MECHANISM OF CHLORAMPHENICOL RESISTANCE IN *E. COLI*
III. THE TOTAL AMINO-ACID COMPOSITION OF CHLORAMPHENICOL
RESISTANT *E. COLI* AND ELECTROPHORETICAL
PATTERN OF ITS β -GALACTOSIDASE

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In a series of investigations with CM*-resistant strains of *E. coli*, we have considered the mechanism of resistance as relevant to the following three possibilities: (I) alteration of metabolism, (II) inactivation of CM, and (III) changes in the permeability. Investigations were made on the possibilities (II) and (III) in the preceding papers (Okamoto, 1959a; Hirokawa *et al.*, 1959). The present report is concerned with the possibility (I).

The primary action of CM on bacterial cells is known to be an inhibition of protein biosynthesis (Wisseman *et al.*, 1954). Obviously, however, CMR strain can synthesize protein and produce induced enzyme in the presence of CM. If CMR cells escape from the action of CM by altering its metabolism according to the possibility (I), it might be expected that the protein(s) synthesized by CMR cells differ(s) from that synthesized by sensitive cells. In an attempt to elucidate this possibility we examined and compared the following two properties of bacterial proteins; (1) the electrophoretical pattern of the induced enzyme β -galactosidase formed by sensitive and CMR strains, (2) the total amino acid composition of the bacterial cells of both strains. Contrary to the above expectation, no significant difference was observed in these properties between the sensitive and resistant strains.

MATERIALS AND METHODS

Strain: *E. coli* BO54 and its CM-resistant strain BO54 CMR were used for the determination of amino acid composition. *E. coli* B and its CM-resistant strain B CMR were used for the examination of the electrophoretical pattern of β -galactosidase (Okamoto, 1959b). Other conditions were described in the preceding reports (Okamoto, 1959a, b).

Chemicals: CM was supplied by Sankyo Co. The starch used in the zone-electrophoresis was a Wako Chemicals Co. product.

Culture medium: (1) The culture medium of BO54 strains for amino acid determination was the medium 1, which was described in the first report (Okamoto, 1959a). (2) The culture medium for the β -galactosidase formation by B strains was as follows:

KH ₂ PO ₄	13.6 g
Casamino acid (Difco, Technical)	5.0 g

* Abbreviations: chloramphenicol (CM); CM-resistant (CMR); 2, 4-Dinitrophenyl- (DNP); o-Nitrophenyl- β -D-galactoside (ONPG); Optical density (OD).

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MgSO ₄ • 7H ₂ O	0.2 g
CaCl ₂	0.01 g
FeSO ₄ • 7H ₂ O	0.0005 g
Lactose	5.0 g

dissolved in 1000 cc of demineralized water and adjusted to pH 7.4. Lactose was added as an inducer as well as an energy source.

Incubation: (1) Culture for amino acid analysis; the BO54 strains were grown in the medium above described at 37°C for 18–24 hours, then 1 cc of this culture was transferred to 400 cc of the same medium distributed in a Roux flask and incubated for 36 hours; the total volume of medium used was 1.2 liter for the sensitive strain and 1.6 liter for the CMR strain. The cells grown were harvested by centrifugation, washed twice with saline and then three times with acetone and finally dried. The yields were 340 mg of sensitive and 240 mg of resistant cells. (2) Culture for β -galactosidase formation; the B strains were grown in the medium shown above by the rocked culture method (Aoyagi and Mizuno, 1959) at 37°C for 6 hours. One cc of this culture was transferred to 75 cc of the same medium and incubated at 37°C with rocking for 12 hours. The reverse T type tubes used in this experiment were 1.8 cm in diameter. CM was added to the final concentration of 100 μ g per cc where used.

Hydrolysis of bacterial cells: Six N HCl was added in the proportion of 1 cc per mg of the dried cells. The suspension was hydrolyzed by heating in a sealed glass-tube at 120°C for 16 hours. The hydrolysate was filtered, HCl was removed *in vacuo*, and pH was adjusted to neutral. Similar results were obtained, when 3N HCl was used for the hydrolysis.

The determination of amino acid composition: According to the quantitative paper chromatography of DNP-amino acids (Levy, 1954; Koch and Weidel, 1956), the ether extractable fraction of DNP-amino acids (hence all amino acids except for arginine, histidine) was analyzed. The following two modifications were made apart from the original method. (1) By the original method, the relative molar ratio of amino acids could be estimated fairly accurately, but, actually, the determination of the absolute value of each amino acid content was somewhat difficult. But, since the acid hydrolysate contains no tryptophan which is completely destroyed during hydrolysis, a known amount of tryptophan added to the hydrolyzed sample can be the absolute control of whole amino acids. Thus, the content of each amino acid could be calculated on the basis of the molar ratio to the amount of recovered tryptophan. Usually 0.4–0.8 μ M of tryptophan per 100 μ g N of sample was chosen. As the factor which should be multiplied for the conversion of OD at 360 m μ to the molar ratio, 1.12 was adopted for DNP-tryptophan on the basis of experiment with standard amino acid solution. The spot of DNP-tryptophan in this paper chromatogram was developed as shown in Fig. 1, and it coincided with the spot of di-DNP-histidine (Levy, 1954). But the amount of di-DNP-histidine was negligible. (2) The spots of DNP-aspartic acid and DNP-glutamic acid were not easily separated by the original method. This difficulty was avoided by the following procedure. After 2-dimensional development was ended, a strip of paper containing the spot of aspartic acid plus glutamic acid was cut off from the chromatogram (Fig. 1) and the spot was redeveloped by acid butanol (5% acetic acid: n-butanol, 1:1) along this strip to the reverse direction; then the two spots were easily separated, DNP-glutamic acid moving faster than DNP-aspartic acid. The filter paper used was Tokyo Filter Paper No. 53.

Total N estimation: The total N of hydrolysate was estimated by micro-Kjeldahl method.

The preparation of β -galactosidase: The cells grown and induced to form β -galactosidase as described above were harvested by centrifugation, washed twice by neutral phosphate buffer, and suspended in 1/45 M phosphate buffer (pH 7.7). This suspension was treated with sonic oscillation (10 KC); disrupted cells were centrifuged down at 10000 rpm for 45 minutes and cell debris was discarded. The supernatant was used as β -galactosidase preparation.

Estimation of β -galactosidase activity: β -galactosidase was estimated by the method of Lederberg (Lederberg, 1950) using ONPG. The unit was expressed by an arbitrary scale.

Electrophoresis of β -galactosidase preparation: Electrophoresis was performed in 1/45 M phosphate buffer, pH 7.7, using starch as the supporter, with an electric current of 1.5 mA/cm², at 10°C for 15 hours. The size of the trough was 1.3 × 3.8 × 40 cm; the sample was placed at a distance from 9 to 10 cm from the negative pole. After the electrophoresis was ended, the starch supporter was cut in pieces of 1 cm width and each piece of starch was eluted with the same volume of phosphate buffer. The eluate was examined as to its protein content by the extinction at 280 m μ with Beckmann spectrophotometer Model U and its β -galactosidase activity

was determined as described above. The recovery of β -galactosidase activity was almost as high as 90 % of the original sample. When tris-buffer was used in place of the phosphate buffer in the electrophoresis, β -galactosidase activity could never be eluted from the starch. Moreover, when the enzyme solution in tris-buffer was mixed with starch and let to stand even without electric charge, β -galactosidase activity diminished gradually with time, whereas protein, *i.e.* the absorption at 280 m μ , could be recovered completely. The cause of this phenomenon remained obscure.

RESULTS

Amino Acid Composition of Sensitive and CMR Strains

As shown in Table 1, there was a remarkable similarity of amino acid composition between the sensitive and CMR strains. Since, as reported previously, the CMR strain was fairly unstable, it was conceivable that under the condition of this experiment the

Table 1. The amino acid composition of *E. coli* BO54 and BO54 CMR

	BO54		CO54 CMR		BO54 CMR (20 μ g/cc CM present)
	Molar ratio to alanine	Amino acid Ng per 16 g of total N	Molar ratio to alanine	Amino acid Ng per 16 g of total N	Molar ratio to alanine
Glutamic acid	0.92	1.11	0.99	1.19	} 2.24
Aspartic acid	1.00	1.22	1.02	1.25	
Alanine	1.00	1.22	1.00	1.23	1.00
Glycine	0.80	0.97	0.89	1.09	0.74
Serine	0.32	0.41	0.28	0.31	0.42
Threonine-Proline	0.70	0.85	0.72	0.87	0.76
Valine	0.73	0.90	0.78	0.93	} 2.14
Leucine-Isoleucine	1.18	1.43	1.26	1.51	
Phenylalanine	0.33	0.41	0.33	0.41	0.38
Tyrosine	0.19	0.25	0.22	0.26	} 0.52
Lysine	0.47	1.14	0.48	1.18	
Cystine	0.03	0.08	0.05	0.12	0.02
Methionine	trace	—	trace		trace

Cells were hydrolyzed with 3N or 6N HCl at 120°C for 16 hours.

Details were given in the text.

culture of CMR cells involved sensitive cells in a considerable proportion. Accordingly, CMR cells grown in the presence of 20 μ g per cc of CM was analyzed by the same procedure. It was shown again that there was no marked difference from the sensitive strain (Table 1).

In the chromatogram of DNP-hydrolysate from CMR strain, a very faint, not yet identified spot was observed near the spot of DNP-cystine (Fig. 1). The significance of this spot is as yet unknown.

The Electrophoretic Pattern of β -galactosidase

As was shown in Fig. 2a, β -galactosidase activity of *E. coli* B, parent strain, had

Fig. 1. Modifications of the paper chromatography of DNP amino acids.

a relatively narrow pattern which showed a sharp peak on the positive side of the starting point. In order to compare the pattern of β -galactosidase of CMR strain with that of wild strain, the preparation from CMR cells was mixed with that from the parent strain in equal amounts, and the electrophoretic pattern of this mixture was observed. As shown in Fig. 2b, an exactly unseparable peak was observed. Similarly, β -galactosidase preparation from CMR cells grown in the presence of $100 \mu\text{g}$ per cc of CM was mixed with that from the parent strain. The electrophoretic pattern of this mixture indicated again the same single pattern as above (Fig. 2c). The pattern of protein may have been variable depending on the experiment, since sometimes a precipitate formed during the storage was discarded.

DISCUSSION

Davis listed up seven possible mechanisms of drug resistance (Davis and Maas, 1952). Among them, the possibilities of inactivation of drug and changes in permeability were discussed in the preceding papers (Okamoto, 1959a; Hirokawa *et al.*, 1959). In this report the remaining five possible mechanisms included in the possibility (I) mentioned under Introduction will be discussed; they are as follows: (1) Alternative metabolic pathway bypassing the inhibited reaction, (2) increased concentration of metabolites that antagonize the inhibitor, (3) increased concentration of enzyme that the drug inhibits, (4) decreased requirement for product of the inhibited metabolic system, (5) formation of an altered enzyme with decreased affinity for the inhibitor, *etc.*

The primary effect of CM on bacterial cells is a specific inhibition of protein biosynthesis. Though much remains still unknown as to the mechanism of protein biosynthesis, it is hardly conceivable that in resistant strains protein synthesis would be performed by some pathway quite different from that of sensitive cells. Especially, the

Fig. 2. The zone electrophoretical pattern of β -galactosidase isolated from *E. coli* B.

2a β -galactosidase isolated from B strain.

2b β -galactosidase mixture isolated from B and [B CMR strain in the ratio 1 : 1.

2c β -galactosidase mixture isolated from B and B CMR (in the presence of CM) strain in the ratio 1 : 1.

Details were given in the text.

possibility (1) above mentioned seems unlikely to be verified. But if such a process could occur at all, it might be probable that protein thus synthesized, especially an induced protein, is different in some way from normal protein. If the mechanism of (2)-(4) listed above is important factors responsible for the resistance of the present CMR strain which was 100 times as resistant as their parent strain, there would be some marked variation of the amino acid composition of bacterial protein, the terminal product of metabolism.

We have carried out the above experiments in view of those presumptions, both for the electrophoretic pattern of the specific protein induced and for the amino acid composition of total bacterial protein. From these data it can be concluded that, at least the high grade resistance of the CMR strains used cannot be explained only by changes of metabolism such as listed above, though it cannot be said that no such changes take place in resistant strains at all. The remaining possible mechanism is that in CMR strains CM could not reach the site of its action and the protein synthesis was performed without the touch of CM. For example, if alterations in permeability as discussed in the preceding papers or the possibility (5) mentioned above prevented CM from its site of action, the findings of this experiment would be easily explained, and this was concordant with the conclusion of the preceding papers.

However, another possibility cannot be denied that in low grade resistance adaptive changes of metabolism may play some role (*e.g.* Merkel and Steers, 1953).

SUMMARY

The amino acid composition of the total bacterial protein of *E. coli* BO54 and of its chloramphenicol resistant strain BO54 CMR was examined by quantitative paper chromatography of DNP-amino acid. There was a marked similarity of amino acid composition between two strains.

E. coli B and its chloramphenicol resistant strain B CMR were induced to form β -galactosidase in the presence of lactose as an inducer. The zone electrophoretic pattern of the β -galactosidase was examined. It was shown that β -galactosidase of sensitive, of chloramphenicol resistant, and of resistant cells grown in the presence of chloramphenicol, showed the same mobility, and peaks of their electrophoretic patterns coincided strikingly with each other.

From these findings it was implied that changes in protein metabolism cannot be an important factor responsible for the high grade resistance of those resistant strains.

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