CHLORAMPHENICOL RESISTANCE OF THREE DIFFERENT FLAVOBACTERIA

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The chloramphenicol resistance of some flavobacteria was investigated comparatively. This resistance can be explained either by acetylation of chloramphenicol to *O*-acetylchloramphenicol *via* constitutively formed acetyltransferases, followed by cometabolic degradation (strain CB 60), or by limited uptake and total degradation (strain CB 6) by inducible enzymes or by other mechanisms (*F. devorans*). The mechanisms of resistance, CM-acetylation, CM-degradation and limited uptake are discussed.

Resistance of bacteria to antibiotics can be caused by alterations of the drug's target site, by enzymatic inactivation or by a limited uptake of the drug. The target site of the antibiotic may have changed in such a manner that only a very weak or even no interaction is possible. Spectinomycin resistance for example is due to a mutation, by which an exchange of amino acids has taken place in the ribosomal protein S 5[1]. Kasugamycin-resistance of *Escherichia coli* is due to the loss of two methyl groups in the *N* 6-position of an adenine residue in the 16 sRNA[2]. An example of the enzymatic inactivation of an antibiotic is the phosphorylation of streptomycin in *E. coli* cells carrying the appropriate R plasmids[3]. However, inactivation may also be the result of the degradation of an antibiotic. This has been observed *e.g.* for chloramphenicol in *Streptomyces* strain 13 S and *Flavobacterium* strain CB 6[4,5]. Finally the antibiotic may be incapable of entering the cell due to permeation resistance. The resistance of Gram-negative bacteria to many antibiotics may be caused to a certain extent by the outer membrane[6]. In the case of tetracycline resistance, however, tetracycline induces the synthesis of a protein which prevents the transport of the antibiotic through the cytoplasmic membrane[7].

We have examined the chloramphenicol resistance of two chloramphenicol-metabolizing flavobacteria, *i.e.* strains CB 6 (34.3 GC mol%, Section I) and CB 60 (65.7 GC mol%, Section II) and of a third strain, *Flavobacterium devorans* (Section II)[8]. The classification of these three strains is preliminary. We would like to emphasize that the genus *Flavobacterium* at present is a collecting ground for Gram-negative yellow pigmented rod-shaped strains rather than a well-defined genus.

Materials and Methods

Strains

Flavobacterium strain CB 6[9]. *Flavobacterium* strain CB 60 (characterization in preparation). *F. devorans* (ATCC 10829) was purchased from the American Type Culture Collection. *Staphylococcus aureus* C 22.1 was kindly provided by Dr. W. V. SHAW, Leicester (Great Britain). The CM resistant

Dedicated to Prof. Dr. A. WACKER on the occasion of his 60th birthday.

Abbreviations: CM = Chloramphenicol
MNNG = N-Methyl-N'-nitro-N-nitrosoguanidine

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strain *F. devorans* CM^R was isolated from a *F. devorans* suspension by repeated selection in YEPD-medium (see below) with increasing CM-concentrations (final concentration 200 μg CM/ml).

Chemicals

Chemicals were acquired from Merck, Darmstadt, Germany, unless otherwise stated. [Methylene-C14]-D-*threo*-chloramphenicol was obtained from NEN Chemicals GmbH, Dreieich, Germany. Chloramphenicol was a gift from Bayer, Leverkusen, Germany.

Media

YEPD-Medium: Per liter deionized water: yeast extract (Oxoid, Difco), 10 g; peptone (Oxoid, Difco), 10 g; glucose, 20 g.

SM-Medium (Penassay-Broth Difco)[10]: Per liter deionized water: beef extract, 1.5 g; yeast extract (Oxoid, Difco), 1.3 g; peptone (Oxoid, Difco), 5 g; glucose, 1 g; NaCl, 3.5 g; K_2HPO_4, 3.86 g; KH_2PO_4, 1.32 g.

CA-Medium: Per liter deionized water: casamino acids, 5 g; glucose, 2 g; NH_4Cl, 1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; KH_2PO_4, 1.5 g; Na_2HPO_4, 3.5 g.

Minimal Medium: Per liter deionized water: KH_2PO_4, 0.875 g; K_2HPO_4, 0.125 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; NaCl, 0.1 g; $CaCl_2 \cdot 6H_2O$, 0.1 g; $(NH_4)_2SO_4$, 0.2 g; glucose, 10 g; boric acid, 0.5 mg; $CuSO_4 \cdot 5H_2O$, 0.04 mg; KI, 0.1 mg; $FeCl_3 \cdot 6H_2O$, 0.2 mg; $MnSO_4 \cdot 4H_2O$, 0.4 mg; $ZnSO_4 \cdot 7H_2O$, 0.4 mg; ammonium molybdate, 0.2 mg. For solid media 16 g agar (Difco) were added per 1 liter medium.

Tris-buffer: Per liter deionized water: Tris(hydroxymethyl)aminomethane, 12.1 g, pH 7.8; $Mg(CH_3COO)_2 \cdot 4H_2O$, 2.14 g; KCl, 3.7 g.

Determination of Growth Curves

The bacteria were cultivated in 30 ml broth at 30°C in a 100-ml-Erlenmeyer flask. Growth was followed by measuring the optical density in an Eppendorf-Photometer at 400~600 nm.

Determination of CM-Inactivation

This determination was carried out by means of the agar diffusion test in Petri plates of 180 mm diameter and a 10 mm layer of YEPD agar with *E. coli* as tester strain. In each of the punched holes 50 μl standard or 50 μl test solution was pipetted. After incubation for 1 day at 37°C the zone diameter was measured.

Uptake of Chloramphenicol

Bacteria were cultivated in 12 ml YEPD-medium at 30°C with shaking. At an optical density of 0.4 ($OD_{400\sim600}$, Eppendorf-Photometer), 0.2 μC [methylene-C14]-chloramphenicol were added directly to the suspension or after dilution with unlabelled chloramphenicol, so that the final concentration was either 3 μg/ml or 100 μg/ml. After different times samples of 2 or 1 ml were taken, filtered immediately (Selectron-Filter, pore size 0.45 μm, Schleicher & Schüll, Dassel, Germany), and washed three times with 3 ml saline. In order to determine the background after centrifugation the cell-free supernatant was treated accordingly. After drying the filters radioactivity was measured in 10 ml toluene scintillation fluid in a scintillation spectrometer Betascint 50, Berthold, Karlsruhe, Germany.

Thin-Layer Chromatography (TLC)

Chloramphenicol and its acetylation products were chromatographed on pre-coated TLC-plates (Silica Gel 60 F-254, Merck, Darmstadt, Germany) in chloroform-methanol (95:5, v/v). The substances were evaluated in a Chromatogramm-Spektralphotometer at 275 nm (KM 3, Carl Zeiss, Oberkochen, Germany). Since no pure *O*-acetyl derivatives were available, we determined their concentrations as follows: At that time when the concentration of the *O*-acetyl derivatives reached its maximum, their total concentration was determined as difference between the original CM-amount and the CM-amount present. At this time the degree of degradation could be neglected. For isolating *O*-acetyl-CM derivatives, silica gel plates H (Merck, Darmstadt, Germany) without fluorescence indicator were used. After chromatography the layers containing the product were scraped off and extracted.

Preparation of 3-*O*-Acetyl-Chloramphenicol

Strain CB 60 was cultivated in SM-medium to an optical density of approx. 1.0 (400~600 nm) and recultivated with 0.5 parts of fresh SM-medium containing 2 mg CM/ml. After shaking for seven

hours at 30°C the cells were centrifuged. The supernatant was extracted three times, each time with 0.5 parts ethyl acetate. The combined organic phases were evaporated. The yellow oily residue was extracted with 0.25 parts of 0.06 M acetic acid and fractionated on a Sephadex G 10 column. The main product was 3-*O*-acetyl-CM.

Products of Chloramphenicol Acetylation in a Crude Cell Extract from Uninduced Strain CB 60-Cells

Strain CB 60-cells were cultivated without CM to the middle of the logarithmic growth phase. The harvested cells were disrupted by grinding with alumina (Alcoa, Aluminium Company of America). The cell extract obtained after centrifugation of the cell paste in tris-buffer was further fractionated by a centrifugation of 120 minutes at 100,000 *g* in an ultracentrifuge (Spinco L2 65 B, Beckman). Samples of 2 ml of the supernatant (12 mg protein/ml) were incubated for 20 minutes at 30°C, pH 7.8, with 0.6 μMol [methylene-C14]-chloramphenicol (10.2 μCi/μMol).

The labelled compounds were extracted three times with ethyl acetate. These samples were co-chromatographed with reference substances in chloroform - methanol (95: 5, v/v)[11] on thin-layer chromatography plates (Silica Gel 60 F-254, Merck). The TLC-plates were scanned with a Berthold TLC-Scanner II. The Rf-values are as follows: Chloramphenicol 0.19, 1-*O*-acetyl-CM 0.36 and 3-*O*-acetyl-CM 0.49.

Results

1. Growth of Strain CB 60 in CM-Containing Complete Medium, Acetylation of CM

CB 60 was characterized as a member of the genus *Flavobacterium* of Section II (characterization in preparation). Since this strain is able to grow in a YEPD-medium with CM-concentrations up to 800 μg per ml, it is extremely resistant. The growth of this strain is retarded for only a certain period of time after adding the antibiotic (Fig. 1). This period of retardation depends on the concentration of the cells in the medium at the time of adding the antibiotic and on the CM-dose. Using the agar diffusion test we can show that CM is inactivated during the lag phase. This is the period of time from the addition of the antibiotic until the normal growth rate is regained.

Fig. 1. Growth of CB 60-culture in complete medium (YEPD) as a function of the CM-concentration. Time of CM-addition marked by an arrow.

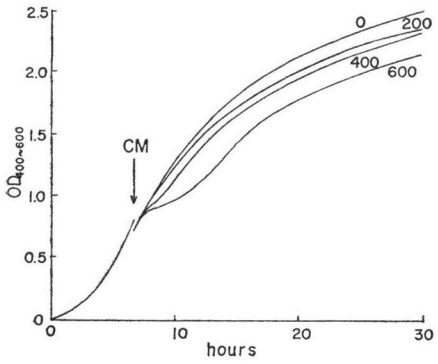


Fig. 2. Growth retardation (latency) as a function of the CM-concentration using CB 60 and CB 6. The bacteria were cultivated in complete medium (YEPD) at 30°C with shaking.

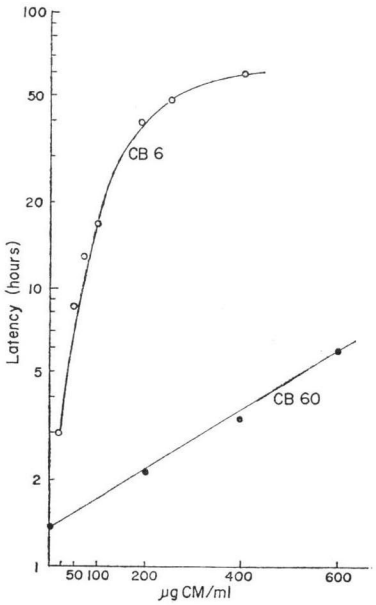


Fig. 3. Influence of CM-concentration (μg/ml) on the growth of CB 60 in SM- and CA-media. Time of addition of the antibiotic marked by an arrow (CA=casein hydrolysate, SM=Penassay Broth).

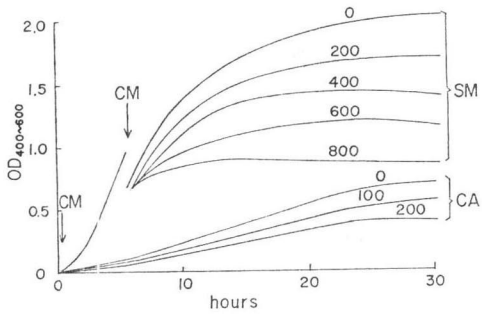
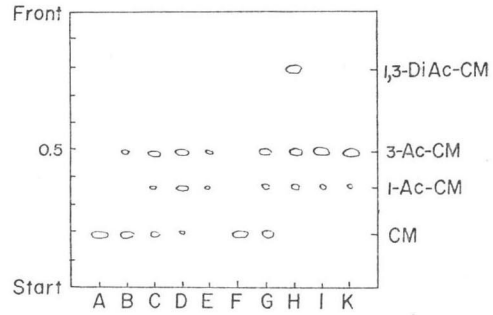


Fig. 4. Thin-layer chromatography of *O*-acetyl-derivatives of CM on Silica Gel 60 F-254 in chloroform - methanol (95: 5, v/v). A~E: Acetylation products of CB 60 in sequence of appearance. F~H: Products of *Staphylococcus aureus*. I and K: Rechromatography of 3- and 1-*O*-acetyl-CM, respectively.



It is also called the latent phase or latency. Plotting the logarithm of latency t versus the CM-concentration (Fig. 2), the relation to the inactivated CM-dose is as follows:

$$\ln t = \text{const.} \cdot [\text{CM}] + \text{const.}\,1$$

In less rich media (SM-medium and CA-medium), however, addition of CM leads to aper manent growth inhibition at concentrations which cause only a transient inhibition of growth in YEPD-medium (Fig. 3). In SM- and CA-medium cells are not immediately inhibited, but grow at a rate dependent on the CM-concentration. At this rate the cultures grow to the stationary phase. The ultimate cell density depends on the amount of the antibiotic previously added. This behaviour resembles that of the CM resistant *F. devorans*. With strain CB 60, however, there is a rapid inactivation of chloramphenicol.

The first transformation product found is 3-*O*-acetyl-CM (Fig. 4) together with small amounts of 1-*O*-acetyl-CM. Both compounds are interconverted spontaneously in a reversible reaction. This is confirmed by the fact that the monoacetates extracted from the TLC-plates give two spots after rechromatography; these spots correspond to both chloramphenicol acetates (Fig. 4). After developing a TLC-plate in a second dimension, the isolated CM-acetates give two spots again showing that 3-*O*-acetyl-CM was converted into 1-*O*-acetyl-CM and *vice versa* even in the silica-gel layer.

As demonstrated by the agar diffusion test and by thin-layer chromatography, the CM-concentration decreases sharply after addition of the antibiotics (Figs. 4 and 5). *O*-Acetyl-CM appears simultaneously. At the time of maximal concentration of CM acetylation products only a small amount of CM is still present in the medium, finally disappearing completely. The concentrations of the acetylated derivatives also diminish without however producing an increase in the CM-concentration in the medium (Fig. 5).

In SM-medium and CA-medium, in which CM causes a permanent inhibition of growth of the CM 60-cells, CM is not totally inactivated (Fig. 6) whereas a complete acetylation of CM is possible in YEPD-medium. The concentration of CM declines at first with simultaneous acetylation of the antibiotic. Then, however, the amount of the biologically active antibiotic increases again (Fig. 6). Apparently, CM is regenerated from its acetylation products. After addition of 800 μg of 3-*O*-acetyl-CM per ml SM-medium to growing cultures of CB 60 (Fig. 7), this compound decomposes with simultaneous retardation of bacterial growth rate. At a concentration of 1,600 μg 3-*O*-acetyl-CM per ml

Fig. 5. Inactivation of CM by acetylation by a CB 60-culture in complete medium (YEPD). The numbers indicate the original CM-concentration in the culture media (μg/ml). Time of CM-addition is shown by an arrow. The upper curve shows the growth of bacteria with 800 μg CM/ml medium.
● 200, ◇ 400, ◆ 600, ○ 800 μg CM or Ac-CM per ml.

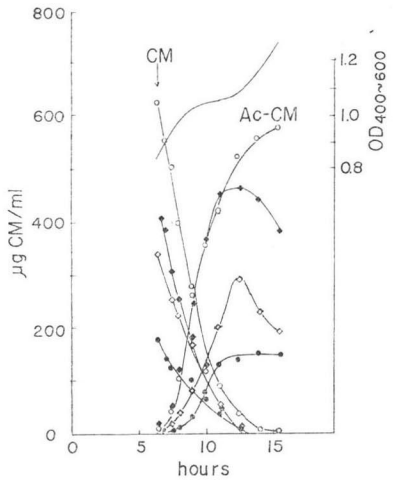


Fig. 6. Acetylation of CM by CB 60 in Penassay Broth. The cultures grew up to an optical density of 1.0 before addition of CM. Time of CM-addition marked by an arrow.
● 200, ◇ 400, ◆ 600, ○ 800 μg CM or Ac-CM per ml

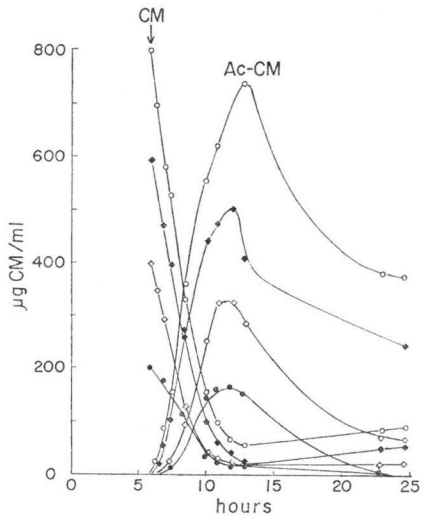


Fig. 7. Effect of *O*-acetyl-CM on the growth of CB 60 in SM-medium.
● 0, ○ 800, ◆ 1600 μg/ Ac-CM per ml.

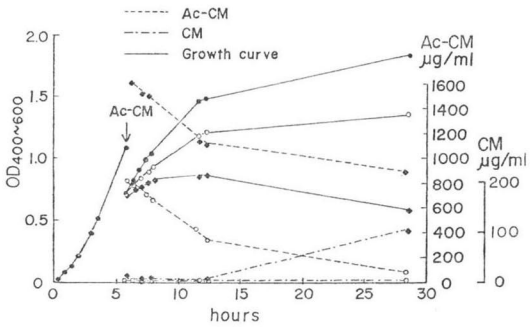
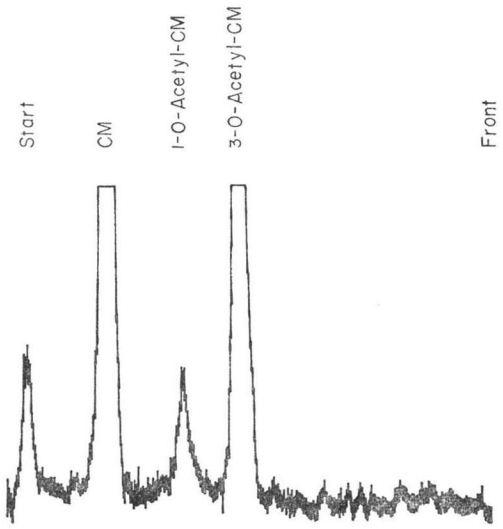


Fig. 8. Acetylation of CM by a cell extract of CB 60. Radio-TLC-scan of a silica gel plate. The reaction mixture consisting of [methylene-C^{14}]-chloramphenicol and of an enzyme extract was incubated for 20 minutes at 30°C. The mixture was extracted and then cochromatographed in chloroform - methanol with unlabelled reference substances.

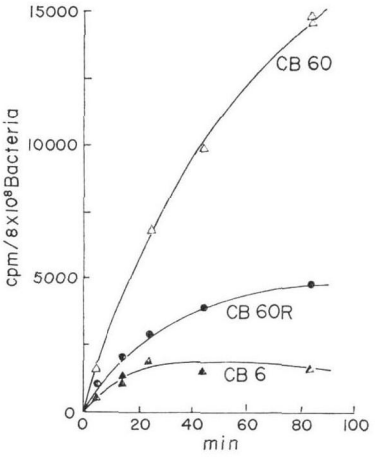


medium, the optical density of the bacteria suspension decreases after 12 hours (Fig. 7). A small amount of biologically active chloramphenicol is formed by decomposition of the acetylation products. First of all CM almost disappears but later on it appears again in higher concentrations. The poor medium does not allow metabolism of the newly formed CM so that growth of the cells is further inhibited. Whether the

CM-acetyltransferase is solely responsible for the deacetylation or whether an esterase takes part in the reaction, is not known. A cell extract of a CB 60-culture not previously exposed to CM, acetylates the antibiotic to the main product 3-*O*-acetyl-CM. Besides that some 1-*O*-acetyl-CM is formed (Fig. 8). The CM-acetyltransferase of CB 60 is synthesized constitutively as are the enzymes of CM-resistant Enterobacteriaceae coded by R-plasmids.

Resistance can be further increased by a reduced uptake of CM into the cell. Fig. 9 illustrates that CB 60R takes up significantly less CM than strain CB 60. CB 60R is considered to be a highly resistant biotype of CB 60, which is able to grow in concentrations of more than 800 μg CM per ml YEPD-medium. Strain CB 60R takes up essentially more CM than the resistant strain CB 6.

Fig. 9. Uptake of [methylene-C^{14}]-chloramphenicol in cells of CB 6, CB 60 and CB 60R growing in complete medium (YEPD) with 3 μg CM/ml in the log phase.



2. Growth of Strain CB 6 in CM-Containing Complete Medium, Degradation of CM

CB 6 was characterized as a *Flavobacterium* which degrades CM sequentially in a definite manner beginning with the oxidation of the primary alcoholic group in the C-3-position[5]. Incubation with increasing CM-concentrations in complete medium results in a more and more prolonged latent phase (Fig. 10). When the bacteria have reached the log phase, their growth curves show identical slopes indicating that the growth rate is not affected by high CM-concentrations. This result is in contrast to the behaviour of the resistant strain *F. devorans* (Fig. 11) and to CB 60 in less nutritive media (SM-medium and CA-medium) (Fig. 3). Plotting the logarithm of latency *versus* the CM-concentration yields a curve which is increasing to an asymptote, representing a critical latency (Fig.

Fig. 10. Growth of CB 6 in complete medium (YEPD) in presence of different CM-concentrations in μg CM/ml.

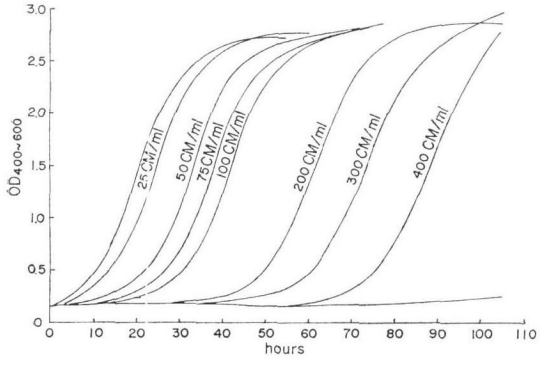


Fig. 11. Growth of two strains of *F. devorans* in complete medium (YEPD) at different CM-concentrations (μg/ml): *F. devorans* (wild type) and *F. devorans* CM^R (resistant).

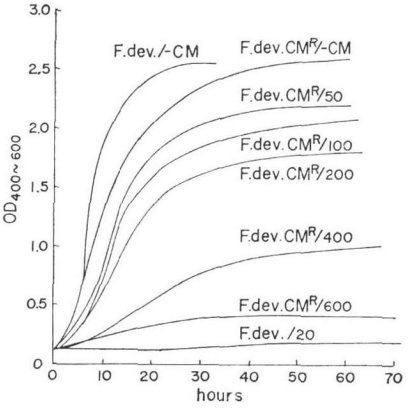


Fig. 12. Growth of CB 6-cultures in complete medium (YEPD) in presence of different CM-concentrations (μg/ml) and decrease of CM-concentration during incubation.

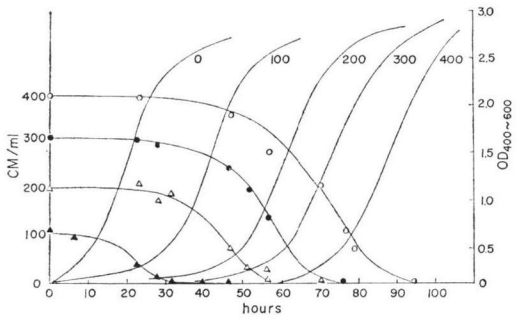
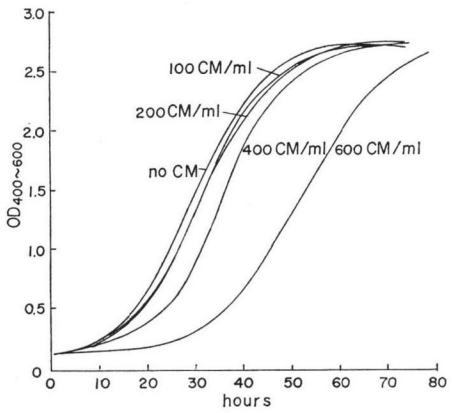


Fig. 13. Growth of CB 6 with CM in complete medium (YEPD) with different CM-concentrations (μg/ml) after repeated transfers on media containing CM.



2). Additional experiments show that the lag phase is also dependent on the growth phase from which the inactivating bacteria are taken. Under comparable conditions, the latency is smallest, if cells of the logarithmic growth phase are incubated with CM.

Chromatographic analysis reveals that the CM-concentration remains unchanged until the bacteria begin to grow; then, however, it quickly declines (Fig. 12). The higher the CM-concentration is at the beginning, the later it starts to decrease. If CB 6 is repeatedly transferred on CM-containing complete or minimal medium and then is incubated in complete medium with different CM-concentrations, growth curves are obtained which are comparable with those obtained from a CM-free CB 6-culture (Fig. 13). A prominent latent phase does not appear here.

3. Growth of *F. devorans* in CM-Containing Complete Medium

As representative strain of three *Flavobacterium* reference strains of Section II we have chosen *F. devorans*. Fig. 11 shows the growth curves of a CM-sensitive and a CM-resistant strain of *F. devorans* in YEPD-complete medium in the presence of different CM-concentrations. In CM-free medium the resistant strain shows a slight growth retardation compared to the sensitive one. While the sensitive wild-type cannot grow at concentrations of 20 μg CM/ml complete medium, the CM-resistant strain is only limited in its maximum cell density by CM. Also the growth rate is reduced at the higher CM-concentrations. Only very weak growth can be observed at 600 μg CM/ml medium. During incubation neither CM-metabolites nor a significant reduction of the CM-concentration could be found.

When comparing the CM-uptake of the resistant and sensitive strains, we found that a considerable proportion of the antibiotic is taken up into the cells but then leaves the cells to a great extent (no data shown). We have not been able to detect a significant difference with regard to the CM-content between the sensitive and resistant strains.

Discussion

The resistance of some flavobacteria to CM was comparatively investigated. While CB 60 is able to acetylate and to degrade CM, CB 6 is only capable of degrading it. Both strains show latent phases dependent on the CM-concentration. For CB 60 a linear correlation between the logarithm of latency and the CM-concentration was found. CB 6 does, however, not show any corresponding correlation. The reason for this finding may be due to the fact that the synthesis of the CM-metaboliz-

ing enzymes is regulated in a different way in these two strains.

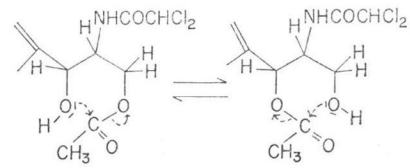
While CM-metabolism by CB 60 starts immediately and is independent of the concentration of the antibiotic (Figs. 5 and 6), CB 6 shows a clear latency of inactivation which is dependent on the CM-concentration (Fig. 12). This can be explained by an induction of degrading enzymes. The lack of the characteristic CM-dependent latent phase, following a previous repeated cultivation of CB 6 in CM-containing media, confirms this induction hypothesis (Fig. 13). Cell extracts decompose CM only when CB 6-bacteria have been previously incubated with CM (K. H. ALTENDORF and F. LINGENS, unpublished results). CB 60, however, synthesizes the metabolizing enzymes constitutively as proven with non-induced cell-free extracts (Fig. 8). Since CB 60 shows a CM-dependent latency, we have to assume that with increasing CM-concentration the number of those cells which contain sufficient enzymes necessary for degradation and survival declines so that the rest of the population needs more and more time for inactivation of the antibiotic. At a very high CM-concentration, no cells are left which can inactivate the antibiotic. If on the other hand CM is finally inactivated, reaching a threshold value at which growth can start again, the culture changes into the log phase. Then growth begins with a growth rate independent of the original CM-concentration in the CB 60-culture.

The CM-metabolizing strains CB 6 and CB 60 are inhibited by CM. Therefore resistance caused by the change of the target site cannot be postulated. The congruency between CM-inactivation and the start of growth for both strains suggests that the inactivation of the antibiotic is the main reason of resistance. If CB 60 is cultivated in a poor medium, CM accumulates again by deacetylation presumably because the less active metabolism causes a slower degradation of the CM-residue. Strain CB 60 is able to degrade CM only in cometabolism; CM, just like glucose, cannot serve as sole carbon source.

Since CM can be taken up immediately and extensively the permeation resistance for CB 60 is of minor importance (Fig. 9). CB 6, however, takes up a small amount of CM at first which presumably induces the synthesis of degrading enzymes. As soon as the CM-concentration has fallen below the inhibition level by degradation, the bacteria start to grow again. MALIK and VINING[12] demonstrated that the CM-producing strain of *Streptomyces venezuelae* also inactivates CM added to the culture and our results proved this to be the case for CB 6 and CB 60 during their lag phase. As soon as a sub-inhibitory concentration is attained, the *Streptomyces* strain grows producing CM at the same time.

The question of whether CB 60 inactivates CM by acetylation in the 1- or 3-position remains. The rapid conversion (transesterification) of 1-*O*-acetyl-CM to 3-*O*-acetyl-CM and *vice versa* leading to a non-enzymatic equilibrium, may be due to a six-membered ring as a transition state. In this state, a nucleophilic attack of the hydroxyl groups may lead to a cyclic conversion of the acetates (Fig. 14). SUZUKI and OKAMOTO[13], when examining the acetylation products of CM from an *E. coli* strain, considered 1-*O*-acetyl-CM to be a non-enzymatic product. According to MIYAMURA *et al.*[14] the CM-acetyltransferase in *Streptococcus faecalis* at first forms 3-*O*-acetyl-CM. This product partially converts spontaneously into 1-*O*-acetyl-CM. The acetylation of CM is not only limited by the amount of CM but also by the concentration of *O*-acetyl-CM. According to PIFFARETTI *et al.*[15] inhibition of protein synthesis by CM is reduced to 40% by monoacetylation. If the CM-activity cannot be further reduced in a CB 60-culture, the bacteria die. In poor media no total detoxification is noted, whereas in rich media complete degradation of CM is observed. Another means of detoxification was found for *Staphylococcus aureus, S. epidermidis* and *Streptococcus faecalis*[14,16,17]. CM is acetylated a second time by these strains forming 1,3-di-*O*-acetyl-CM which is almost biologically inactive (Fig. 15). 1,3-Di-*O*-acetyl-CM is formed by the acetyltransferase of *Streptococcus faecalis* using 1-*O*-acetyl-CM stemming from a spontaneous rearrangement reaction[14]. The acetylation proceeds in the same way as the formation of 3-*O*-acetyl-CM and is effected by the same type of enzyme[14]. Even after a longer incubation time no di-*O*-acetyl-CM can be detected in CB 60-cul-

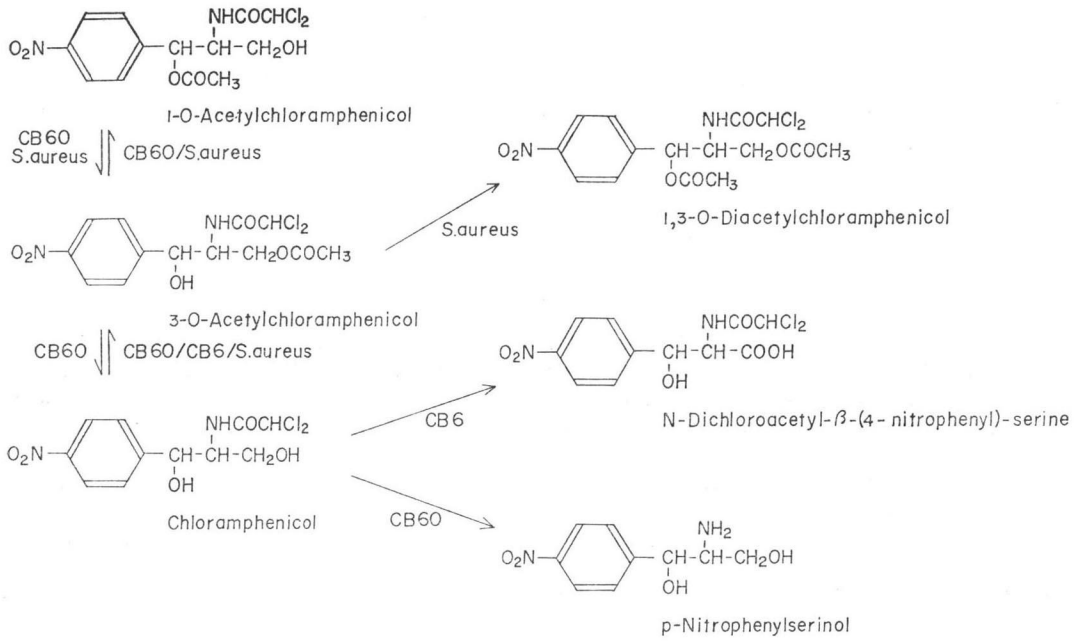
Fig. 14. Hypothetical mechanism of spontaneous transesterification of the mono-*O*-acetylchloramphenicol compounds.



3-O-Acetylchloramphenicol 1-O-Acetylchloramphenicol

Fig. 15. Possibilities of inactivation of CM.

Monoacetylation and diacetylation by *Staphylococcus aureus*. CM-monoacetylation and metabolism by CB 60 and CM-degradation by CB 6. The first CM-degradation product detected is stated here.



tures. It still has to be clarified whether the lack of 1,3-di-*O*-acetyl-CM is due to a higher substrate specificity of the CB 60-acetyltransferase or to other effects.

Examination with radioactively labelled CM and cochromatography shows that *N*-dichloroacetyl-*p*-nitrophenylserine, the first degradation product of CM by CB 6[5], is not formed by CB 60. However, we did find *p*-nitrophenylserinol (unpublished results). The appearance of this compound and the deacetylation of the *O*-acetyl-CM to CM do not support the possibility that degradation of *O*-acetyl-CM proceeds *via* the mixed anhydride O_2N–φ–CH(OH)–CH(NHCO–$CHCl_2$)CO–$OCOCH_3$ by oxidation of the CM residue. We therefore assume a degradation of the *O*-acetyl-CM *via* CM (Fig. 15). A decrease below the level of inhibition of protein synthesis is achieved *via* acetylation of CM by CB 60 and *via* degradation of CM by CB 6. As soon as CM falls short of this inhibitory concentration, *de novo* protein synthesis of degrading enzymes induced by CM can start. CM-degrading enzymes of CB 60 eliminate the antibiotic, which is in a constant equilibrium with the acetylated derivatives, so that finally also the mono-*O*-acetyl-CM has vanished. At the same time, the constitutively formed acetylating enzymes keep down the CM-concentration in the culture medium. Thus the growth can start sooner with CB 60 than with CB 6. Because of the high inactivation rate, more CM can be taken up into CB 60 cells than into CB 6 cells (Fig. 9). A further increase of resistance against CM is possible by a reduction of the uptake (Fig. 9).

A simple explanation for the growth characteristics of wild type *F. devorans* and the resistant biotype *F. devorans* CM^R at different CM-concentrations is a limitation of the CM-concentration in the cell. At higher concentrations in the medium, the antibiotic enters the cell in amounts that cause an early start of the stationary phase. The slight increase of the growth curve depending on the CM-concentration resembles the growth of CB 60 in poor complete media. In these media, CB 60 hardly inactivates CM: Our results do not show any significant CM-inactivation for *F. devorans*. It was not possible to obtain CM-metabolizing mutants from this strain by mutagenization with MNNG comparable to the inactivating activity of CB 6 or CB 60.

According to PESTKA[18] impermeability is the main cause for the CM-resistance besides acetylation. CM-uptake experiments in *F. devorans* show that the antibiotic obviously is removed out of the

cell by active transport after a short period of uptake. Such mechanisms are also discussed for the resistance to tetracycline[19]. Up to now we have not been able to demonstrate that the resistance of *F. devorans* CM^R is based on differences in the uptake or in the transport of CM as shown for CB 60/CB 60R. *F. devorans* CM^R neither has the ability to inactivate by acetylation nor the ability to degrade the antibiotic. Whether there is really a reduced susceptibility of the ribosomes to CM, a reduced binding of the antibiotic to protein L12 of the 50 S-ribosomal subunit[20], or an unknown factor remains to be investigated. Further experiments are required to establish whether cell-free protein synthesis is inhibited by CM.

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