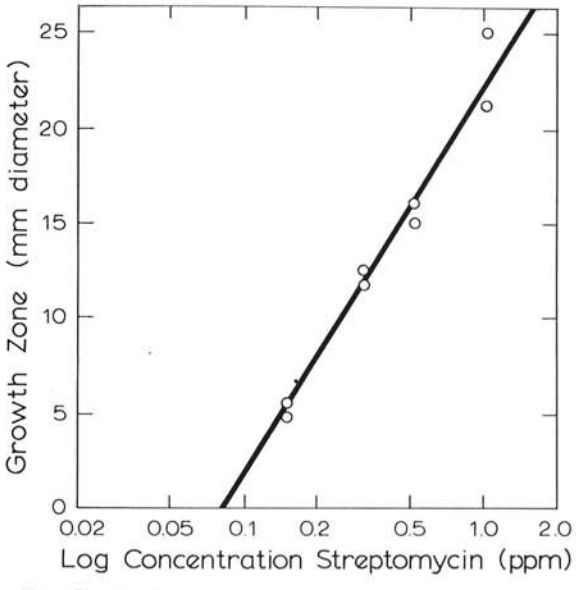

Fig. 1. Sensitivity of a streptomycin-dependent strain of *Escherichia coli* to streptomycin expressed as diam of growth zones about cylinders (diam of cylinder subtracted) containing different concentrations of antibiotic. Each point represents the mean of two replications.

the cylinder (mean of 8 replications) was 9 mm when phosphate was used. This figure, based on the sensitivity tests (Fig. 1), indicated that 80% of streptomycin in the pear preparation was recovered. Similar data were obtained in three separate experiments.

Residue tests.—Analyses of streptomycin residues were made with fruit from *Pyrus communis* L. 'Bartlett' pears collected from three ranches. Concentrations of streptomycin sulfate, 0.9-6.1 oz/acre (0.7-4.9 oz active streptomycin) had been applied to the trees 13-17 times. Spray applications were terminated 30 days prior to harvest. Two-lb. samples of fruit were collected from 40 plots, each with 3 replications, and frozen. The fruit was prepared and assayed according to the previously described method. The control consisted of processing nonsprayed pears and adding streptomycin sulfate to half the samples (0.18 ppm final concentration).

Bacterial growth did not occur about any of the cylinders, except with extracts from control pears where streptomycin had been added. This indicated that if streptomycin was present in the fruit, it was below 0.15 ppm, the sensitivity limit of the bioassay.

DISCUSSION.—Care must be taken to insure that the cultures remain in a highly dependent state if a streptomycin sensitivity level of 0.15 ppm is required. The sensitivity test should be performed each time the culture is prepared for the bioassay, as the culture becomes a heterogeneous population of cells after several mass transfers. Colonies selected from such a culture often varied in degree of dependency to streptomycin.

The principle of the increased sensitivity of a bioassay using streptomycin-dependent bacterium instead of a streptomycin-sensitive bacterium involves the action of streptomycin on ribosomes (3, 5). The present evidence is that streptomycin inactivates normal 30-S ribosome function by binding to the ribosome, but activates 30-S ribosomes of streptomycin-dependent mutants. Possibly only a small fraction of the ribosomes in a cell need be activated for growth of a streptomycin-dependent strain in comparison to the large fraction of ribosomes that must be inhibited to kill sensitive cells.

LITERATURE CITED

1. HEATLEY, N. G. 1949. The assay of antibiotics, p. 110-199. *In* H. W. Florey et al. [ed.] Antibiotics, Vol. I. Oxford Univ. Press, N. Y.
2. KEIL, H. L., & R. A. WILSON. 1968. Relation of streptomycin sprays to fire blight control and apple fruit residue. Plant Dis. Reptr. 52:259-262.
3. LIKOVER, T. E., & C. G. KURLAND. 1967. Ribosomes from a streptomycin-dependent strain of *Escherichia coli*. J. Mol. Biol. 25:497-504.
4. SPOTTS, C. R., & R. Y. STANIER. 1961. Mechanism of streptomycin action on bacteria: A unitary hypothesis. Nature 192:633-637.
5. WEISBLUM, B., & J. DAVIES. 1968. Antibiotic inhibitors of the bacterial ribosome. Bacteriol. Rev. 32:493-528.