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## THE EFFECT OF ANTIMICROBIAL AGENTS ON ANAEROBIOSIS AND INHIBITION OF PROTEIN SYNTHESIS.

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### Abstract

Antimicrobial agents were known to interact with DNA gyrase, based on their decreasing activity and concentrated on the effect of cadmium on the biodegradative process performed by the activity of the enzyme were estimated. Cadmium were chosen because they are often found as contaminant. Bacteriostatic and bactericidal activities were measured with wild-type cells and isomerase mutants of organisms for ciprofloxacin, formation of DNA gyrase complexes. Nalidixic acid, norfloxacin and ciprofloxacin, were lethal for cultures growing aerobically, and the bacteriostatic activity of each antibiotic was unaffected by anaerobic growth. However, lethal activity was distinct for each antibiotic with cells treated aerobically with chloramphenicol or grown anaerobically. Nalidixic acid failed to kill cells under both conditions; norfloxacin killed cells when they were grown anaerobically but not when they were treated with chloramphenicol, ciprofloxacin killed cells under both conditions but required higher concentrations than those required with cells grown aerobically. The entry of nalidixic acid into cells of *E. coli* was not dependent upon protein synthesis. The lethal action of nalidixic acid also was controlled by transfer of treated cells to drug-free medium. Antibiotic activity against *Escherichia coli* was examined during aerobic growth, aerobic treatment with chloramphenicol, and anaerobic growth. However, lethal chromosome fragmentation, detected as a drop in viscosity in the absence of SDS, occurred with nalidixic acid treatment only under aerobic conditions in the absence of chloramphenicol. The radiation of sublethal dose of 4.0 kGy at rate of 0.75 kGy/min. was shown as ( NS ) non significant result.

### Introduction

DNA breaks are released from the protein-mediated constraint present in ternary complexes. At rapidly lethal antibiotic concentrations, supercoils cannot be maintained (Chen, C. R., 2009) and fragmented DNA is obtained under conditions that allow resealing at bacteriostatic drug concentrations (Malik, M., 2010).

Anaerobiosis is potentially useful for the study of antibiotic lethality, since it affects gyrase and supercoiling (Hsieh, L.-S., 2010) and it allows antibiotic (oxolinic acid) to form ternary complexes. Moreover, fluoroquinolones kill *Escherichia coli* under some anaerobic conditions (Zhao, X., 2010), structure-activity relationships have not been established for anaerobic activity.

Rapid antibiotic mediated cell death, which does not appear to require interruption of active DNA replication fork movement (Zhao, X., 2010), occurs in two ways: one that requires ongoing protein synthesis and one that does not (Howard, B. M., 2009). The relative contribution of each of these two lethal pathways depends on antibiotic structure, the lethal actions of nalidixic and oxolinic acids are blocked by chloramphenicol, an inhibitor of protein synthesis (Chen, C. R., and Lewin, C., 2009). In contrast, fluoroquinolones, such as ciprofloxacin, do not require ongoing protein or RNA synthesis to kill cells (Chen, C. R., and Lewin, C., 2009). Whether other perturbations of bacterial metabolism distinguish the two pathways is (NS) non significant. As a result, antibiotic concentrations sufficient to block replication do not relax chromosomal DNA supercoiling (Snyder, M. 2009). When the antibiotic is removed, the breaks are readily resealed (Gellert, M. and Sugino, A., 2007) and the inhibitory effects of the compounds are reversed (Goss, W., Malik, M. and Pohlhaus, J., 2010).

Irreversible events that lead to rapid cell death occur at higher antibiotic concentrations (Chen, C. R., and Malik, M., 2010). We have proposed that rapid cell death arises from chromosome fragmentation that occurs when double-strand In the present study we examined antibiotic mediated lethality after passing an anaerobic gas mixture through a growing culture of *E. coli*. The gas mixture had no effect on the ability of the antibiotic to block growth. However, it allowed only fluoroquinolones to kill the cells. The effects of anaerobiosis and inhibition of protein synthesis on antibiotic lethality provided a way to classify the antibiotics into groups, to distinguish bacteriostatic action from bactericidal action and to correlate chromosome fragmentation with rapid cell death.

### **Materials and Methods**

The MICs were measured by incubation of  $10^4$  to  $10^5$  cells/ml in liquid medium containing serial twofold dilutions of a vancomycin at 37°C either with shaking (2ml cultures in 2.5 cm diameter tubes) for aerobic cultures or in sealed tubes (1.5cm diameter tubes) following 20 min of treatment with the anaerobic gas mixture. To measure lethal action, cells were grown aerobically with shaking at 37°C in liquid medium to mid-log phase. The cells were split into 2ml portions, with one portion grown under aerobic conditions with shaking and the other portion grown in a sealed tube treated with the anaerobic gas mixture.

Bacterial strains, culture conditions, and vancomycin susceptibility:

The *E. coli* strains used in the study were HM100 from Culture collection Faculty of Agriculture Ein-Shams University and the following derivatives of HM100 constructed by P1-mediated transduction: KD1373 (ParC Ser-80 to Leu),

KD2750 (GyrA Ser-83 to Leu and Asp-87 to Tyr), and KD2329 (GyrA Ser-83 to Leu, Asp-87 to Tyr, and ParC Ser-80 to Leu). Introduction of the mutations was confirmed by nucleotide sequence determination of the quinolone resistance-determining regions for *gyrA* and *parC* (Friedman, S. M., and Yoshida, H., 2010). Strains were grown on LB agar (Miller, J., 2011) or in LB liquid medium containing 1% glucose and 100 mM equimolar sodium phosphate buffer (pH 6.8). Exponentially growing cultures were shifted to anaerobic conditions by passing a mixture of 85% N<sub>2</sub>, 10% H<sub>2</sub>, and 5% CO<sub>2</sub> through the culture (the gas mixture was scrubbed by passage through a powdered zinc suspension in 20 μM phenazine methosulfate (pH 4) and then bubbled through the culture (Hsieh, L.-S., 2010).

After 20 min, aerobic or anaerobic solutions of a vancomycin were added, the cultures were mixed with a Vortex mixer, and incubation was continued for 2 h with (anaerobic) or without (aerobic) passage of the anaerobic gas mixture. Cells were diluted in ice-cold LB medium, applied to LB agar plates lacking drug, and incubated aerobically overnight at 37°C on agar plates to determine the number of cfu, which was expressed relative to the number of cfu at the time of antibiotic addition, measurement of killing in the absence of protein synthesis, chloramphenicol was added to 20 μg/ml 10 min prior to addition of a antibiotic. Cultures that were aerated by shaking or by passage of atmospheric air bubbles exhibited the same sensitivity to killing by nalidixic acid.

#### Antimicrobial agents:

Nalidixic acid, norfloxacin, and chloramphenicol were obtained from Sigma-Aldrich; ciprofloxacin was obtained from Bayer Corp. and PD161144 was obtained from Pfizer ( Pharm.). The antibiotics were dissolved in 0.1 ml of 1 N NaOH at 1/10 of the final volume, followed by the addition of sterile water to give a final concentration of 10 mg/ml. Stock solution aliquots were kept at -20°C for several weeks during the experiments. Aliquots were used only once; after the aliquots were thawed, dilutions were prepared with sterile distilled water.

#### Detection of chromosome fragmentation by viscosity:

Bacterial cells were gently lysed by incubation with lysozyme and nonionic detergents at 20°C for 2 to 3 min, as described previously (Snyder, M. 2009). Cells grown anaerobically were lysed under anaerobic conditions. Serial, twofold dilutions of cell lysates (0.2 ml) were transferred to glass tubes (10 by 75 mm) with minimal shearing. Pancreatic RNase was added to 20 μg/ml; the samples were then treated at 80°C for 2 min to unfold the chromosomal DNA, chilled on ice, and brought to 20°C in a water bath. A 0.025ml glass microcapillary pipette (catalog no. 71900-25; Kimble Glass Co.) was placed in the lysate samples, and the time to fill the

capillary, after subtraction of the time for buffer alone to fill the capillary. Use of viscosity to detect chromosome fragmentation was previously validated by sedimentation measurements (*Malik, M., 2010*). In addition, a dramatic decrease in viscosity was observed after addition of DNase (1  $\mu\text{g/ml}$  for 10 min) to lysates grown either aerobically or anaerobically.

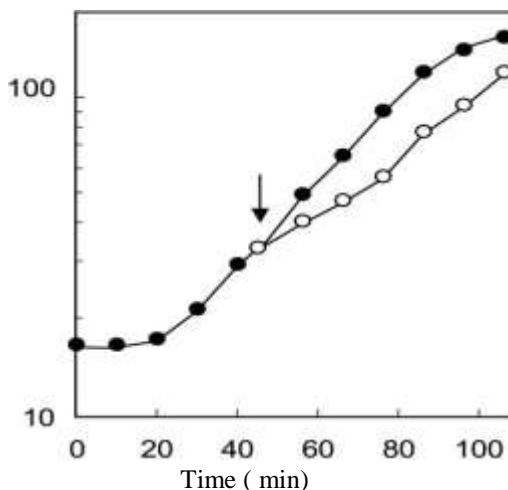
#### Radiation exposure:

The source of radiation was a gamma cell-40 for biological irradiation installed at National Centre for Radiation Research and Technology, Nasr City, Cairo ,Egypt. The radiation dose was a sublethal dose of 4.0 Gy at rate of 0.75 Gy/min.

### Results and Discussion

#### Effect of anaerobic growth on bacteriostatic activity of antibiotic :

An anaerobic gas mixture was passed through an exponentially growing culture of wild-type *E. coli* (strain PD161144), growth slowed modestly (Relative turbidity & Time/min) where recorded in (Fig.1). The bacteriostatic actions (MICs) of nalidixic acid, norfloxacin, ciprofloxacin, and PD161144 (see Fig. 2 for the compound structures) .



**Fig 1:**

Effect of anaerobic conditions on growth of *E. coli*. Wild-type strain PD161144 was grown under aerobic conditions (filled circles), at the time ( min.) indicated by the arrow and relative turbidity ( 10-100 ) part of the culture was shifted to anaerobic conditions .

The lethal activity of the antibiotic was quickly inhibited (% of survival & Time/min) were recorded in (Fig. 2). Addition of chloramphenicol also quickly blocked

lethal activity ( Fig. 2 ) (Crumplin, G. C., 2009). Thus, lethal factors synthesized under aerobic conditions appear to be unstable and/or are not synthesized under anaerobic conditions.

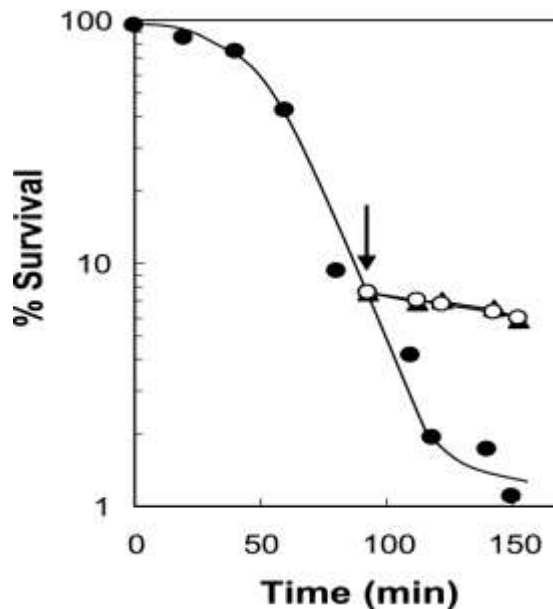
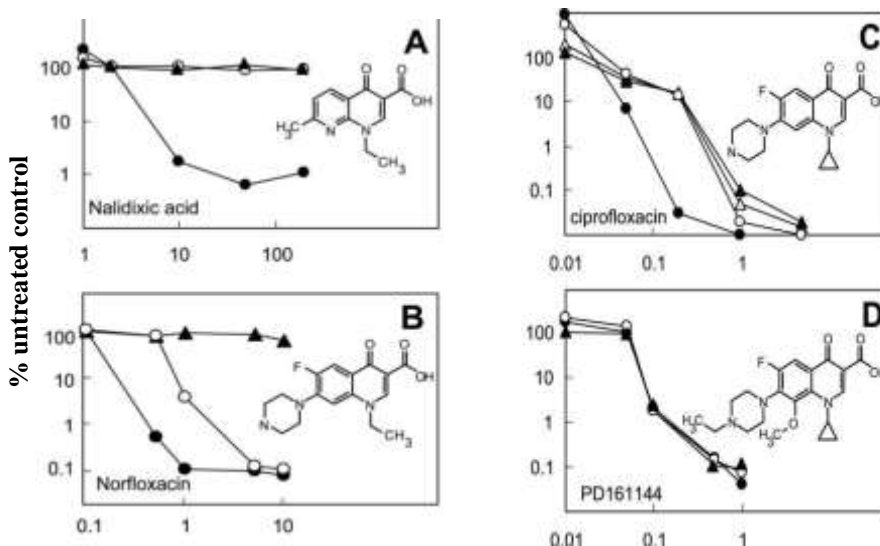


Fig 2:

Chloramphenicol and anaerobic effects on antibiotic mediated lethality: Exponentially growing cells (strain HM100) were treated with 50 µg/ml nalidixic acid (10 times the MIC; filled circles), and after 90 min (arrow) a portion.



**Fig 3 :** Antibiotic ( µg/ml )

Effect of anaerobic conditions on antibiotic lethality: Exponentially growing *E. coli* (strain PD161144) was treated with nalidixic acid (A), norfloxacin (B), ciprofloxacin (C), or PD161144 (D) for 2 h under aerobic conditions (filled circles), anaerobic, (cfu % untreated control & antibiotic µg/ml) were recorded in (Fig 3). For ciprofloxacin, we determined the inhibition of DNA synthesis of HM100 (*recA*<sup>+</sup> *gyrA*<sup>+</sup> *parC*<sup>+</sup>) at the MIC (0.25 µg/ml) (Malik, 2010) compared to that of (*recA*<sup>+</sup> *parC* Phe80) at the MIC (1 µg/ml) (Fig.3A).

Effect of anaerobic growth of chloramphenicol on antibiotic mediated lethality:

To compare the abilities of the antibiotics to rapidly kill *E. coli* under anaerobic conditions, the anaerobic gas mixture was passed through cultures of exponentially growing cells for 20 min. Then, nalidixic acid, norfloxacin, ciprofloxacin, or PD161144 was added anaerobically at various concentrations, followed by passage of the anaerobic gas mixture for 2 h. The cells were diluted and plated on drug-free agar to determine the number of viable cells present in the culture. A portion of each aerobically growing culture was also treated with chloramphenicol for 10 min before addition of an antibiotic to measure the effect of inhibition of protein synthesis on antibiotic lethality. Both anaerobic growth and treatment with chloramphenicol blocked the killing of *E. coli* by nalidixic acid (Fig.3 A). The lethal activity of norfloxacin was blocked by chloramphenicol but not by anaerobic growth, although a higher norfloxacin concentration was required for anaerobic lethality (Fig. 3 B). Ciprofloxacin killed the *E. coli* cells anaerobically and in the presence of chloramphenicol, but increased ciprofloxacin concentrations were required (Fig.3 C

). With ciprofloxacin the two turbations appeared to act on the same process, since a combined treatment (Fig. 3 C) had the same effect as either treatment alone. Neither anaerobic growth nor chloramphenicol treatment affected the activity of a C-8-methoxy fluoroquinolone (Fig.3 D). An antibiotic concentration higher than that observed under aerobic conditions is required to kill cells (Fig. 3).

These data indicate that the antibiotics fall into four categories with respect to their ability to kill cells shifted to anaerobic conditions or treated with chloramphenicol (summarized in Table 1). The rate at which a shift to low oxygen tension blocked lethality was measured by growing the cells aerobically, treating them with nalidixic acid for 90 min. These concentrations also generated a similar plateau of inhibition at 30 min. The rate of inhibition of DNA synthesis for HM100 (61% inhibition at 3 min) were lower than that for (82% inhibition). When the *recA*-deficient derivatives of HM100 were compared using the same concentrations of ciprofloxacin as for the *recA*<sup>+</sup> strains, a similar but more pronounced pattern was seen, with more rapid inhibition in the *parC* mutant, in which interaction of the drug was with gyrase (36% inhibition at 3 min).

Effects of anaerobic growth and chloramphenicol treatment on antibiotic mediated chromosome fragmentation.

**Table 1. Effect of anaerobic growth on bacteriostatic and bactericidal activities of antibiotics :**

Antibiotics	Bacteriostatic activity (MIC $\mu$ g/ml)		Bactericidal activity			
	Aerobic	Anaerobic	Aerobic	Saline	Anaerobic	CL
Nalidixic acid	5	5	+	-	-	-
Norfloxacin	0.075	0.075	+	+	+	-
Ciprofloxacin	0.01	0.01	+	+	+	+
PD161144	0.08	0.08	+	ND	+	+

Data were obtained with *E. coli* strain PD161144.

Abbreviations: CL, chloramphenicol; ND, not determined; -, no activity; +, lethal activity.

Unaffected by the shift to anaerobic conditions (Table1). Since the MIC is likely to reflect the formation of gyrase-DNA complexes (Chow, R., and Snyder, M.,



2009) the data in Table 1 suggest that anaerobic growth has little effect on ternary complex formation.

*E. coli* cells are gently lysed in the absence of proteases or ionic detergents, the nucleoid retains its structure and the lysates have very low viscosities. Disruption of the nucleoid structure by treatment with RNase and/or mild thermal treatment causes the solutions to become very viscous (Drlica, K., 2012). Drug-gyrase-DNA ternary complexes form anaerobically, a result that is consistent with growth inhibition (Table 1). These results confirmed that nalidixic acid formed ternary complexes under anaerobic conditions, but the complexes were not processed to release the DNA breaks that fragmented the chromosomes. The fluoroquinolone PD161144 was expected to behave differently from nalidixic acid and kills *E. coli* under anaerobic conditions. Antibiotic mediated chromosome fragmentation detected by lysate viscosity. Wild-type *E. coli* (strain PD161144) was grown exponentially under aerobic or anaerobic conditions and then treated for 2 h with 50 µg/ml nalidixic acid (Nal; 10 times). The effect of chloramphenicol on antibiotic mediated changes in lysate viscosity.

**Table 2 : Prevalence of Antibiotic - resistant *E. coli* from healthy patient in Hospitals and Culture collection.**

Antibiotic	conc (mg/L)	Kasr el-Einy (H.)	Al-Azhar (H.)	Ein-Shams (H.)	Mansoura (H.)
		n = 230	n = 149	n= 99	n=100
Norfloxacin	25	43	48	78	89
Ciprofloxacin	4	1	1	15	8
Chloramphenicol	25	30	8	45	82
Vancomycin	16	3	4	10	2
Nalidexic	25	52	56	86	90
Fluoroquinolone	8	33	32	76	89

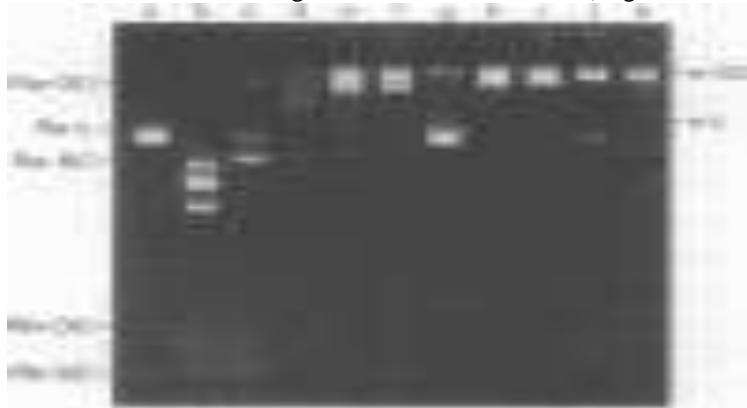
H. Hospital. U, urban, NU, non-urban

The anaerobic growth allowed nalidixic acid, the prototype antibiotic, to block growth (Table 1 and 2 ) and to trap gyrase on DNA, as indicated by an SDS-dependent drop in cell lysate however, lethal action (Fig. 3 A). Norfloxacin killed *E. coli* under anaerobic conditions, but not in the presence of chloramphenicol (Fig.3B). Ciprofloxacin and PD161144 were lethal anaerobically and in the presence of chloramphenicol (Fig.3C&D) .The concentration of ciprofloxacin needed to kill cells was increased by anaerobic and chloramphenicol treatments, with the two treatments having no additional effect when they were combined (Fig3C). The lethal action of the C-8-methoxy compound PD161144 was unaffected by anaerobic

growth or chloramphenicol (Fig.3D). Thus, anaerobic experiments support the two-step scheme (*Malik, M., 2012*) . The slow inhibition of DNA synthesis at growth-inhibitory concentrations suggests that a subset of more distantly distributed complexes is physiologically relevant for drug action and is unlikely to be located immediately in front of the DNA replication .

While the experiments described above indicate that fluoroquinolones can kill *E. coli* cells exposed to an anaerobic gas mixture, they do not resolve an earlier controversy over whether the compounds are lethal when a different method for anaerobiosis is used (*Cooper, M. A., Lewin, C., and Morrissey, L., 2012*). The anaerobic lethalities of fluoroquinolones may be sensitive to experimental conditions as shown in ( Fig 4). The effects of anaerobiosis on quinolone lethality and cell lysate viscosity (Fig.3A and D) support the conclusion that chromosome fragmentation is responsible for rapid quinolone-mediated cell death. In earlier work we showed (i) that nucleoids can maintain supercoils when they are isolated from *E. coli* cells treated with antibiotics under bacteriostatic conditions but not bactericidal conditions (*Chen, C. R., 2011*); (ii) that chromosome fragmentation, measured by sedimentation analyses, occurs during the same time frame as cell death (*Malik, M., 2012*); and (iii) that chromosome fragmentation fails to occur when cell death is blocked by chloramphenicol (*Malik, M.,2012*). We emphasize that these data do not address the contributions of other processes, such as lethal filamentation (*Goss, W., 2012*) and induction of toxin-antitoxin systems (*Hazan, R., 2011*), to cell death.

On the other hand, the Biochemistry find that Cadmium were chosen because they are often found as contaminant. The data represent averages and standard deviations of estimations original substrate as shown in ( Fig. 4 & Table 3).



**Fig 4 : Antibiotic - resistance in *E. coli* from healthy patient in hospitals and culture collection .**

**Table : 3 Activity of enzymes supplemented with cadmium ( 0 and 2 mM Cd ).**

	5 days		7days		9 days	
	0 mM Cd	2 mM Cd	0 mM Cd	2 mM Cd	0 mM Cd	2 mM Cd
Endo-1,4- $\beta$ -glucanase	67 $\pm$ 15	116 $\pm$ 11	65 $\pm$ 26	127 $\pm$ 20	38 $\pm$ 9	39 $\pm$ 12
Exo-1,4- $\beta$ -glucanase	17 $\pm$ 7	23 $\pm$ 7	1 $\pm$ 1	40 $\pm$ 11	6 $\pm$ 1	8 $\pm$ 4
1,4- $\beta$ -Glucosidase	203 $\pm$ 21	314 $\pm$ 58	29 $\pm$ 8	183 $\pm$ 46	37 $\pm$ 4	42 $\pm$ 8
Endo-1,4- $\beta$ -xylanase	31 $\pm$ 9	9 $\pm$ 7	49 $\pm$ 19	13 $\pm$ 9	41 $\pm$ 6	58 $\pm$ 22
1,4- $\beta$ -Xylosidase	4 $\pm$ 1	4 $\pm$ 0	5 $\pm$ 1	12 $\pm$ 3	6 $\pm$ 1	5 $\pm$ 1
1,4- $\beta$ -Mannosidase	6 $\pm$ 3	0 $\pm$ 0	37 $\pm$ 7	13 $\pm$ 5	65 $\pm$ 8	38 $\pm$ 6

In control treatments, the enzyme during the whole steady state fermentation process, whereas the activities of 1,4- $\beta$ -glucosidase and exo-1,4- $\beta$ -glucanase were highest during substrate isolation. All three studied enzymes were more active in Cd-containing cultures and high activity of 1,4- $\beta$ -glucosidase and exo-1,4- $\beta$ -glucanase were also recorded on 5days. In the steady state decomposition phase, the activities of all enzymes in Cd-treated and untreated flasks were the same. Significant xylanase and 1,4- $\beta$ -mannosidase activities were present in Cd-free cultures. As with enzyme, a stable xylanase activity was present at all three sampling times, whereas the production of 1,4- $\beta$ -mannosidase increased with the time of cultivation. Low levels of 1,4- $\beta$ -xylosidase in the cultures were also detected. The presence of Cd affected negatively the activities of xylanase and 1,4- $\beta$ -mannosidase, the activity of 1,4- $\beta$ -xylosidase seemed to be unaffected. The Poly B-411 decolorization activity of culture extracts was the highest on 7days, corresponding to highest activities ( *Saito, T.,2010* ). It was 75 $\pm$ 3% per day in Cd-supplemented flasks and 70 $\pm$ 3% per day in control flasks, reflecting the higher activity at 2 mM Cd. Although the activity on 5 days and 9 were comparable to the activity on 7 days, the decolorization of Poly B-411 was only between 2 and 30%, higher in the presence of Cd.

The source of radiation was a gamma cell-40 for biological irradiation :

The irradiated (4Gy) % of change in the 4 days gives 0.415 $\pm$  0.070 % of change +60.0 , 0.405 $\pm$  0.080 % of change +50.0, 0.375 $\pm$  0.050 % of change +40.0 and 0.345 $\pm$ 0.035 % of change +35.0 , where the F. ratio was 0.75 and gives Non significant (NS ) ( *Kumerova, A.O., 2010*).

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## المخلص العربى

تأثير المضادات الميكروبية على البكتيريا اللاهوائية وتثبيتها للبناء البروتينى .

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قسم المنتجات الطبيعية و قسم البحوث الصحية - المركز القومى لبحوث وتكنولوجيا الاشعاع - هيئة الطاقة الذرية .

تم فحص المضادات الميكروبية النشطة على البكتيريا اللاهوائية خلال النمو الهوائى لمعالجة النمو الهوائى والنمو اللاهوائى مع الكلورامفينيكول . كانت النورفلوكساسين ، حمض الناليديكسيك والفاكوميسين سيبروفلوكساسين قاتلة لمطفرة النمو الهوائى ، ونشاط جراثيم من كل لم يتأثر النمو اللاهوائى، ومع ذلك كان النشاط قاتل و متميز لكل المضادات مع الخلايا المعالجة و التى نمت هوائيا أو لاهوائيا مع الكلورامفينيكول . فشل حمض الناليديكسيك لقتل الخلايا تحت كل الظروف . النورفلوكساسين قتل الخلايا عندما كانت تزرع لا هوائيا ولكن ليس عندما كانوا يعاملون مع الكلورامفينيكول . سيبروفلوكساسين قتل الخلايا تحت كل الشروط المطلوبة ولكن نمت مع الخلايا هوائيا مع تركيزات اعلى من تلك المطلوبة و الميثوكسى الفلوروكينولون كذلك قاتل على حد سواء فى ظل جميع الظروف. بعد المعالجة مع حمض الناليديكسيك و التحول الى ظروف لاهوائية او اضافة الكلورومفينيكول منعت موت الخلايا بسرعة اكبر. كما لوحظ تكوين مركب دى ان ايه - جبريز كمضادات و كبريتات الصوديوم دوديسيل و التى تعتمد على انخفاض اللزوجة فى الخلية و التى تمت خلال النمو الهوائى واللاهوائى بحضور او غياب الكلورومفينيكول. جميع اختبارات المضادات كونت مركب عكسى كمضاد بكتيرى مكونا جزئى الذى ان ايه اثناء النمو الهوائى واللاهوائى وتوقف البناء البروتينى و بناء عليه :

فأن المقدرة على تكسير الكروموسومات و سرعة قتل الخلايا تحت ظروف تعتمد على المضادات. وتم قياس الجرعات المختلفة ومدى تأثيرها مع تحديد الجرعة قبل المميته للاشعاع ب 4.0 و بمعدل 0.75 . ومن قبل ذلك تم عمل دراسة من وجهة النظر الكيمياء الحيوى على نشاط الانزيم مطبقا على عنصر الكادميوم والحصول على النتائج المؤدية لذلك .