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Occurrence of Chloramphenicol-acetylating Enzymes in Various Gram-negative Bacilli

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The occurrence of a chloramphenicol-acetylating enzyme, similar to that found in *Escherichia coli*, carrying an R factor was investigated in various gram-negative bacilli. The acetylated products of chloramphenicol were identified by chromatography and quantitatively assayed after benzene extraction. The investigated strains were of the *Salmonella-Arizona* group, the *Klebsiella-Aerobacter* group, *Serratia marcescens*, the *Proteus* group, and *Pseudomonas aeruginosa*, most of which were isolated from 1947 to 1957. Both chloramphenicol-sensitive and -resistant strains were included, but none of them was able to transfer chloramphenicol resistance by conjugation. In the *Proteus* group, a significant level of a chloramphenicol-acetylating enzyme was found in most strains, whether they were sensitive or resistant to chloramphenicol; the resistant strains showed higher levels of the enzyme. Some chloramphenicol-sensitive strains lacked this enzyme. Only the sensitive strains containing the enzyme could easily produce chloramphenicol-resistant mutants with higher enzyme activity. Thus, the chloramphenicol resistance of this group can be reasonably explained on the basis of the chloramphenicol-acetylating enzyme. All of the *Pseudomonas aeruginosa* strains were resistant to chloramphenicol, and most strains showed low levels of the enzyme (which, however, did not appear sufficient to explain their resistance). All of the strains of the other groups (except one strain of *Enterobacter cloacae*) lacked the enzyme, although most strains of the *Klebsiella-Aerobacter* group and of *S. marcescens* were resistant to chloramphenicol. With respect to the origin of the resistance gene of the R factor, it is noteworthy that the strains of *Proteus mirabilis* isolated in 1947 possessed this enzyme before the discovery of chloramphenicol.

In a previous paper (6), we reported that the multiply drug-resistant strains of *Escherichia coli* carrying an R factor produce enzymes which inactivate chloramphenicol, dihydrostreptomycin, and kanamycin. The mode of inactivation of chloramphenicol was shown to be by acetylation of hydroxyl groups (9; and Suzuki and Okamoto, J. Biol. Chem., *in press*). In relation to the origin of the R factor and the role of these enzymes in the mechanisms of drug resistance in other resistant bacteria, it seemed of interest to study the distribution of these enzymes in various organisms. The ease of the detection of the chloramphenicol-acetylating enzyme led us to study the occurrence of this enzyme in other bacterial species. We previously reported a similar chloramphenicol-inactivating enzyme in chloramphenicol-resistant strains of *Staphylococcus aureus* from clinical sources (10). Miyamura and Oketani reported inactivation of chloramphenicol by cell suspensions of various gram-negative bacilli and *Staphylococcus aureus* (3). In the present work, we studied the occurrence of chloramphenicol-acetylating enzymes in several stock strains of enteric bacilli and *Pseudomonas aeruginosa*, most of which were isolated between 1947 and 1957.

MATERIALS AND METHODS

Strains. E. coli K-12 CS2, its derivative carrying an R factor determining resistance to five drugs, K-12 R5, and a strain of *Proteus mirabilis* with an R factor, F67 (NR1), were described previously (6, 7). Other strains used are listed in Table 1, which also describes the year of their isolation, their sensitivity to chloramphenicol, and the occurrence of the chloramphenicol-acetylating enzyme. The strains investigated were: 2 strains of *Salmonella*, 2 of *Paracolobactrum arizonae*, 2 of *E. freundii*, 8 of the *Klebsiella-Aerobacter* group, 2 of *Serratia marcescens*, 15 of the *Proteus* group, and 5 of *Pseudomonas aeruginosa*. Although the choice of the strains for this study was in favor of chloramphenicol-resistant strains, the above-mentioned strains included chloramphenicol-sensitive strains, chlor-

amphenicol-resistant strains isolated from them by single-step selection, and chloramphenicol-resistant strains isolated as such from natural sources (Table 1). Most of the strains were isolated before 1957, and some strains of *Proteus mirabilis* were isolated in 1947, just before the discovery of chloramphenicol. None of the strains showed transferability of chloramphenicol resistance in conventional mating experiments (7).

Determination of sensitivity to chloramphenicol. A strain was considered resistant to chloramphenicol if it could grow on Penassay agar (Difco) plates containing 50 μg of chloramphenicol per ml (Table 1). For a more accurate determination of chloramphenicol sensitivity, 5 ml of peptone-glucose medium (5) containing various concentrations of chloramphenicol was inoculated with about 5×10^7 cells of an overnight culture and incubated at 37 C with shaking for about 3 hr. The growth in each tube was determined by measuring the turbidity, and the concentration of the drug that effected a 50% inhibition of growth (ID_{50}) was determined.

Preparation of cell-free extracts. Cells were grown overnight as thick cultures on several Penassay agar plates (containing 10 μg of chloramphenicol per ml in the case of chloramphenicol-resistant strains), and were harvested by scraping. The cells were washed twice with tris(hydroxymethyl)aminomethane (Tris) chloride buffer (*p*H 7.8) containing 0.01 M magnesium acetate, 0.06 M KCl, and 0.006 M 2-mercaptoethanol; the cells were then suspended in an equal volume of the same buffer and disrupted by passage through a French pressure cell at 420 kg/cm². Deoxyribonuclease (5 μg/ml) was added to the disrupted-cell suspension, which was centrifuged at 10,000 × *g* for 20 min, and the supernatant fluid was used as the crude extract.

The 100,000 × *g* supernatant fraction (S100) of *E. coli* K-12 CS2, which had no chloramphenicol-acetylating activity, served as a source of the acetyl coenzyme A-synthesizing system, and was prepared as described previously (6).

Chemicals. Chloramphenicol was a gift from Yamanouchi Pharmaceutical Co. ^{14}C-labeled chloramphenicol, 18.0 mc/mmole, and sodium acetate-2-^{14}C were purchased from The Radiochemical Centre, Amersham, England. Adenosine-5′-triphosphate (ATP) and coenzyme A (CoA) were products of the Sigma Chemical Co., St. Louis, Mo.

Detection and assay of chloramphenicol-acetylating activity with the cell-free preparation. The reaction mixture contained: 0.1 M Tris chloride buffer (*p*H 7.8), 0.01 M magnesium acetate, 0.06 M KCl, 5 μmoles of ATP, 0.04 μmole of CoA, 0.5 mg (protein) of the S100 fraction of *E. coli* K-12 CS2 (included to insure a sufficient supply of the acetyl CoA-synthesizing system), ^{14}C-labeled chloramphenicol (about 3×10^4 counts/min) plus an appropriate amount of nonlabeled chloramphenicol (10 to 200 μg), and the crude extract of the strain to be tested (containing about 1 mg of protein), in a total volume of 0.5 ml. The reaction mixture was incubated at 37 C. At intervals (5 to 60 min), an 0.1-ml sample was pipetted into 0.4 ml of water at 95 C, incubated at this temperature for 5 min, and then cooled. This solution was extracted three times with 0.5-ml volumes of benzene. The ^{14}C counts extracted with benzene were measured with a windowless gas-flow counter. Experiments with untreated ^{14}C-chloramphenicol or monoacetylated ^{14}C-chloramphenicol (the product of the *E. coli* R5 inactivating reaction, which was chromatographically identified) showed that about 90% of the monoacetylated derivative of chloramphenicol and about 10% of the unchanged chloramphenicol were extracted by the benzene. Since, at the early stage of the reaction, the product is almost exclusively the monoacetylated derivative of chloramphenicol, and the diacetylated derivative is negligible, the net counts of acetylated chloramphenicol (X) could be roughly estimated by the formula $X = (10B - T)/8$, where B is the benzene-extracted counts and T is the total counts of input chloramphenicol in an 0.1-ml sample (which could be estimated by extracting with two 0.75-ml volumes of ethyl acetate). The percentage of acetylated chloramphenicol versus the total chloramphenicol originally present was given by $(X/T) \times 100$, and hence the amount of chloramphenicol acetylated could be estimated. The enzyme activity was expressed by the amount of chloramphenicol acetylated per minute per milligram of enzyme protein.

Assay of chloramphenicol-acetylating activity with growing cells. Cells were grown in 1 ml of peptone-glucose medium at 37 C with shaking to about 2×10^8 cells per ml, and then ^{14}C-chloramphenicol (about 3×10^4 counts/min, 0.14 μg) with nonlabeled chloramphenicol (0 to 20 μg) was added. Growth was continued and at intervals (from one to several hours) 0.1-ml samples were pipetted into 0.4 ml of water at room temperature, and the mixture was immediately extracted with benzene as described above. Heating of the mixture was omitted, since this hindered quantitative extraction. This method gave information about the actual process in growing cells which involved the permeation process of chloramphenicol and the supply of acetyl CoA. The amount of acetylated chloramphenicol was determined from the formula presented above. Within the range of chloramphenicol used (0.14 to 20 μg), the percentage of chloramphenicol acetylated by a given strain per given time interval remained constant, the rate of acetylation increasing with the concentration of chloramphenicol in the medium. Apparently the chloramphenicol-acetylating capacity of the cells was not saturated at the concentration of chloramphenicol used. Therefore, this method could be used to compare acetylating activity in different strains only when the concentration of chloramphenicol added was kept constant.

Thin-layer and paper chromatography of the reaction products. The reaction mixture or the culture medium which contained about 10,000 counts/min of ^{14}C-chloramphenicol was extracted twice with two volumes of ethyl acetate. The extract was concentrated by evaporation and spotted on a sheet of silica gel (Eastman Kodak Co.) or a strip of filter paper (Toyo Roshi, no. 51A, 2 by 40 cm). The thin-layer chromatogram was developed for 20 min with benzene-methanol-water (60:35:5; lower phase), and the paper chromatogram was developed for 2 hr with benzene-methanol-water (98:2:2; upper phase); both were

scanned by a windowless gas-flow chromatogram-scanner. The R_F values of chloramphenicol, its 3-*O*-monoacetyl and 1,3-*O*,*O*-diacetyl derivatives were about 0.2, 0.3, and 0.6 in the former system and 0.1, 0.7, and 0.9 in the latter system, respectively.

Bioassay of the paper chromatogram for antibiotic activity. The paper strip was placed on an agar plate which had been overlayered with 4 ml of soft agar seeded with 4×10^8 cells of chloramphenicol-sensitive *E. coli*, and the strip was held in this position for 10 min at room temperature. After removal of the strip, the plate was incubated overnight at 37 C and the antibiotic activity of the radioactive spots of the chromatogram was assessed by determining the inhibition of the indicator bacteria.

Test for the incorporation of acetate into chloramphenicol derivatives. The reaction mixture was almost the same as that used for the assay of chloramphenicol acetylation, except that the mixture contained ^{14}C-labeled acetate (about 5×10^5 counts/min) and 200 μg (about 0.6 μmole) of nonlabeled chloramphenicol and the total acetate was reduced to 3 μmoles per ml. The reaction mixture was incubated at 37 C for 30 min, heated at 95 C for 5 min to stop the reaction, and twice extracted with two volumes of ethyl acetate. The radioactivity incorporated into the ethyl acetate-extracted fraction was measured; a portion of the extract, containing about 10,000 counts/min, was subjected to thin-layer chromatography, and the chromatogram was scanned.

RESULTS

General procedure for examination of the chloramphenicol-acetylating enzymes. Chloramphenicol-acetylating activity was usually determined by the benzene extraction method with cell-free preparations. In most cases, paper or thin-layer chromatography of the reaction mixture was also performed to confirm the results. When the benzene extraction method gave positive results, the chromatograms showed two radioactive peaks (in addition to the chloramphenicol peak), which corresponded to 3-*O*-acetyl and 1,3-*O*,*O*-diacetyl derivatives of chloramphenicol (Fig. 1), the latter being absent in weakly positive cases. The pattern was essentially the same as that observed in *E. coli* with R factor (Suzuki and Okamoto, J. Biol. Chem., *in press*). Bioassay of the paper chromatogram confirmed that these acetylated derivatives were antibiotically inactive. The reactions were shown to depend on the presence of ATP and CoA. (Acetate was always present.) In the experiment on acetate incorporation with the *P. mirabilis* F 67/CM preparation, it was shown that ^{14}C-labeled acetate was significantly incorporated into the ethyl acetate-extracted fraction only in the presence of nonlabeled chloramphenicol; in the thin-layer chromatogram of the extract, the radioactive peak coincided with 3-*O*-acetyl-chloramphenicol.

From all of these results, it can be concluded

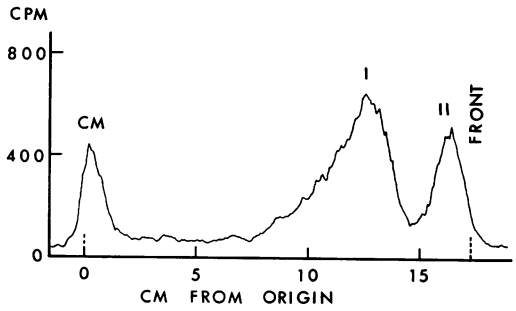

FIG. 1. *Paper chromatography of the reaction products formed by the cell-free preparation of Proteus mirabilis NP 4. The reaction mixture contained 100 μg of chloramphenicol. Incubation was for 30 min at 37 C. CM, chloramphenicol; I, monoacetyl derivative of chloramphenicol; II, diacetyl derivative of chloramphenicol.*

that the enzymatic activity measured by the benzene extraction method was similar to that of the chloramphenicol-acetylating enzymes found in *E. coli* carrying an R factor.

Usually, acetylating activity was also examined with growing cells, and the results were identical to those obtained with cell-free preparations (Fig. 2 and 3).

Occurrence of the chloramphenicol-acetylating enzyme in gram-negative bacilli. The occurrence of the chloramphenicol-acetylating enzyme among the tested strains, and rough estimates of the specific activities of the crude extracts, are presented in Table 1. Other than the strains of *Proteus* and *Pseudomonas aeruginosa*, only one strain (*Aerobacter cloacae* 858-53/CM) was found to have chloramphenicol-acetylating activity, even though most strains of the *Klebsiella-Aerobacter* group and *Serratia marcescens* were resistant to chloramphenicol. On the other hand, most strains of *Proteus* (except *P. vulgaris*), including those sensitive to chloramphenicol, showed significant activities of the enzyme. Also, most strains of *P. aerugionsa*, all of which were resistant to chloramphenicol, had very weak but definitely positive chloramphenicol-acetylating activity (Fig. 3).

Chloramphenicol-acetylating enzyme and chloramphenicol resistance in the Proteus group and P. aeruginosa. Chloramphenicol-acetylating activity was either negative or weak in strains sensitive to chloramphenicol and was strongly positive in chloramphenicol-resistant strains of *Proteus* (except for *P. inconstans* 47). A more detailed study was carried out with *P. mirabilis* strains; among three chloramphenicol-sensitive strains, two (F 67 and NP 4) possessed weak acetylating activity, whereas the other one (NP 3) did not. The former two differed from NP 3 in their behavior to chloramphenicol in that they produced resistant

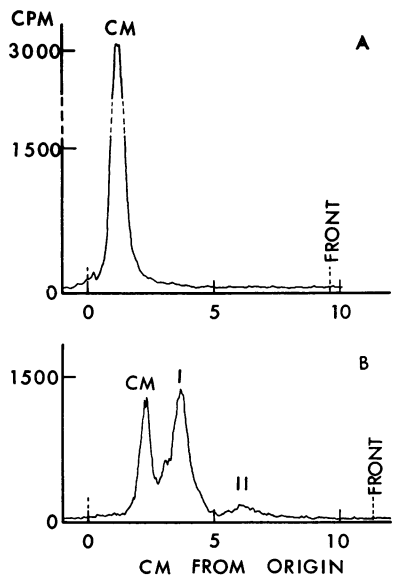

FIG. 2. *Thin-layer chromatography of the reaction products of growing cells of Proteus mirabilis strains. (A) P. mirabilis NP 3 (lacking the chloramphenicol-acetylating enzyme) was grown in the presence of ^{14}C-chloramphenicol (0.14 μg per ml) for 4 hr. (B) P. mirabilis F 67/CM was grown in the presence of ^{14}C-chloramphenicol (3 μg per ml) for 4 hr. All chloramphenicol was converted to acetylated derivatives. The reference ^{14}C-chloramphenicol was added to the ethyl acetate extract just before chromatography. CM, chloramphenicol; I, monoacetyl derivative of chloramphenicol; II, diacetyl derivative of chloramphenicol.*

colonies on an agar plate containing 50 μg of chloramphenicol per ml at a frequency of about 2.5×10^{-5}; under the same conditions, NP 3 never produced such single-step resistant colonies. Chloramphenicol-resistant strains obtained in this way from F 67 and NP 4 (F 67/CM and NP 4/CM) showed about 10 times higher chloramphenicol-acetylating activity than did their sensitive parental strains.

The cell-free preparations used in the above study were prepared from cells grown overnight on Penassay agar plates containing 10 μg of chloramphenicol per ml. To examine the effect of chloramphenicol in the medium on the level of the enzyme, cells of F 67/CM, which were maintained on Penassay agar plates containing 50 μg of chloramphenicol per ml, were transferred to Penassay Broth (Difco) without chloramphenicol and grown overnight. The cells were inoculated in Penassay Broth, with or without chloramphenicol (10 μg/ml), and grown to the late logarithmic phase (about 5 hr) with shaking. Cell-free preparations were prepared from these two cultures, and the chloramphenicol-acetylating enzyme activities were compared. Both preparations showed similar levels of the enzyme (acetylation of about 20 μg of chloramphenicol per min per mg of protein of the crude extract). Thus, the level of chloramphenicol-acetylating enzyme in *Proteus* F 67/CM seems to be independent of prior growth with chloramphenicol.

As reported earlier, *P. mirabilis* can receive R factor from *E. coli*, and such a derivative of F 67 carrying an R factor [F 67 (NR 1)] was used in a previous study (7). F 67 (NR 1) showed a much higher level of chloramphenicol resistance and a correspondingly higher level of chloramphenicol-acetylating activity than did *P. mirabilis* strains without R factor. The quantitative data on the levels of chloramphenicol sensitivity and the activity of the chloramphenicol-acetylating enzyme of *P. mirabilis* and other strains are shown in Table 2.

In most strains of *P. aeruginosa*, chloramphenicol-acetylating activity was very weak but definitely positive (Tables 1 and 2). However, even in the most active strain in this respect (Ps 2), which showed approximately the same level of chloramphenicol resistance as did *P. mirabilis* F 67/CM, the enzyme activity of the extract was

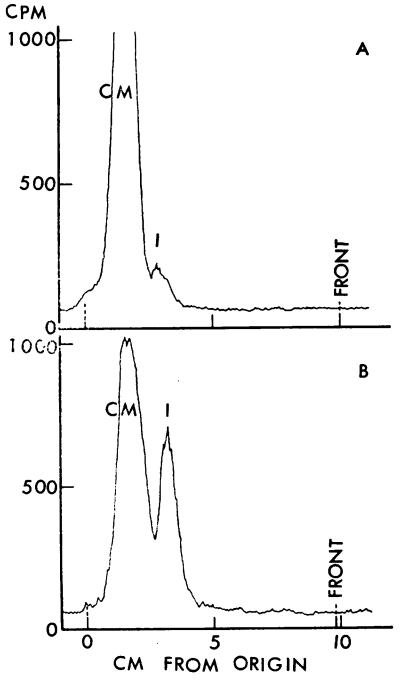

FIG. 3. *Thin-layer chromatography of the reaction products of growing cells of Pseudomonas aeruginosa. P. aeruginosa was grown overnight at 37 C in medium with ^{14}C-chloramphenicol (5 μg per ml). (A) P. aeruginosa Ps 1; (B) P. aeruginosa Ps 2.*

TABLE 1. *Chloramphenicol-acetylating activity of the strains investigated*

Strain	Sensitivity to chloramphenicol[a]	Date of isolation	Reference	Chloramphenicol-acetylating activity[b]
Salmonella typhimurium 1406	s	1934		−
S. gallinarum 416	s	1934		−
Paracolobactrum arizonae D. C. 5	s	1941		−
P. arizonae 3592-56	s	1947		−
Escherichia freundii 5218-51	s	1957		−
E. freundii SR 724	s	1957		−
Klebsiella 3	r*	1952		−
Klebsiella 25	r	1952		−
Klebsiella 42	r	1952		−
Aerobacter aerogenes 90	s	1955		−
A. aerogenes 214	s	1955		−
A. aerogenes 214/CM	r*	1955		−
A. cloacae 868-53/CM	r*	1953	8	+
A. cloacae 12	s	1953		−
Serratia marcescens Nsm 26/CTS	r*	1957		−
S. marcescens Nsm 1-2/CM	r*	1961		−
Proteus morganii 1092	s	1956	SS[c]	+
P. morganii 1096	s	1956	SS[c]	+
P. morganii 1093/CM	r	1956	SS[c]	++
P. morganii 1095/CM	r	1956	SS[c]	++
P. rettgeri 15	s	1957	4	+
P. rettgeri 16	r	1957	4	++
P. vulgaris NP 2 (F 280)	s	1947	2	−
P. vulgaris NP 5	s	Unknown		−
P. mirabilis F 67	s	1947	2	+
P. mirabilis F 67/CM	r*	1947	2	++
P. mirabilis NP 3 (U 510)	s	1947	2	−
P. mirabilis NP 4	s	Unknown		+
P. mirabilis NP 4/CM	r*	Unknown		++
P. inconstans 47	r	1954	1	+
P. inconstans 61	r	1954	1	++
Pseudomonas aeruginosa Ps 1	r	Unknown		±
P. aeruginosa Ps 2	r	Unknown		+
P. aeruginosa Ps 8	r	Before 1950	H[d]	+
P. aeruginosa Ps 9	r	Before 1950	H[d]	−
P. aeruginosa Ps 10	r	Before 1950	H[d]	±

[a] Symbols: s = sensitive; r = a resistant strain, isolated from a natural source, which could grow on a Penassay agar plate containing 50 μg of chloramphenicol per ml; r* = a resistant mutant obtained by single-step selection on a Penassay agar plate containing 50 μg of chloramphenicol per ml.

[b] Symbols: − = absent; ± = barely detectable; + = acetylation of 0.5 to 2 μg of chloramphenicol per min per mg of protein of the crude extract; ++ = acetylation of 10 to 90 μg of chloramphenicol per min per mg of protein of the crude extract.

[c] I. Sasagawa and K. Sato, 31st Meet. Japan. Soc. Bacteriol., 1958.

[d] Y. Homma, *personal communication.*

even lower than that of the sensitive *P. mirabilis* F 67; this low level of activity was also observed with growing cells (Fig. 3). By growing Ps 2 in peptone-glucose broth containing chloramphenicol, a variant with a higher level of chloramphenicol resistance (with a 50% inhibition concentration of 40 μg/ml) was easily obtained. The extract of this highly resistant strain showed the same low activity of chloramphenicol acetylation as that of the parent Ps 2.

DISCUSSION

The present study revealed that most strains of the *Proteus* group, with the exception of *P. vulgaris*, whether sensitive or resistant to chloramphenicol, showed significant levels of the chloramphenicol-acetylating enzyme, similar to that found in *E. coli* carrying the R factor. The possibility that all those tested strains [except for F 67 (NR 1), of course] had been infected by an

TABLE 2. *Chloramphenicol sensitivity and chloramphenicol-acetylating activity in representative strains*

Strain	ID_{50} of chloramphenicol[a]	Rate of chloramphenicol acetylation by crude extracts[b]
	(μg/ml)	
Escherichia coli		
K-12 CS2	0.6	0[c]
K-12 R5	100	100[c]
Proteus mirabilis		
N P3	1	0[d]
F 67	1	1–2[e]
F 67/CM	8	20
F 67 (NR 1)	100	50–200[f]
Klebsiella- Aerobacter		
Klebsiella KL 25	8	0
A. cloaca 868-53/CM	2.5	0.3
Pseudomonas		
Ps 1	5	±
Ps 2	8	0.25

[a] Estimated in peptone-glucose medium.
[b] Expressed as micrograms per minute per milligram of protein.
[c] Activity of the S100 fraction.
[d] Less than 10^{-10} colonies per cell on chloramphenicol (50 μg/ml)-agar.
[e] About 2.6×10^{-5} resistant colonies per cell on chloramphenicol (50 μg/ml)-agar.
[f] Variable depending on the growth phase.

R factor containing a gene for resistance is unlikely because of their date of isolation and because their properties (e.g., the lack of transferability of resistance and the lower level of resistance and enzyme activity) differed from the usual R factor-containing strains. The possibility, however, that some of them were carriers of atypical R factors cannot be definitely excluded. The discovery of chloramphenicol-acetylating enzyme in a *P. mirabilis* strain which was isolated before use of chloramphenicol became common is especially interesting, as it relates to the origin of the drug-inactivating enzyme and of the chloramphenicol resistance gene of the R factor.

The behavior of strains of *P. mirabilis* and presumably other species of this group (i.e., the sensitivity or the level of resistance to chloramphenicol and the tendency to produce resistant variants) can well be explained by the level of the enzyme activity present. Thus, the chloramphenicol-acetylating enzyme is probably the common cause of chloramphenicol resistance in this group. Most strains of this group can develop a moderate level of resistance without intervention of exogenous R factor, and they can also acquire a higher level of resistance by receiving an R factor; in either case, the basis of the chloramphenicol resistance is the chloramphenicol-acetylating enzyme. As for *P. vulgaris*, only two strains, both sensitive to chloramphenicol, were investigated, and both lacked the enzyme, as did *P. mirabilis* NP 3. Further studies are necessary to determine whether this is an invariable feature of this species.

The very weak activities of chloramphenicol acetylation in most strains of *P. aeruginosa* are apparently insufficient to explain their resistance to chloramphenicol; other mechanisms (e.g., low permeability to chloramphenicol, a different kind of chloramphenicol-inactivating enzyme) should also be operating in this species. The significance of the chloramphenicol-acetylating enzyme of this organism remains obscure. The significance of the weak activity of the enzyme in only one strain of the *Klebsiella-Aerobacter* group (*A. cloacae* 868-53/CM) is also unknown and, of course, other mechanisms should be searched for to explain the chloramphenicol resistance in this group as well as in *S. marcescens*.

Miyamura and Oketani (3) studied the inactivation of chloramphenicol by cell suspensions of various gram-negative bacteria and *S. aureus* isolated from clinical sources in recent years (1960 to 1962). Our results are generally in accordance with theirs. Some discrepancies which do exist can be easily explained by the difference in the sensitivity and specificity of the methods used. A remarkable difference between their results and ours is that they observed chloramphenicol inactivation in almost all chloramphenicol-resistant strains of *Klebsiella*. Since the number of strains investigated in our study was limited, it is possible that our negative results with members of this group do not represent the general situation. However, an equally likely possibility is that the resistant strains studied by Miyamura and Oketani might be those infected with R factor, since they were isolated from recent clinical material and since *Klebsiella* can receive R factor. These authors did not comment on this point.

The chloramphenicol-acetylating enzyme of the *Proteus* group produces mono- and diacetyl derivatives of chloramphenicol, as does the enzyme of *E. coli* strains possessing the R factor. The chloramphenicol-acetylating enzyme from *S. aureus* is different in that it does not produce the diacetyl derivative, even when an extensive monoacetylation is observed (10). The situation is not clear with the enzyme of the *Pseudomonas* strains, since the activity was too weak to allow any con-

clusion concerning the occurrence of the diacetylating reaction, which is very much slower than the monoacetylating reaction (Suzuki and Okamoto, J. Biol. Chem., *in press*). We have not been able to establish whether one or two enzymes are involved in these two reactions.

Our studies have revealed a rather wide distribution of the chloramphenicol-acetylating enzyme in various bacteria (strains carrying R factor, chloramphenicol-resistant staphylococci, the *Proteus* group, and *P. aeruginosa*), and the presence of at least two other kinds of drug-inactivating enzymes (for dihydrostreptomycin and kanamycin; 6) in *E. coli* carrying R factor; their distribution in other bacteria remains to be studied. These findings, together with our knowledge of penicillinase, suggest that drug-inactivating enzymes may play more important roles in bacterial drug resistance than heretofore expected.

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