DIFFICIDIN AND OXYDIFFICIDIN: NOVEL BROAD SPECTRUM ANTIBACTERIAL ANTIBIOTICS PRODUCED BY *BACILLUS SUBTILIS*

III. MODE OF ACTION OF DIFFICIDIN

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The mode of action of difficidin (DIF) was investigated. Upon addition of DIF to log phase cultures of *Escherichia coli*, growth ceased immediately and small round cells accumulated after 30 minutes incubation. No cell lysis was observed. DIF was rapidly bactericidal to both growing and stationary phase cultures, and inhibited protein synthesis more rapidly than RNA, DNA, or cell-wall synthesis in growing cells. Protein synthesis was also inhibited in a cell-free system. The frequency of natural mutation to resistance in *E. coli* was less than 1 in 10^{10} cells.

A screening program for new antibacterial antibiotics led to the discovery of a new broad spectrum antibiotic, difficidin (DIF), [16-dihydroxyphosphinyloxy-5,17-dimethyl-4-methylene-22-(3-methyl-3,5-hexadienyl)oxacyclodocosa-7,9,11,17,19-pentaene-2-one], produced by a *Bacillus subtilis* strain isolated from soil. Preceding papers in this series have described the isolation, structure determination, and antibacterial spectrum of this antibiotic[1,2]. In this report, we describe mode of action studies of DIF that suggest the antibiotic rapidly inhibits protein synthesis in *Escherichia coli*; however, other properties of the drug suggest that activity at the cell membrane may also be involved.

Materials and Methods

Strains and Media

Escherichia coli K-12 strains MB 4827, (C_{600}, *galK*$^-$), and MB 4196 (SCH62, HfrH, *thi*) were used in these experiments. Cultures were maintained at room temp on either Brain Heart Infusion Agar (Difco) or Luria-Bertani (LB) agar slants with weekly transfer. All standard plate counts were performed with LB agar. All experiments in liquid culture and all MIC determinations were performed using M9 medium[3].

MIC Determination

MICs were assayed by a conventional broth dilution method[4] using M9 medium.

Antibiotics

DIF (Merck & Co., Inc.; stock solution, 7.3 mg/ml in MeOH), chloramphenicol (CAM, Calbiochem; 25 mg/ml in MeOH), rifampicin (RIF, CIBA Pharmaceutical Company; 10 mg/ml in MeOH), nalidixic acid (NAL, Aldrich; 10 mg/ml in 0.1 N NaOH), benzylpenicillin (PEN, Lilly; 20 mg/ml in H_2O), streptomycin (STR, ICN; 16 mg/ml in H_2O) and polymyxin B (PMB, ICN; 10 mg/ml in H_2O) were prepared as indicated. DIF was stored at -70°C. All other antibiotic stock solutions were stored at -20°C.

Radionuclides

L-[^{14}C]Amino acid mixture 0.1 mCi/ml, L-[^{3}H]amino acid mixture 1.0 mCi/ml, [6-^{3}H]thymidine

1.0 mCi/ml (15.1 Ci/mmol) and [5,6-^{3}H]uracil 1.0 mCi/ml (42.0 Ci/mmol) were obtained as sterile aqueous solutions from New England Nuclear. [2,6-^{3}H]Diaminopimelic acid (DAP) 1.0 mCi/ml (281 mCi/mmol) was obtained from Amersham.

Measurement of Radioactive Precursor Incorporation

The total synthesis of RNA, DNA, protein and peptidoglycan were measured by quantitating the incorporation of radiolabeled precursors (uracil, thymidine, amino acids and DAP, respectively) into acid insoluble material. An overnight culture of MB 4827 was diluted 1:200 into M9 and incubated at 37°C, 250 rpm, for approximately 2 hours until the optical density at 600 nm (OD_{600}) reached 0.1. Depending on the experiment, various radioactive and nonradioactive precursors were then added to the culture. Aliquots of the culture (usually 10 ml) were then transferred to prewarmed 50 ml sterile plastic tubes and the tubes were incubated at 37°C, 250 rpm, for a total of 180 minutes. Drug was added 60 minutes after the addition of the radioactive precursors to the culture.

Growth was monitored by measuring the OD_{600} of the culture. The incorporation of radioactive precursors into acid insoluble material was monitored by spotting triplicate 20 μl samples of each culture onto Whatman GF/C glass fiber filters that had been presoaked in 10% TCA and dried. The filters were washed by vacuum filtration once with 5% TCA, three times with ice cold water, and once with 100% ethanol. After the filters had dried, they were placed in vials, covered with scintillation cocktail and counted in an LKB liquid scintillation counter.

Inhibition of Protein Synthesis *In Vitro*

Preparation of S-30 Extract: The effect of DIF on protein synthesis *in vitro* was assayed using an "S-30" extract according to the method of NIRENBERG and MATTHAEI[5], and MODOLELL[6]. Briefly, *E. coli* strain MB 4827 was grown to late log phase in LB broth, harvested by centrifugation and the cell pellet was rapidly frozen and stored at −70°C. The frozen pellet was ground with alumina, extracted with standard buffer (60 mM ammonium chloride, 10 mM magnesium acetate, 6 mM 2-mercaptoethanol and 10 mM Tris-HCl, pH 7.8) and treated with DNase. Alumina and cell debris were removed by centrifugation at 20,000×g for 15 minutes, and the supernatant was clarified by centrifugation at 30,000×g for 45 minutes. The "S-30" extract was then dialyzed against fresh standard buffer for 5 hours, aliquoted, rapidly frozen, and stored at −70°C. The ribosome concentration of the extract was typically 8~9 mg protein/ml (OD_{600} 1.3).

Purification of Messenger RNA (mRNA): mRNA was purified from bacteriophage MS2. *E. coli* strain MB 4196 was infected with MS2 at an initial multiplicity of infection of 1.0. The infected cells were incubated for 3 hours at 35°C and rapidly chilled. Phage was precipitated from the culture with 6% PEG 6000 and 0.5 M NaCl at 4°C overnight, and harvested by centrifugation at 12,000×g for 2 hours. The phage pellet was resuspended in 10 mM Tris-HCl, pH 7.5, and banded to equilibrium in a cesium chloride gradient (Type V Ti65 rotor, 47,000 rpm, 20 hours, 20°C). The visible band of phage was collected, diluted with 10 mM Tris buffer, pelleted by centrifugation at 100,000×g for 3 hours, and resuspended in 1% SDS, 20 mM EDTA. RNA was purified from the phage by successive extraction with phenol and chloroform, followed by ethanol precipitation. The final concentration of RNA was 13 mg/ml, and the preparation was stored at −70°C.

In vitro protein synthesis using the S-30 extract and MS2 mRNA was performed as described by MODOLELL[6]. Assay mixtures contained 50 mM Tris-HCl (pH 7.8), 60 mM ammonium chloride, 10 mM magnesium acetate, 10 mM reduced glutathione, 1 mM ATP, 0.02 mM GTP, 5 mM potassium phosphoenolpyruvate, 30 μg/ml pyruvate kinase, 0.05 mM amino acids (complete), 0.05 mCi [^{3}H]-amino acids, 10 μl S-30 extract (final concentration 1.6 μg/ml) and 10 μl MS2 mRNA (diluted 1:8 in 50 mM Tris-HCl, pH 7.8) in a final volume of 100 μl. Following incubation at 34°C, 1 ml of 5% TCA was added, and the assay tubes were incubated at 80°C for 20 minutes. Material insoluble in hot TCA was collected on glass fiber filters, washed with 5% TCA, 100% ethanol, and counted. Incorporation was complete by 20 minutes at 34°C, and reached 13,000 cpm per assay tube. Translation of endogenous message accounted for 5~10% of the total protein synthesized. All assays were performed in triplicate.

Determination of Mutation Frequency

The frequency of natural mutation to resistance was determined using *E. coli* strains MB 4827 and MB 4196. Cells were grown overnight in LB broth, concentrated by centrifugation, and 10^9 cells were spread onto the surface of each of 20 LB plates containing 70 μg/ml DIF. The plates were incubated for 72 hours at 35°C, and resistant colonies were picked and transferred to a fresh LB plate containing DIF (70 μg/ml). An isolated colony of each mutant was then transferred to an LB slant without DIF. The MIC of DIF for each mutant was determined by broth dilution. In some experiments, cells were mutagenized with ethyl methanesulfonate (EMS, 2%, 2 hours, 24°C) prior to plating on LB agar.

Results

Effects of DIF on MB 4827

To determine the effects of DIF on the growth of log phase cultures of *E. coli*, the optical density and microscopic morphology of the cultures were monitored following the addition of drug at 10× the MIC (MIC 3.5 μg/ml). PEN and CAM were included for comparison. Results are shown in Fig. 1. Immediately following addition of DIF, the OD_{600} of the culture stopped increasing, indicating that growth had ceased. CAM, an inhibitor of protein synthesis, had a similar effect. Addition of PEN to the culture caused the OD_{600} to decrease, indicating cell lysis. Microscopic examination of the treated cultures revealed small round cells, but no evidence of cell lysis or bizarre cell forms.

To assess the effects of DIF on cell viability, the drug was added at several concentrations to log phase cultures of MB 4827. At the times indicated, samples were removed, diluted and plated on LB agar. DIF was rapidly bactericidal at concentrations at and above the MIC (Fig. 2).

DIF was added to a stationary phase culture of MB 4827 to assess the effects of DIF on non-

Fig. 1. Growth of *Escherichia coli* MB 4827 in M9 medium was monitored by optical density measurements as described in Materials and Methods.

The culture was divided into four portions when the OD_{600} reached 0.100, and drugs were added to each portion 60 minutes later.

■ 35 μg/ml DIF, △ 40 μg/ml CAM, ▲ 200 μg/ml PEN, ○ no drug. Arrow indicates time of drug addition.

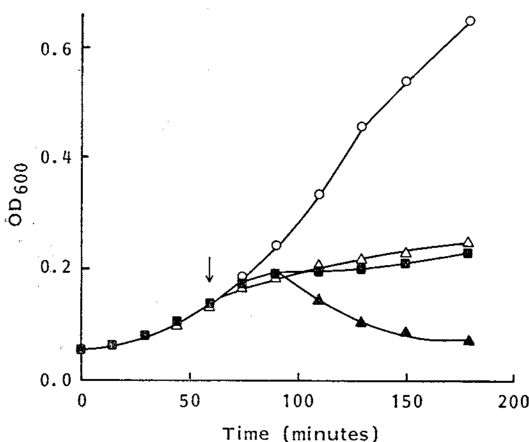
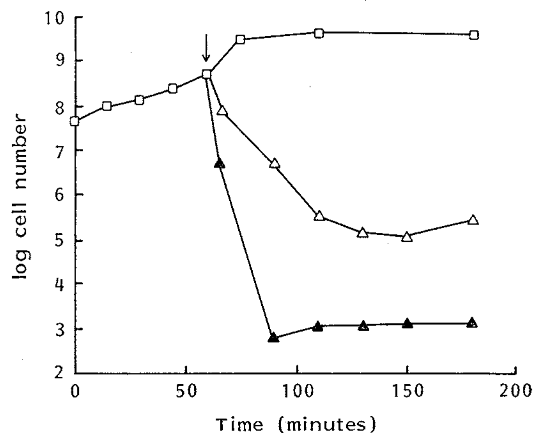


Fig. 2. Effects of DIF on viability of *Escherichia coli* MB 4827.

DIF was added to a log phase culture of *E. coli* MB 4827 in M9 medium and samples were withdrawn at the times indicated, diluted, and plated on LB agar to determine cell viability.

▲ 35 μg/ml DIF, △ 3.6 μg/ml DIF, □ no drug. Arrow indicates time of drug addition.



growing cells. The cells were incubated for 15 minutes, diluted in M9 medium, and plated on LB plates. For comparison, similar experiments were performed with STR, an antibiotic that exhibits a bactericidal effect only on growing cells. While STR had no effect on cell viability under these conditions (the viable count of both STR treated and untreated cultures was 3.5×10^9 cells/ml), DIF decreased the viable count by almost two logs (7.5×10^7 cells/ml). It is possible that the effect of DIF on stationary phase cells was due to adherence of the drug to the bacterial cell, causing a relatively high concentration of drug to be carried to the plate with the cells. To investigate this possibility, a stationary phase culture was incubated for 15 minutes with DIF or STR as above, but the cells were diluted in a variety of diluents prior to plating. The results shown in Table 1 indicate that M9, LB, and taurocholate reduced the apparent killing by 10-fold. However, none of the diluents completely ablated the killing effect of DIF on resting cells.

Effects of DIF on RNA, DNA, Protein and Cell-wall Synthesis

Experiments were designed to assess the effects of DIF on total RNA, DNA, protein and cell-wall synthesis in MB 4827. To determine which process was affected most rapidly by the drug, log phase cultures were prelabeled with radioactive precursors for 60 minutes prior to the addition of DIF. Total incorporation of precursor was monitored throughout the preincubation period, and for a total of 120 minutes following the addition of drug. The optical density of the drug treated cultures was also monitored as a measure of cell growth. Known inhibitors were included in each experiment and the effects of DIF were compared to the effects of these known antibiotics.

In preliminary experiments in which STR (an inhibitor of protein synthesis) was added to cultures at a final concentration of 6.5 μg/ml (the MIC level), growth and amino acid incorporation slowed but did not stop immediately. However, when the concentration of drug was increased 3- to 10-fold, both growth and amino acid incorporation stopped almost immediately. All antibiotics were, therefore, used at concentrations 5- to 10-fold above the MIC to magnify effects.

Upon addition of DIF or STR, incorporation of labeled amino acids stopped immediately. Incorporation continued when NAL (an inhibitor of DNA synthesis) or cycloserine (CYS, an inhibitor of cell-wall synthesis) were added (Fig. 3). The rapid inhibition of protein synthesis was observed even when

Table 1. Bactericidal effects on stationary phase cells[a].

Diluent	cfu/ml	
	STR (40 μg/ml)	DIF (35 μg/ml)
Distilled water	2.0×10^9	6.5×10^6
M9 medium	3.5×10^9	7.5×10^7
LB broth	2.9×10^9	6.3×10^7
Taurocholate	2.5×10^9	7.9×10^7
Zwittergent	3.7×10^8	6.3×10^6
Triton X 100	1.1×10^9	4.5×10^7
Tween 20	2.0×10^7	4.9×10^6

[a] A stationary phase culture of *Escherichia coli* MB 4827 was treated with STR or DIF at the concentrations indicated. After 15 minutes incubation, the cells were diluted in the various diluents and plated on LB plates.

Fig. 3. [^{3}H]Amino acid incorporation of cultures.
□ No drug, ○ 35 μg/ml DIF, △ 50 μg/ml NAL, ● 64 μg/ml STR, ■ 150 μg/ml CYS. Arrow indicates time of drug addition.

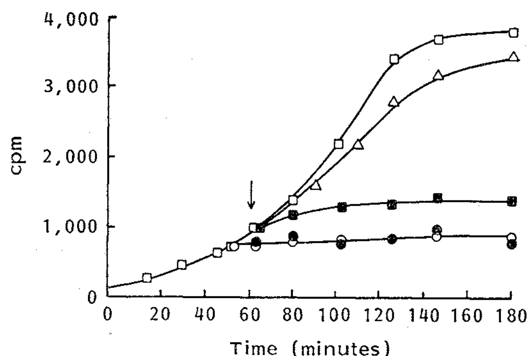


Fig. 4. [³H]Uracil incorporation of cultures.

□ No drug, ○ 35 μg/ml DIF, ● 50 μg/ml RIF, ▲ 40 μg/ml CAM, △ 50 μg/ml NAL. Arrow indicates time of drug addition.

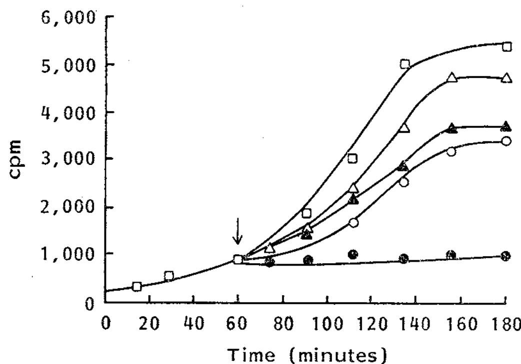


Fig. 5. [³H]Thymidine incorporation of cultures.

□ No drug, ○ 35 μg/ml DIF, △ 50 μg/ml NAL, ● 50 μg/ml RIF. Arrow indicates time of drug addition.

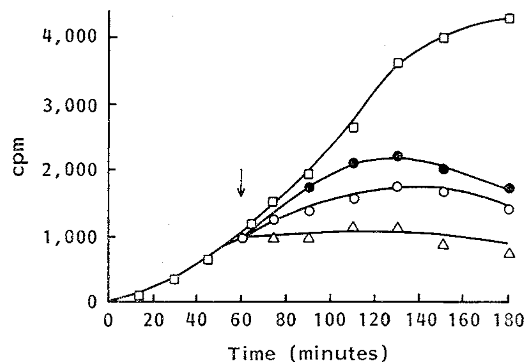


Fig. 6. [³H]DAP incorporation of cultures.

□ No drug, ○ 35 μg/ml DIF, ▲ 150 μg/ml CYS, ● 64 μg/ml STR, △ 50 μg/ml NAL. Arrow indicates time of drug addition.

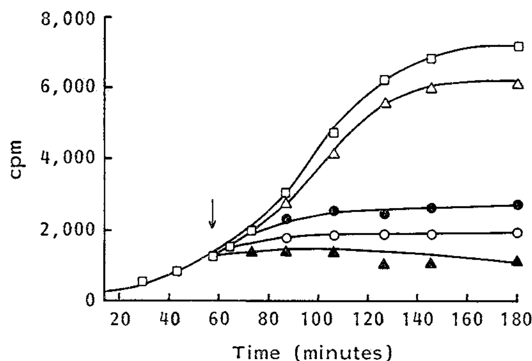
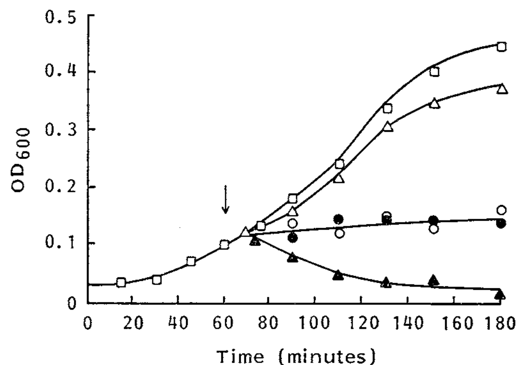


Fig. 7. Optical density of cultures.

□ No drug, △ 50 μg/ml NAL, ○ 50 μg/ml RIF, ● 40 μg/ml CAM, ▲ 150 μg/ml CYS. Arrow indicates time of drug addition.



DIF was added to the culture at concentrations as low as 2 μg/ml (the MIC). However, when DIF was added to cultures preincubated with labeled uracil, the synthesis of RNA did not stop as rapidly as it did in the presence of RIF, an inhibitor of RNA synthesis (Fig. 4). Similarly, DNA synthesis and cell-wall synthesis were inhibited more rapidly by NAL and CYS, respectively, than by DIF (Figs. 5 and 6). The effects of these antibiotics on cell growth (OD_{600}) are shown in Fig. 7. PMB, a membrane-active antibiotic, inhibited the incorporation of all precursors. These results strongly suggest that a primary mode of action of DIF is inhibition of protein synthesis.

Inhibition of Protein Synthesis *In Vitro*

An *in vitro* protein synthesis assay was established using an "S-30" extract of *E. coli* and mRNA purified from bacteriophage MS2. At 2 μg/ml, DIF inhibited 100% of the incorporation of labeled amino acids into protein. The antibiotic inhibited 30% of the label incorporation at 0.1 μg/ml and did not inhibit incorporation at levels below 0.05 μg/ml. DIF inhibited the incorporation of label directed by both endogenous and exogenous message.

Development of Resistance to DIF

To determine the frequency of resistance to DIF, *E. coli* cells were plated on LB agar containing 70 μg/ml DIF, and incubated for 72 hours. Resistant clones were picked and retested for DIF resistance. The frequency of natural mutation to resistance was very low (10^{-10}) for both MB 4196 and MB 4827. When cells were treated with 2% EMS for 2 hours prior to plating, the resistance frequency was increased by only 2-fold. Many DIF-resistant clones grew more slowly than the parent strain. No DIF-resistant clones were cross-resistant to STR or CAM.

Discussion

The results of this study suggest that DIF inhibits protein synthesis in *E. coli*. The drug inhibited protein synthesis more rapidly than RNA, or DNA synthesis *in vivo*, and inhibited protein synthesis in a cell-free system at concentrations comparable to the MIC (2 μg/ml vs. 3.5 μg/ml). Although DIF rapidly inhibited the incorporation of [^{3}H]DAP into the bacterial cell-wall, the drug did not cause cell lysis as would be expected for cell-wall active antibiotics. This suggests that the observed inhibition of cell-wall synthesis is due to the general inhibition of cell growth by DIF.

These studies have also shown that DIF is apparently bactericidal to resting cells, and that the development of resistance in *E. coli* is very rare. These characteristics may indicate that DIF binds rapidly and irreversibly to an intracellular target. However, they may also indicate that the antibiotic has effects on the cell in addition to inhibition of protein synthesis. For example, these characteristics are also consistent with effects on the cell membrane. Further studies are necessary to address this question. DIF-resistant cells will be a useful tool for future genetic and biochemical analyses of both primary and secondary effects of the drug.

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