MICROBIAL DEGRADATION OF ERYTHROMYCINS A AND B

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Growing cultures, as well as broken and lyophilized cells of pseudomonas 56 were found to degrade erythromycin A, and lyophilized cells inactivated erythromycins A and B. The enzyme system involved in this degradation was constitutive and the enzyme level in the cells could be increased about 8-fold when oleandomycin or erythromycin B was added to the growth medium. The ability of whole or broken cells to inactivate erythromycin A was completely lost when these preparations were boiled, and the erythromycin A-inactivating activity was localized in the cell membrane fraction. The lyophilized cells did not degrade oleandomycin, methymycin, tylosin, a mixture of leucomycins, josamycin, or maridomycin III.

The ability of microorganisms to transform or inactivate macrolide antibiotics has recently begun to be studied. FELDMAN *et al.*[1] noted the disappearance of erythromycin from the culture media of steroid-transforming strains of *Streptomyces* and *Nocardia*, and NAKAHAMA *et al.*[2,3] have reported the deacylation and hydroxylation of maridomycin and josamycin.

The purpose of our study was the isolation of a microorganism capable of enzymatic inactivation of erythromycin A. Such an enzyme may be a useful tool for specific bioassays.

Materials and Methods

Antibiotics.

The erythromycin A, erythromycin B, erythralosamine, desosamine, and erythronolide B were obtained from Abbott Laboratories through the courtesy of Dr. W.J. CLOSE. The oleandomycin was obtained from the Pfizer Corporation, and the methymycin was obtained from Ms. B. STEARNS of the Squibb Institute for Medical Research. The tylosin was the gift of the Lilly Research Laboratories and the leucomycin mixture of the Ayerst Laboratories (through the courtesy of Dr. C. VEZINA). The josamycin was obtained from Yamanouchi Pharmaceutical Company Ltd. through the cooperation of Dr. A. KITAI of the Sanraku-Ocean Company. The maridomycin III was obtained from Takeda Chemical Company through the generosity of Dr. M. ISONO. All antibiotics were used as received (without purification) as chromatographic analyses showed the presence of only one antibiotic component (except for the leucomycin).

Culture media.

Pseudomonas 56 was grown in a medium containing yeast extract (Difco), 10 g, KH_2PO_4, 2.28 g, K_2HPO_4, 5.79 g, $(NH_4)_2SO_4$, 0.5 g, and distilled water q.s. 1 liter unless otherwise indicated.

Bioassay methods.

Bioassays were carried out using *Staphylococcus aureus* FDA 209 P or *Sarcina lutea* as the test organisms in an agar diffusion assay. A two-layer agar system was employed to enhance the sensitivity of the bioassays. Difco Antibiotic Medium 1 was used for the bioassays using *S. aureus* and Difco Antibiotic Medium 11 for those assay using *S. lutea*. Paper discs (12.7 mm from Schleicher and Schuell) were used as reservoirs for the antibiotic solutions,

and incubation was overnight at 37°C for the *S. aureus* and 30°C for the *S. lutea*. The antibiotic inhibition zones were read manually using a vernier calipers (graduated in 0.1 mm). The precision of the bioassays was below ±15 % when 3 discs were used for each antibiotic solution.

Chemical assay method.

A colorimetric assay was developed based on our previously described method:[4] A 1-ml sample of test solution at pH 8 (containing about 200 mcg of erythromycin) was transferred to a conical glass-stoppered centrifuge tube at 0°C; 4.0 ml of ethylacetate was then added, the tube stoppered and shaken on a Vortex mixer for 15 seconds (Scientific Industries, Inc.); 1.0 ml of the ethylacetate layer was transferred to a 18×150 mm Pyrex test tube and the solvent removed by a current of filtered air at room temperature. To this tube was the added 2.0 ml of 6 N sulfuric acid and 1.0 ml of the arsenomolybdate color reagent.[5] The tube was then thoroughly mixed and incubated in a boiling water bath for 15 minutes to develop the color. Then, 0.5 ml of water was added and the contents of the tube cooled to room temperature. The blue color was measured at 660 nm in a Bausch and Lomb Spectronic 20 spectrophotometer. The response of this assay was linear between 0 and 80 mcg erythromycin A per tube, 0 and 60 mcg of erythromycin B, and 0 and 60 mcg of leucomycin. Assay with this reagent for methymycin, oleandomycin, and tylosin was less satisfactory since the extraction was not so efficient or the acid degradation of the antibiotic did not yield products reacting with the arsenomolybdate.

Results and Discussion

Isolation of Erythromycin Degrading Organism.

Soil dilutions were added to tubes of the yeast extract-$(NH_4)_2SO_4$-salts medium supplemented with 100 mcg/ml of erythromycin A, and the tubes were placed on a rotary shaker in a 37°C incubator. Culture 56 was found to have the ability to 'remove' the antibiotic from the medium (as determined by bioassay) under conditions where the antibiotic activity of the uninoculated medium did not drop appreciably. This culture was shown to be a Gram-negative rod belonging to the genus *Pseudomonas*. It grows well when incubated in shaken culture over the temperature range 27°~37°C, and will not grow at 41°C. Culture 56 is not able to metabolize carbohydrates (in the standard taxonomic test media), has a requirement for lipid which is satisfied by oleic acid, and will grow well in media containing acid hydrolyzed casein as C and N source. It has been maintained both on agar slants and as a frozen suspension (in a liquid N refrigerator).

Inactivation of Erythromycin by Growing Cultures.

Study of the inactivation of erythromycin A by culture 56 grown in buffered media in shaken flasks showed that marked inactivation occurred when the pH of the medium was at pH 7.0 (or higher) under conditions where chemical inactivation of the antibiotic was minimal. Addition of 80 mcg/ml of erythromycin A to the culture just prior to the culture attaining maximum absorbance resulted in complete inactivation of the antibiotic in 6 hours. (An uninoculated flask of the identical medium under the same conditions of pH, incubation temperature (35°C), and agitation showed no significant loss of bioactivity). In similar studies using erythromycin B, oleandomycin, methymycin, and tylosin, no degradation of the antibiotic occurred (as determined by bioassay).

Inactivation of Erythromycin by Resting Cells.

Cells of culture 56 obtained by centrifugation of an 18 hour culture grown at 30°C were washed with 50 mM phosphate buffer (pH 7.0) and resuspended in the buffer to give twice the original concentration. Ten ml of the suspension were mixed with 5 mg of erythromycin A and placed in a 45°C-water bath. Samples were withdrawn every hour for analysis and showed that while 90 % of the bioactivity was lost after 6-hour incubation, only 30 % of the bioactivity of samples from a tube containing the same amount of erythromycin and boiled cells disappeared.

Inactivation of Erythromycin and Other Macrolide Antibacterial Antibiotics by Broken Cell Preparation.

Disrupted cell preparations were used to investigate whether the permeability of cell preparations influenced the rate of and substrate specificity of inactivation. The most effective preparations were made by sonicating washed cells (50 mM phosphate buffer, pH 7.0) at 0°C with a Bronson Sonicator for 10×3 seconds. This procedure resulted in a broken cell preparation that had greater inactivation ability than resting cells. Preparations made by crushing the cells in an Aminco French Press, by hand grinding with sand and by freezing and thawing were less effective than the sonicated cells. Addition of dithiothreitol (to a final concentration 0.1 mM) resulted in higher inactivation rates. The optimum pH for inactivation of erythromycin A by the broken cell preparations was pH 8.0, and highest rates of strictly biological inactivation were obtained when the preparations were incubated at 45°C.

A differential centrifugation study using the active sonicated cell preparations suggested that the inactivation enzymes were not cytoplasmic: All of the inactivation activity was found in the pellet when the sonicated preparations were centrifuged at $6,000\times g$ for 20 minutes. Attempts to solubilize this activity were unsuccessful. In a study using spheroplasts (made by EDTA-lysozyme treatment of cells in sucrose solutions) the erythromycin A inactivating activity was associated with the membranes collected when the spheroplasts were lysed in distilled water.

Induction of Erythromycin A Inactivating Activity.

The time course of erythromycin A inactivating enzyme synthesis in growing cultures was studied in experiments testing various compounds as enzyme inducers. The enzyme levels were expressed as follows:

$$\text{Enzyme units} = \frac{\text{change in absorbance at 660 nm (arsenomolybdate assay) in 30 min.}}{\text{mg cell dry weight}}$$

Some of the data collected in this study are summarized in Fig. 1. Erythromycin B and oleandomycin were effective in increasing the erythromycin A-inactivating enzyme system level in the growing culture about 8-fold. The effect of these and other inducers on cell mass production was not significant as shown in the data presented in Table 1.

Preparation of Stable Enzyme Preparations.

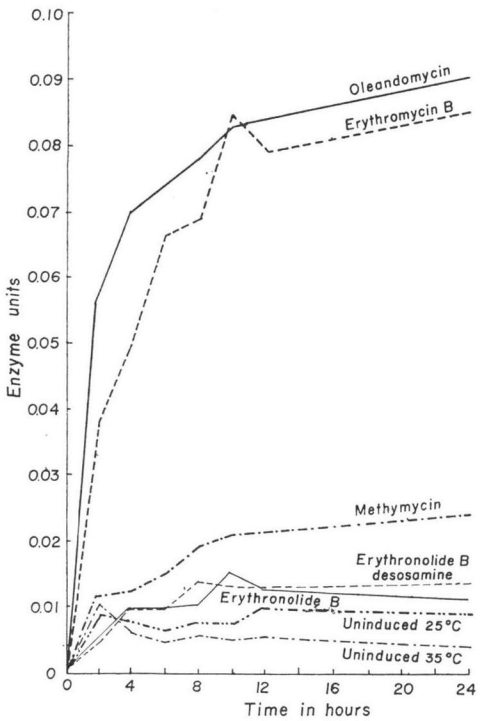
Study of the erythromycin A inactivating ability of sonicated cells showed that preparations frozen at −20° for 24 hours and thawed at 30°C lost all activity. Acetone-dried cells and sonicated cells were also inactive. Lyophilized cells prepared by freezing 12-hour old cultures (enzyme

Table 1. Induction in *pseudomonas* 56 of erythromycin A inactivating enzyme system activity by macrolide antibiotics (at 25°C).

Inducer compound*	Relative enzyme level obtained	Maximum cell yield, mg/liter (dry weight)
None	1.0	1,800
Erythronolide B	1.22	1,920
Erythronolide B-desosamine	1.27	1,650
Methymycin	2.00	1,850
Erythromycin B	7.50	2,140
Oleandomycin	8.00	2,230

* inducer added to give final concentration of 25 mg/liter cultures grown in yeast extract medium.

Fig. 1. Induction of erythromycin A-inactivating enzyme system in *pseudomonas* 56 cells by macrolide antibiotics and related substances.



induced with oleandomycin) in pH 8 phosphate buffer (100 mM) followed by lyophilization resulted in active powders. These lost about 50 % of their activity when stored at −20° for 4 months.

Substrate Specificity of Lyophilized Cell Preparations.

The lyophilized cells inactivated 100 % of the erythromycin A added (500 mcg antibiotic per ml) to the cell suspension (2 mg/ml) in 30 minutes as measured by bioassay and the arsenomolybdate assay. When erythromycin B was added, 40 % was inactivated as determined by chemical assay, while the bioassay showed only 15 % inactivated in a 120-minute exposure time. Less than 10 % inactivation was noted when methymycin, tylosin, oleandomycin, josamycin, or maridomycin III were added to the lyophilized cells and incubation was continued for 120 minutes (chemical assay).

Identification of Erythromycin A Inactivation Products.

The products of the inactivation of erythromycin A by growing cells of *pseudomonas* 56 were recovered from the incubation mixture by the following procedure: (1) Residual erythromycin A was extracted from the pH 8 solution by extraction with ethylacetate. (2) The aqueous phase was lyophilized, dissolved in pH 10 morpholine buffer, and desalted at pH 7 by passage over a pH 9 AG 1-X2 anion exchange resin (using volatile buffer system). (3) Removal of volatile buffer by evaporation. The concentrated material was examined by thin-layer chromatography on Eastman Chromagram (cat. 6060) plates with results as shown in Table 2. Among the products which appear to be present are erythronolide, erythralosamine, and two unknown products. Incubation of erythronolide B, desosamine, erythralosamine B,

Table 2. Mobility of erythromycins, chemical degradation products, and enzyme inactivation products in thin-layer chromatography.

Material examined	Rf in indicated solvent system			
	System A	System B	System C	System D
Erythromycin A	0.35	0.07	0.67	0.11
Erythromycin B	0.34	0.07	0.66	not tested
Erythronolide B	0.67	0.31	0.83	0.61
Erythralosamine	0.58	0.17	0.55	0.24
Desosamine	0.22	0.06	0.21	0.08
Product A	0.78	0.63	0.79	0.79
Product B	0.69	0.31	0.83	0.61
Product C	0.60	0.18	0.55	0.24
Product D	0.07	0.0	0.33	0.0~0.06

Support: Eastman Chromagram (cat. no. 6060).
Solvent systems: A. $CHCl_3$ - MeOH - acetone (10:3:1)
B. Benzene - MeOH - toluene (85:10:5)
C. *n*-BuOH - AcOH - H_2O (4:1:5)
D. *n*-Hexane - benzene - MeOH - EtOAC - acetone (30:25:10:20:10)
Location of organic materials detected by spraying with $CeSO_4$ solution.

and erythromycin B with the cell suspension did not result in significant degradation. Erythromycin A was shown not to be altered when subjected to the same product isolation procedures.

It appears then that *Pseudomonas* 56 constitutively synthesizes an enzyme system which is capable of degrading erythromycin A (and to a lesser extent erythromycin B) under conditions of pH and temperature where little chemical inactivation occurs. The specificity of this enzyme system for erythromycin A over other macrolide antibacterial antibiotics may be useful for inactivating erythromycin in antibiotic mixtures.

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