Effect of Growth Conditions on Chlorine Sensitivity of *Escherichia coli*[1]

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Escherichia coli grows luxuriantly on rich media like nutrient broth or nutrient agar; however, minimal media fully satisfy all its nutritional demands as the organism can use glucose and ammonium salts as sole sources of carbon and nitrogen. During studies on the disinfective power of chlorine, we found considerable differences in susceptibility to the disinfectant between cells growing in minimal media and cells from nutrient agar. In this report the nature of the differences is described and the mechanism involved is discussed.

MATERIALS AND METHODS

Media. The following media were used:

(1) Nutrient agar: Peptone (Difco),[2] 10 g; NaCl, 5 g; $Na_2HPO_4 \cdot 12H_2O$, 2.5 g; Bovril,[3] 3 g; Bacto-agar (Difco), 20 g; distilled water, 1000 ml.

(2) Minimal agar (according to Davis and Mingioli in 1950): K_2HPO_4, 7 g; KH_2PO_4, 3 g; Na_3-citrate$\cdot 3H_2O$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $(NH_4)_2SO_4$, 1 g; Bacto-agar (Difco), 20 g; distilled water, 1000 ml. Glucose was sterilized separately and added aseptically to the medium to contain finally 0.2 per cent.

(3) Minimal agar + 1 per cent peptone (Difco).

(4) Minimal agar + 0.3 per cent Bovril (Bovril).

(5) Minimal agar + 2 per cent yeast extract (Difco).

Bacteria. Cultures of *E. coli* strain B/r[4] grown for at least three consecutive passages on the above mentioned media were taken. Bacteria grown on nutrient agar were designated N and those grown on minimal agar as MG. The inoculum consisted of cells obtained from 18- to 24-hr-old cultures. The cells were washed 3 times with bi-distilled water and diluted to give a barely visible suspension in a Coleman Junior spectrophotometer[5] (optical density (OD) 0.02 at 500 mμ). This suspension was further diluted 5-fold in the disinfection mixture. For determination of SH groups, suspensions of an OD of 0.8 were prepared and these were diluted 2-fold in the disinfection mixture.

Chlorine. For the purpose of disinfection, a concentrated NaOCl solution was prepared (approximately 10,000 to 15,000 parts per million (ppm)). The chlorine concentration was determined by iodimetric titration, and the chlorine diluted as desired. All experiments and dilutions were performed in tubes (washed with acid) covered with aluminum caps.

Disinfection. Chlorine was added to the bacteria suspended in liquid minimal medium and incubated at 37 C. The inoculum consisted of approximately 1 to 2×10^7 bacteria (a barely visible suspension diluted 5-fold). After time intervals, aliquots were withdrawn and $Na_2S_2O_3$ (final concentration, 200 ppm) was added to neutralize the excess of chlorine.

Plating. After neutralization with $Na_2S_2O_3$ the mixture was kept at 1 C. Various dilutions were plated on nutrient agar and results read after incubation of the plates for 48 hr at 37 C.

Determination of SH groups. Total protein SH groups were determined by the ferricyanide method described by Barron (1951), with sodium lauryl sulfate as denaturant. Readily available protein sulfhydryl groups, "enzymatic SH," were determined after hydrolysis with dilute HCl, according to Dubnoff *et al.* (1956); 4 ml of a bacterial suspension (OD, 0.4) in minimal liquid medium were incubated for 2 hr with appropriate chlorine concentrations. Thereafter, the excess of chlorine was neutralized by addition of 0.64 mg of $Na_2S_2O_3$, and the cells (collected by centrifugation) were used for the determination of "enzymatic" and total protein SH.

RESULTS

In the following experiment, bacteria grown on minimal media (MG) and others grown on nutrient agar (N) were exposed to chlorine for various time intervals. The number of survivors was determined by plating.

Table 1 shows that bacteria previously grown on nutrient agar (N) are much less affected by chlorine than are cells from minimal agar (MG). With the increase of the rate of disinfection, the survival rate of N cells increased up to 5000 fold, as compared to that of MG cells (tables 1 and 2).

Starvation did not appreciably affect the sensitivity of cells. Although depletion of the nitrogen reserves

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[2] Difco Laboratories, Inc., Detroit, Michigan.

[3] Meat extract manufactured by Bovril, Ltd., London, England.

[4] The strain was obtained in 1950 from Dr. E. Witkin, The Biological Laboratories, Cold Spring Harbor, Long Island, New York.

[5] Coleman Instruments, Inc., Maywood, Illinois.

caused a 3- to 5-fold increase in sensitivity of broth-grown cells, no essential change in resistance towards chlorine was achieved by this procedure (table 3).

To establish which of the constituents of the nutrient agar was responsible for the increased resistance of the N cells, bacteria were grown on minimal media supplemented with various ingredients of nutrient broth, and then exposed to chlorine.

As can be seen (table 4), growth on minimal agar supplemented with peptone or Bovril increased survival of *E. coli* only to a small extent, whereas yeast extract was much more effective. Bacteria grown on optimal concentrations of yeast extract were almost as resistant as those from nutrient agar.

TABLE 1

*Effect of chlorine on bacteria from nutrient or minimal agar**

Source of Bacteria	Disinfection Time	Bacterial Counts		Percentage of Survivors	Relative Sensitivity†
		Chlorine-treated cells	Control (without chlorine)		
	hr				
N	1	2.4×10^6	1.2×10^7	20.00	181
MG	1	1.3×10^4	1.2×10^7	0.11	
N	2	2.5×10^5	1.2×10^7	2.08	2080
MG	2	1.8×10^2	1.2×10^7	0.001	

* Washed *Escherichia coli* cells from nutrient agar (N) and minimal agar (MG) suspended in minimal liquid medium were exposed to 0.2 ppm chlorine. After various time intervals, aliquots were removed and the excess of chlorine neutralized by addition of 200 ppm $Na_2S_2O_3$. Various dilutions were plated on nutrient agar as described in Methods.

† Relative sensitivity is defined as the ratio of survival of N cells to MG cells under standardized conditions (that is, chlorine concentration, exposure time, size of inoculum, and so forth).

TABLE 2

*Sensitivity differences of MG and N cells at different disinfection rates**

Source of Bacteria	Disinfection Time	Chlorine Concentration	Bacterial Counts		Percentage of Survivors	Relative Sensitivity†
			Chlorine-treated cells	Control (without chlorine)		
	hr	*ppm*				
N	2	0.3	2.2×10^5	2.0×10^7	1.10	4400
MG	2	0.3	5.0×10	2.0×10^7	0.00025	
N	2	0.2	1.0×10^6	2.0×10^7	5.00	142.8
MG	2	0.2	7.0×10^3	2.0×10^7	0.035	
N	2	0.1	1.1×10^7	2.0×10^7	55.00	3.6
MG	2	0.1	3.0×10^6	2.0×10^7	15.00	
N	2	0.06	2.5×10^7	2.0×10^7	125.00	1.9
MG	2	0.06	1.3×10^7	2.0×10^7	65.00	

* Washed *Escherichia coli* N and MG cells were exposed to different chlorine concentrations. After 2 hr the excess of chlorine was neutralized by addition of $Na_2S_2O_3$. Various dilutions were plated as described in Methods.

† For explanations see table 1.

To find the factors responsible for the differences in susceptibility to chlorine, some biochemical systems known to be affected by chlorine were sought. Since chlorine is known as an SH poison, exerting its action by oxidizing sulfhydryl enzymes (Knox *et al.*, 1948), it seemed most appropriate to test for the SH content of chlorine treated bacteria. Cells harvested from the different media were exposed to chlorine, and thereafter total protein SH and "enzymatic SH" were determined (table 5).

Untreated cells from both media contain approximately the same amount of SH. Chlorine treatment

TABLE 3

*Effect of starvation on Escherichia coli sensitivity towards chlorine**

Source of Bacteria	Disinfection Time	Chlorine Concentration	Bacterial Counts		Percentage of Survivors
			Chlorine-treated cells	Control (without chlorine)	
	hr	*ppm*			
MG	1	0.3	1.0×10^2	1.8×10^7	0.00055
MG starved	1	0.3	7.0×10	1.8×10^7	0.00038
N	1	0.3	1.0×10^5	1.8×10^7	0.55
N starved	1	0.3	1.8×10^4	1.7×10^7	0.10
MG	2	0.3	2.4×10	1.8×10^7	0.00013
MG starved	2	0.3	1.2×10	1.8×10^7	0.00007
N	2	0.3	5.0×10^3	1.8×10^7	0.027
N starved	2	0.3	1.5×10^3	1.7×10^7	0.0088

* Washed *Escherichia coli* N and MG cells were starved for nitrogen compounds by growing them for 5 hr at 37 C with constant shaking in a minimal medium from which $(NH_4)_2SO_4$ was omitted. After starvation, the cells were resuspended in the complete minimal medium and exposed to 0.3 ppm chlorine. The disinfection rate of starved cells was compared to that of unstarved *E. coli* of both strains. After exposure for 1 and 2 hr, the excess of chlorine was neutralized and survivors plated.

TABLE 4

*Effect of nutrient broth ingredients on the chlorine sensitivity of Escherichia coli**

Growth Medium	Chlorine Concentration	Disinfection Time	Bacterial Counts		Percentage of Survivors
			Chlorine-treated cells	Control (without chlorine)	
	ppm	*hr*			
Nutrient agar.....	0.3	2	1.3×10^6	1.7×10^7	8.0
Minimal agar.....	0.3	2	6.0×10	6.0×10^6	0.001
+ Bacto-peptone, 1 per cent	0.3	2	1.3×10^3	1.8×10^7	0.007
+ Bovril, 0.3 per cent.....	0.3	2	1.5×10^3	1.2×10^7	0.012
+ Yeast extract, 2 per cent...	0.3	2	2.3×10^5	2.3×10^7	1.0

* *E. coli* was grown for at least 3 consecutive passages on the different media. The cells were washed, suspended in liquid minimal medium, and exposed for 2 hr to 0.3 ppm chlorine. After neutralization of the excess of chlorine with $Na_2S_2O_3$, cells were plated.

resulted in a reduction of the SH content. However, MG cells showed a more striking reduction. The higher the chlorine concentration used, the greater were the differences in intact SH groups between the cells.

Discussion

The experiments described above show that by maintaining *E. coli* on nutrient agar, cells were obtained which were more resistant to chlorine than if kept on minimal medium. The differences between the cells were particularly striking when an excess of chlorine was used; under the latter conditions cells from nutrient agar were almost 5000 times more resistant than cells from minimal medium. Differences between the cells were also encountered when SH groups were determined after exposure to chlorine. Application of the disinfectant resulted in a reduction of SH; however, broth-grown cells maintained a higher SH level (after exposure to large amounts of chlorine) than MG cells. When limiting amounts of chlorine were used, the differences in SH content between the cells grown in different media were small, and so were the differences in survival rate. Apparently a threshold chlorine concentration unaffected by previous growth history is required to exhibit antibacterial action.

Growth on nutrient agar seemed to allow the cells to survive in the presence of relatively high concentrations of chlorine. As chlorine tests were performed with carefully washed cells, it was assumed that the increased resistance of N cells was due to substances firmly bound to the cell. (The latter might be absent or present in reduced amounts in cells grown on minimal media.)

These hypothetical cell-bound components may exert their protective action by (a) accelerating repair mechanisms of broth-grown cells; (b) binding chlorine nonspecifically and thereby diminishing its attraction to active sites; (c) inhibiting the penetration of chlorine into the cell.

Broth-grown cells were found to contain larger amounts of nucleic acids than cells from minimal agar (Roberts *et al.*, 1955). These findings were in accord with the first assumption as excess of nucleic acids might be effective in repairing injured enzymatic systems. However, no differences in nucleic acid concentration between the strains were detected. Moreover, the quantitative differences in SH content found immediately after application of chlorine seem to rule out a repair mechanism.

If the increased resistance of broth-grown cells to chlorine was due to nonspecific binding of the disinfectant, we would expect to find increased amounts of chlorine-binding substances in the N cells. It is generally assumed that aromatic amino acids and SH groups of proteins might be the sites to which chlorine is bound in the cell. However, no significant differences in protein (measured as tyrosine) and in SH content in untreated N and MG cells were found. Thus, no direct proof in favor of the second assumption can be given.

Still, another possibility is the occurrence of a substance near the cell surface of broth-grown cells which affects penetration of certain toxic agents (for example, chlorine). In preliminary experiments with radioactive iodine, differences were found in the amount of iodine absorbed by the different strains. MG cells took up more iodine than broth-grown cells. We hope, therefore, that it will be possible to explain the differences between the cells by use of radioactive chlorine. It is intended to pursue the study in this direction.

TABLE 5

*Total and "enzymatic SH" content of cells before and after chlorine treatment**

Chlorine Concentration	Exposure Time	"Enzymatic SH"		Total Protein SH	
		N cells	MG cells	N cells	MG cells
ppm	*hr*	*μg cysteine*			
15.0	2	7.5	2.6	34.0	0
10.0	2	13.6	7.6	244.0	28.0
7.5	2	16.0	7.4	631.2	204.0
5.0	2	16.3	13.3	653.2	600.0
2.5	2	16.6	17.0	672.0	679.2
1.25	2	17.0	17.0	653.2	630.0
Control 1		17.2	17.0	679.2	663.6
Control 2		17.0	17.0	681.0	665.0

* Washed cells from both media (4 ml, OD 0.40) suspended in minimal liquid medium, were incubated at 37 C with different chlorine concentrations. After 2 hr the excess of chlorine was neutralized by addition of 0.64 mg of $Na_2S_2O_3$. Cells were collected by centrifugation. Total protein SH and "Enzymatic SH" were determined. Two controls were used: (1) cells untreated with chlorine; (2) cells to which, at zero time, a thiosulfate-neutralized chlorine solution was added.

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Summary

Escherichia coli maintained in nutrient agar shows an enhanced resistance (up to 5000-fold) towards chlorine in comparison to cells grown on minimal agar. The higher the concentration of chlorine used, the more marked were the differences between the cells.

As a result of chlorine application to cells grown on nutrient agar and minimal agar, differences in "enzymatic" and total protein SH are observed.

Nitrogen starvation did not diminish appreciably the resistance of broth-grown cells.

Possible explanations for the enhanced resistance to chlorine of *E. coli* grown on nutrient agar are discussed.

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Production of Fumaric Acid by *Rhizopus arrhizus*[1]

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The formation of appreciable quantities of fumaric acid by fermentation is largely confined to organisms of the order *Mucorales* and in particular to the genus *Rhizopus*. Production of fumaric acid by these fungi has been reviewed by Foster (1949, 1954), whose papers may be consulted for prior references. Fumaric acid production is not strictly a one-product fermentation. Under limited conditions selected strains of *Rhizopus* produce primarily fumaric acid, but usually ethanol is present together with a substantial proportion of acids other than fumaric. In general, weight yields of fumaric acid have been reported as high as 40 to 50 per cent of the sugar fermented, glucose being the most commonly employed carbohydrate. Optimal conditions for the production of fumaric acid have not previously been clearly defined for either surface or submerged fermentations. According to published data, the level of various components of the fermentation medium such as carbohydrate, potassium, iron, magnesium, zinc, and copper can greatly influence the yield. The purpose of this paper is to describe the optimal conditions for the production of fumaric acid in shaken flask culture.

MATERIALS AND METHODS

The compositions of the media employed in this work are summarized in table 1. The two media (C and D) are optimum for the laboratory-scale production of fumaric acid by the two strains of molds used.

The organisms were cultured on slants of medium A for 5 to 7 days at 33 C to obtain a massive crop of spores. The spores were washed from slants of medium A with 30 ml of a dilute detergent solution into 90 ml of medium B contained in a 300-ml indented Erlenmeyer flask. The spores were germinated in medium B by incubation for 18 hr at 33 C on a rotary shaker. Three- to five-ml quantities of medium B containing germinated spores were used as inocula for each 100 ml of the fermentation medium. The quantity of germinated

TABLE 1

Summary of media used for fumaric acid production by Rhizopus species

Ingredient (g/1000 ml final vol)	Inoculum		Fermentation	
	A Sporulation	B Germination	C Glucose	D Molasses
Commercial glucose*	4.0	15.0	Varied	—
High-test molasses	—	10.0	—	Varied
Crude lactose	6.0	—	—	—
Glycerol	10.0 ml	—	—	—
Urea	0.6	1.0	1.0†	0.9†
Peptone	1.6	—	—	—
Corn steep liquor	1.0 ml	3.0 ml	0.5 ml	—
KH_2PO_4	0.4	0.3	0.3	0.1
$MgSO_4 \cdot 7H_2O$	0.3	0.25	0.4	0.15
$ZnSO_4 \cdot 7H_2O$	0.088	0.066	0.044	0.044
Ferric tartrate	—	0.01	0.01	—
$FeSO_4 \cdot 7H_2O$	0.25	—	—	—
$CuSO_4$	0.005	—	—	—
$MnSO_4 \cdot 4H_2O$	0.05	—	—	—
KCl	0.4	—	—	—
NaCl	40.0	—	—	—
Agar	30.0	1.0	—	—
Corn starch‡	—	30.0	—	—
Methanol†	—	—	1.5%	1.5%
$CaCO_3$	—	—	Varied	Varied

* Corresponds closely to glucose monohydrate.

† Sterilized separately and added aseptically.

‡ Thick boiling corn starch made up separately and added to basal salts medium before autoclaving.

[1] Presented at the 58th General Meeting of the Society of American Bacteriologists, Chicago, Illinois, April 27 to May 1, 1958.

[2] Agricultural Research Service, U. S. Department of Agriculture.