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CURING ANTIBIOTIC RESISTANCE IN *BACTEROIDES* SPECIES BY AMINOACRIDINES AND ETHIDIUM BROMIDE

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Summary

Three clinical isolates of *Bacteroides fragilis* and one faecal isolate of *B. thetaiotaomicron* resistant to one or more of the antibiotics chloramphenicol, erythromycin, clindamycin and tetracycline, were cured of their resistance markers by treatment with subinhibitory levels (16 μg/ml) of acriflavine, acridine orange and ethidium bromide. Chloramphenicol, erythromycin and clindamycin resistance markers were cured *en bloc* after exposure to the agents for 24 hr but elimination of tetracycline resistance markers required longer incubation (17 – 21 days) with the reagents. The minimum inhibitory concentrations of the antibiotics for the *Bacteroides* strains before and after elimination are compared. Elimination of these antibiotic resistances indicates that the resistance markers are located as extrachromosomal plasmids (R-factors). The emergence of plasmid-mediated antibiotic resistances may compromise treatment of bacteroides infections and may also provide a reservoir of antibiotic resistance in the intestinal flora.

Résumé

Nous avons effacé les indicateurs de résistance de trois isolètes cliniques de *Bactéroides fragilis* et une isolète fécale de *B. thétaiotaomicron* résistantes à une ou plusieurs des antibiotiques chloramphénicole, érythromycine, clindamycine et tétracycline en les traitant par des niveaux sous-inhibitoires (16 μg/ml) d'acriflavine, d'orange acridine et de bromure d'éthidiume. Les indicateurs de résistance de chloramphénicole, érythromycine et clindamycine ont été effacés en bloc après une mise à nu aux agents pendant 24 hr mais pour éliminer les indicateurs de résistance de la tétracycline, il a fallu plus de temps d'incubation (17–21 jours) avec les réactifs. Nous avons comparé les concentrations inhibitoires minimes des antibiotiques pour les genres *Bactéroides* avant et après l'élimination. L'élimination de ces résistances antibiotiques montre que les indicateurs de résistance se trouvent comme des plasmides extrachromosomiques (facteurs-R). L'émergence des résistances antibiotiques plamides-médiates peut compremettre le traitement des infections bactéroides et peut aussi fournir un réservoir de résistance antibiotiques dans la flore intestinale.

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Introduction

Several workers have shown that resistance in *Bacteroides* spp. can be determined by plasmids, extrachromosomal resistance factors (R-factors) which are transferable to sensitive recipient bacteria independently of the chromosomal genes (Privitera, Dublanchet & Sebald, 1979; Tally *et al.*, 1979; Welch, Jones & Macrina, 1979; Rotimi, Duerden & Hafiz, in press). Sensitive variants may arise from resistant strains by spontaneous loss of the R-factors as a result of segregation or replication errors (Falkow, 1975). The rate of spontaneous loss can be increased by exposure to various chemical and physical agents (Hashimoto, Kono & Mitsuhashi, 1964; Bouachaud, Scavizzi & Chabbert, 1968). Some aminoacridines (acriflavine and acridine orange) eliminate R-factors from both entero-

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bacteria and *Staphylococcus aureus* at low frequency (Hashimoto, Kono & Mitsuhashi, 1964; Jyssum, 1971). Ethidium bromide, a phenanthridinium trypanocide and antiviral agent, is a powerful eliminator of the heritable competence factor from genetically competent variants of *Neisseria meningitidis* (Jyssum, 1971) and of plasmid-mediated antibiotic resistance from many bacteria (Bouachaud *et al.*, 1968).

The present investigations were undertaken to demonstrate that *Bacteroides* spp. with plasmid-mediated antibiotic resistance could be cured of their resistances by exposure to acridine orange, acriflavine and ethidium bromide.

Materials and methods

Bacterial strains

The resistant *Bacteroides* spp. used were four clinical isolates obtained from the Bacteriology Department, Royal Hallamshire Hospital, Sheffield. They were: *B.thetaiotaomicron* strain M 1022a resistant to chloramphenicol (Chl^r), clindamycin ($Clind^r$) and erythromycin (Ery^r); *B.distasonis* strain M 1022b Chl^r $Clind^r$ Ery^r; and *B fragilis* strains M 512 Ery^r and M 5162 Chl^r Ery^r and tetracycline resistant (Tet^r). All the strains contained plasmids (Rotimi, 1980). The strains were identified by the methods of Duerden *et al.*, (1980) and Rotimi, Faulkner & Duerden (1980).

Culture media

The liquid medium for all curing experiments was a modification of the BM medium of Nash (see Deacon, Duerden & Holbrook, 1978) with cooked meat particles and supplemented with menadione (1 μg/ml), haemin (5 μg/ml) and L-cysteine hydrochloride (2 μg/ml). The basic solid media were BM agar (Holbrook, Ogston & Ross, 1978), blood agar (Oxoid Blood Agar base No. 2, with 10% defibrinated horse blood) and DST agar (Oxoid) with 5% lysed blood. The selective media were BM agar containing the following antibiotics chloramphenicol (10 μg/ml), clindamycin (5 μg/ml), erythromycin (8 μg/ml) or tetracycline (8 μg/ml).

Anaerobic incubation

The anaerobic procedure of Collee *et al.* (1972) was followed in all essential aspects. BTL anaerobic jars (Baird & Tatlock Ltd.), and Whitley 13-plate alloy jars and 48-plate stainless steel jars were equipped with one (small jar) or three (large jars) 4-g sachet of room temperature catalyst. The incubation atmosphere was 90% H_2 + 10% CO_2 (BOC Special Gases, Deer Park Road, London) and the plates were incubated at 37°C for 48 hr. A slope of Simmons citrate medium seeded with *Pseudomonas aeruginosa* was incubated in each jar as a control for anaerobiosis.

Chemical reagents

The curing agents tested were acriflavine, AF, acridine orange, AO, (George T. Gurr Ltd., London, SW6) and ethidium bromide, EB, (BDH Chemicals Ltd., Poole, Dorset).

Sensitivity tests

Antimicrobial susceptibility of the bacterial strains was determined initially by the disk diffusion method with inocula of 10^5 cfu/ml of the test strains. The minimum inhibitory concentrations (MICs) were determined by seeding 0.02 ml of a 10^{-2} dilution of overnight cultures of test strains on to DST agar containing doubling dilutions of filter-sterilized antibiotic solutions (0.25 – 128 μg/ml): the MIC was the lowest concentration that did not permit visible growth of bateria after 24 hr anaerobic incubation.

Determination of subinhibitory concentration

The subinhibitory concentrations of the three dyes was determined in a pilot study with *B.fragilis* strain M 5162. A series of doubling dilutions of acriflavine, acridine orange and ethidium bromide were prepared in modified BM broth in universal bottles to give final concentrations of 0.1, 0.2, 0.4, 0.8, 2, 4, 8, 16 and 32 μg/ml. The media were then autoclaved at 121°C for 20 min. The test strain was grown on BM agar incubated anaerobically at 37°C for 24 hr and a few colonies were emulsified in sterile phosphate

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buffered saline (PBS; pH 7.3) to give a density of *ca* 10^4 organisms/ml; 0.1 ml of the suspension was seeded into each set of nine dilutions of the reagents and into a control bottle of BM medium without reagent. The bottles were incubated anaerobically at 37°C for 24 hr. 0.01 ml of each dilution was subcultured on to fresh BM agar and plated out by the standard method of Gillies & Dodds (1976) and the density of growth noted after anaerobic incubation for 24 hr.

Curing experiments

Curing experiments were also carried out with the *B.fragilis* strain M 5162 at the dilutions used for the determination of the subinhibitory concentration.

The subinhibitory concentration of the three dyes was used for these experiments. The test *Bacteroides* strains were suspended in PBS to a density of *ca* 10^5 organisms/ml; 0.01 ml was seeded into three bottles of BM medium containing the predetermined subinhibitory levels of the dyes and into a fourth bottle of plain BM broth as a control. The bottles were incubated anaerobically for 24 hr at 37°C and thereafter reincubated and subcultured on to plain BM agar every 24 hr for periods of up to 21 days. Wherever possible 25 – 50 individual colonies were taken from each plate (test and control) and streaked on to (a) selective media containing the appropriate antibiotics and (b) non-selective BM agar; all were incubated anaerobically. Colonies that were inhibited by the selective media were subcultured from the non-selective BM agar into BM broth to provide the inoculum for MIC determinations (as above). The frequency of loss of antibiotic resistance was calculated as the percentage of colonies from the treated cultures that were sensitive variants.

Results

The effect of dyes on growth of B.fragilis *strain M 5162*

The effect of the three dyes on *B.fragilis* strain M 5162 in BM broth culture was similar. There was no reduction in growth at concentrations below 8 μg/ml. At a concentration of 8 μg/ml of each agent, there was a small reduction in the density of growth of subcultures on BM agar but there was a marked reduction in growth of the strain at a concentration of 16 μg/ml of each dye. There was no growth in the presence of any dye at a concentration of 32 μg/ml even after prolonged incubation. On the basis of these results the subinhibitory concentration was taken as 16 μg/ml and this concentration was used in all subsequent curing experiments with the other test strains.

Effect of dye concentration on curing of resistance — B.fragilis *strain M 5162*

Resistance was not eliminated from *B.fragilis* strain M 5162 in experiments with concentrations of the three dyes <0.4 μg/ml. There was a significant difference between the effect of ethidium bromide, acriflavine and acridine orange at concentrations of 0.4 μg/ml – 16 μg/ml. Chl^r and Ery^r markers were eliminated by ethidium bromide at a concentration of 0.4 μg/ml at low rate (14%). Ethidium bromide was more effective than acriflavine in eliminating these resistances at concentrations between 0.04 and 16 μg/ml and acriflavine was much more effective than acridine orange (Table 1). Tet^r marker was not cured by any of the three dyes at any concentration up to 16 μg/ml in 24 hr.

TABLE 1. Effect of different concentrations of aminoacridines and ethidium bromide on curing of resistance from *B.fragilis* strain 5162

Concentration of agent (μg/ml)	Percentage cure of Chl^r Ery^r markers with		
	*EB	AF	AO
0.1	0	0	0
0.2	0	0	0
0.4	14	0	0
0.8	40	32	6
2.0	50	36	18
4.0	78	60	30
8.0	100	76	38
16.0	100	94	72
32.0	†NG	NG	NG
Control	0	0	0

*EB = ethidium bromide
AF = acriflavine
AO = Acridine orange
†NG = no growth

Elimination of antibiotic resistances from test strains

Chl^r Ery^r and $Clind^r$ markers were completely

TABLE 2. The effect of subinhibitory concentrations of acriflavine, acridine orange and ethidium bromide on the elimination of antibiotic resistance from four *Bacteroides* strains after 24 hr incubation

Test strains	†Resistance *pattern*	No. of colonies sensitive to the given antibiotic out of twenty-five colonies after treatment with 16 μg/ml of reagents															
		Chloramphenicol				Erythromycin				Tetracycline				Clindamycin			
		*AF	AO	EB	C	AF	AO	EB	C	AF	AO	EB	C	AF	AO	EB	C
B. fragilis M 5162	Chl Ery Tet	24	8	25	0	23	13	25	0	0	0	0	0	...	...	...	...
B. *fragilis* M 516	Ery	...	...	...	0	7	4	8	0	...	...	...	...	...	...	...	...
B. thetaiotaomicron M 1022a	Chl Ery Clind	24	14	24	0	22	15	25	0	...	...	...	...	22	15	25	0
B. distasonis M 1022b	Chl Ery Clind	22	12	25	0	23	16	24	0	...	...	...	...	21	14	25	0

*AF = acriflavine
AO = acridine orange
EB = ethidium bromide
C = negative control

†Chl = chloramphenicol
Ery = erythromycin
Tet = tetracycline
Clind = clindamycin

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eliminated after incubation for 24 hr with subinhibitory concentrations (16 μg/ml) of ethidium bromide and were eliminated from 92 – 94% of cultures treated with acriflavine; there was only 54% elimination with acridine orange (Table 2). However, the solitary Ery^r marker was eliminated from *B.fragilis* strain M 512 at a frequency of only 12 – 24% by the three dyes. In general Chl^r Ery^r and Clind^r markers were eliminated *en bloc* from *B.thetaiotaomicron* strain M 1022a and *B.distasonis* strain M 1022b. However, only Chl^r and Ery^r were lost together from *B.fragilis* strain M 5162 after incubation for 24 hr. The Tet^r marker was not eliminated from this strain by subinhibitory concentrations of any of the dyes during the first 17 days of incubation. The frequency of loss of Tet^r on the 18th day was 24% and 30% with acriflavine and ethidium bromide respectively, rising to 40% and 42% on the 21st day. Tet^r was not eliminated by acridine orange even after prolonged incubation up to 21 days. There was no spontaneous loss of antibiotic resistance in the control cultures throughout the period of incubation. None of the cured strains reverted to resistance after passage on plain media or media containing subinhibitory concentrations of the antibiotics.

MICs of test strains

Table 3 shows the MICs of the antibiotics for the test strains before and after growth in media containing acriflavine, acridine orange and ethidium bromide. All the sensitive variants became very sensitive to all the antibiotic to which they were formerly resistant. The control cultures remained resistant throughout the incubation period.

Discussion

The proof that a phenotypic trait such as antibiotic resistance is plasmid-mediated may be established only by correlating the presence of the plasmid with the expression of the trait. Evidence for this correlation may be achieved by curing the baterial strains of their plasmid-mediated resistance phenotype with mild mutagens such as acriflavine, acridine orange, and ethidium bromide that have a selective effect upon plasmid DNA. Antibiotic resistance was eliminated from all the test strains of *Bacteroides* spp. at high frequency by these dyes. Increasing both the concentration of the subinhibitory levels and the duration of incubation increased the rate of elimination of antibiotic resistance. This supports the findings of Tomeda *et al.* (1968) that the population of drug-susceptible cells increased with the duration of incubation and the concentration of sodium dodecyl sulphate (SDS).

The mechanism of curing appears to be the insertion or intercalation of aminoacridine or ethidium bromide molecules between adjacent DNA base pairs causing an extension or unwinding of the phosphodiester backbone (Waring, 1965). The selective effect of these reagents on extrachromosomal (plasmid) DNA is not clear but Hudson & Vinograd (1967) showed a significant difference between drug fixation by linear and circular DNA. Hahn & Ciak (1972) suggested that intercalation eliminates closed circular plasmid DNA because of the subsequent production of unnatural supercoiled molecules that are unable to replicate.

Bouachaud *et al.* (1968) found that ethidium bromide eliminated resistance factors from *E.coli* at high frequency with good reproducibility. The results of the present study with *Bacteroides* spp.

TABLE 3. MICs of Antibiotics for the test strains before and after elimination of resistance by *AF, AO and EB

Test strains	Resistance markers	MICs of the given antibiotics (μg/ml)							
		Before elimination				After elimination			
		Chl	Ery	Clind	Tet	Chl	Ery	Clind	Tet
B.fragilis M5162	Chl Ery Tet	64	64	0.5	64	0.5	0.5	0.5	1.0
B.fragilis M512	Ery	1	64	0.5	2	0.5	0.5	1.0	1.0
B.thetaiotaomicron M1022a	Chl Ery Clind	32	64	64	0.5	0.5	1	<0.25	0.5
B.distasonis M1022b	Chl Ery Clind	64	64	32	1	0.5	0.5	<0.25	1

*EB = ethidium bromide
AF = acriflavine
AO = Acridine orange

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were similar. Acriflavine was almost as good as ethidium bromide but acridine orange was less effective, although all showed good activity against the R-factors once they had been mobilized (unpublished observation).

Privitera *et al*. (1979) were unable to cure Tet^r from bacteroides by treatment with subinhibitory concentrations of acridine orange, proflavine, ethidium bromide, promethazine or chlorpromazine after 24 hr incubation at 34°C but the present study showed that Tet^r could be eliminated from *Bacteroides* spp. but only after prolonged incubation.

The results reported in this study of the elimination of Clin Ery Chl Tet resistances indicate an extrachromosomal location for these resistance genes in clinical and faecal isolates of the *B.fragilis* group of *Bacteroides*. The emergence of these R-factors may complicate the treatment of infections due to these organisms and may also provide a reservoir of antibiotic resistance in the intestinal flora. This may arouse particular concern in many developing countries such as in Nigeria, where antibiotics may be purchased readily from the chemist shop and where self-medication is common place (Osoba, 1979). The widespread misuse of antibiotics may create a strong selective pressure for the emergence of resistant strains in the faecal population with serious consequences.

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