THE POSSIBLE ROLE OF CATABOLIC PLASMIDS IN BACTERIAL STEROID DEGRADATION

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1. Introduction

The correlation between the presence of extra chromosomal DNA in bacteria and the ability of such bacteria to degrade unusual substrates is a relatively new observation. Observations of this kind reported so far have been almost exclusively with *Pseudomonas* spp. The degradation of salicylate [1], camphor [2], naphthalene [3], toluene and xylene [4,5] by *Pseudomonas putida*; octane by *Pseudomonas oleovorans* [6] and chlorophenoxy acids [7] by *Alcaligenes paradoxus* have all been shown to be plasmid inherited. Not all the enzymes responsible for the degradation of these substrates are necessarily plasmid coded. The genes which code for the enzymes of octane degradation in *Pseudomonas oleovorans* are distributed between a plasmid and the main chromosome [6].

The study of plasmid genetics in the genus *Pseudomonas* has advanced rapidly in recent years [8,9]. Plasmids have been isolated and characterised from *Pseudomonas aeruginosa* [10] and *Pseudomonas putida* [11].

It is the purpose of this publication to present evidence for plasmid-mediated steroid degradation in 37 different bacterial isolates.

2. Materials and methods

2.1. *Reagents and bacterial isolates*

Deoxycholic acid and lithocholic acid were obtained from Roussel (Paris). Taurocholic acid, glycocholic acid, cholic acid, chenodeoxycholic acid, hyodeoxycholic acid, cholesterol and β-sitosterol were all obtained from Koch Light. 12α-hydroxy-3-oxo-pregana-1,4-dien-20-carboxylic acid and 12β-hydroxy-androsta-1,4-dien-3,17-dione were isolated from the degradation of deoxycholic acid by *Pseudomonas* spp. NCIB 10590 [12]. Mitomycin C was obtained from Sigma. General reagents were of Analar grade and obtained from BDH and all solvents were redistilled before use.

The bacterial cultures used (table 1) were isolated from silage and faeces on deoxycholic acid as a sole carbon source under both aerobic and anaerobic conditions [13]. Each bacterial isolate was maintained on slopes of deoxycholate agar and Oxoid nutrient agar at 4°C.

2.2. *Media preparation*

The aerobic metabolism of steroids by bacteria

Table 1
Identification of the bacterial isolates

Number of bacterial isolates	Identification
1 strain	*Pseudomonas aeruginosa*
13 strains	*Pseudomonas fluorescens*
8 strains	*Pseudomonas maltophilia*
3 strains	*Pseudomonas spp.*
3 strains	*Escherichia coli*
5 strains	*Staphylococcus aureus*
2 strains	*Acinetobacter calcoaceticus*
1 strain	*Klebsiella ozaenae*
1 strain	*Klebsiella pneumoniae*

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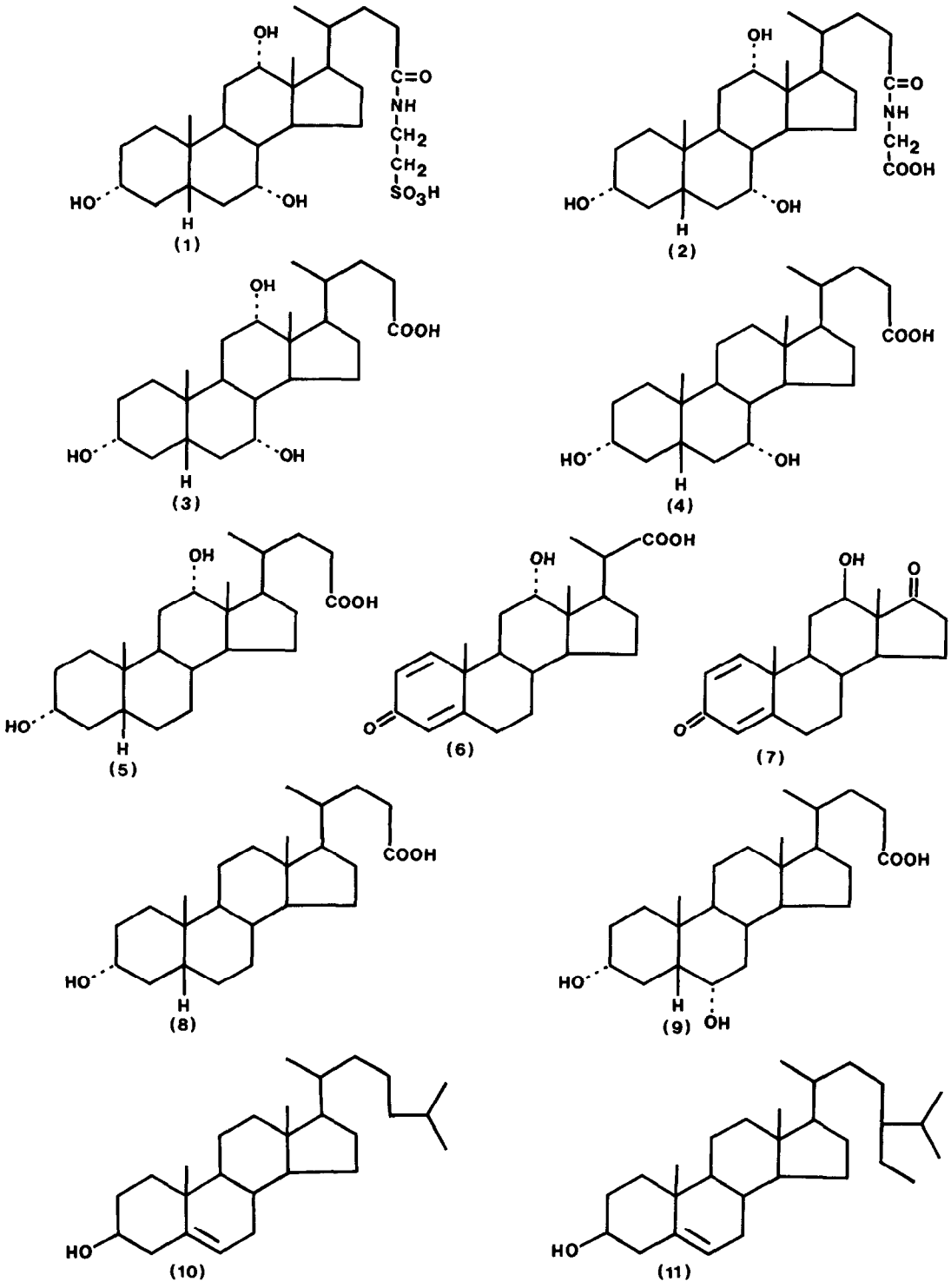

Fig.1. Substrates used to demonstrate bacterial steroid degradation.

was carried out using buffered mineral salts media comprising: steroid carbon source (1.0 g), K_2HPO_4 (1.6 g), KH_2PO_4 (0.4 g), KNO_3 (1.0 g), $FeSO_4 \cdot 7\,H_2O$ (2.5 mg), $ZnSO_4 \cdot 7\,H_2O$ (2.5 mg), $MnSO_4 \cdot 4\,H_2O$ (2.5 mg), $MgSO_4 \cdot 7\,H_2O$ (0.1 g) and distilled water to one litre (pH 7.2). Solidified medium was prepared by the addition of 10.0 g Oxoid agar no. 1. All bile acids and 12α-hydroxy-3-oxo-pregna-1,4-dien-20-carboxylic acid were prepared as their sodium salts. Cholesterol and β-sitosterol were prepared as fine suspensions by first dissolving in a few millilitres of ether, followed by slow addition of the ether solution to distilled water. The ether/water mixture was then subjected to agitation with an ultraturax homogeniser causing dispersal of the sterol into fine particles and evaporation of the ether. 12β-hydroxyandrosta-1,4-dien-3,17-dione was first dissolved in dichloromethane, followed by addition of the dichloromethane solution to distilled water. The dichloromethane/water mixture was then steamed for 10 min causing evaporation of the dichloromethane leaving the steroid in solution. All the media constituents were autoclaved before use.

2.3. *Steroid degradation*

Thirty seven bacterial strains (table 1) were tested for their ability to degrade taurocholic acid (1), glycocholic acid (2), cholic acid (3), chenodeoxycholic acid (4), deoxycholic acid (5), 12α-hydroxy-3-oxo-pregna-1,4-dien-20-carboxylic acid (6), 12β-hydroxyandrosta-1,4-dien-3,17-dione (7), lithocholic acid (8), hyodeoxycholic acid (9), cholesterol (10) and β-sitosterol (11) (fig.1) as sole carbon sources after being maintained on deoxycholate agar and nutrient agar. The cultures, after inoculation, were incubated at 28°C. The degradation products were separated by thin layer chromatography on silica gel GF_{254} in methanol/dichloromethane (1:9, v/v) and detected by ultraviolet light and by spraying with anisaldehyde reagent [14].

2.4. *Plasmid 'curing'*

Using similar methods to those in [6] 'curing' of the steroid degradative ability of 7 isolates was attempted. Nutrient broth (2 ml) was supplemented with mitomycin C (at 5 μg increments to 50 $\mu g\ .ml^{-1}$) in 5 ml sterile tubes. After inoculation with $\sim 10^5$ cells, the tubes were incubated at 28°C with shaking for 65 h. The cultures were then diluted in saline (1 in 10 serial dilutions) and plated on nutrient agar. After incubation the plates were replicated to deoxycholate agar. The proportion of colonies unable to grow on deoxycholic acid was noted in each case. Colonies growing on nutrient agar but not deoxycholic acid (5) were subcultured to a series of plates containing 1–11 as sole carbon sources and their ability to grow was noted.

3. Results

Each bacterial strain (table 1) showed the ability to degrade all the steroid substrates (fig.1) when maintained on deoxycholate agar (table 2). However, except for deconjugation of taurocholic acid and glycocholic acid and some evidence of hydroxysteroid dehydrogenation, 30 of the isolates lost the ability to degrade the steroid substrates when maintained on nutrient agar (table 2). The other 7 isolates still capable of degrading all the steroid substrates when maintained on nutrient agar (table 2) were shown to be 'cured' of this ability by mitomycin C (tables 2,3). Mitomycin C was shown to 'cure' all the 7 isolates, from 5–7% (table 3), of the ability to degrade all the steroid substrates apart from the ability to deconjugate taurocholic acid and glycocholic acid and to carry out hydroxysteroid dehydrogenation.

4. Discussion

The strains of bacteria tested for their ability to degrade steroid substrates can be divided into two distinct groups. One group (30 isolates) readily lost the steroid degradative ability when maintained on rich media. This suggests the loss of genetic material necessary to enable the isolates to utilise steroids as substrates. It is probable that the genetic material is present in these isolates in the form of non-compatible plasmids [15]. This means that the plasmids will only exist in the bacterial cell under selection pressure, that is when the bacterial cell is maintained on a steroid carbon source. The other group (7 isolates) showed $< 1\%$ loss of the steroid degradative ability when maintained on rich media. It is probable that the relevant genetic material is present in these

Table 2
The ability of the bacterial isolates to degrade steroid substrates

Substrate (fig.1)	Number of isolates showing degradative ability			
	After maintenance on deoxycholate agar	After maintenance on nutrient agar		After 'curing' with mitomycin C (7 only)
Taurocholic acid (1)	37	7c	30p	7p
Glyocholic acid (2)	37	7c	30p	7p
Cholic acid (3)	37	7		0
Chenodeoxycholic acid (4)	37	7		0
Deoxycholic acid (5)	37	7		0
12α-hydroxy-3-oxo-pregna-1,4-dien-20-carboxylic acid (6)	37	7		0
12β-hydroxyandrosta-1,4-dien-3,17-dione (7)	37	7		0
Lithocholic acid (8)	37	7		0
Hyodeoxycholic acid (9)	37	7		0
Cholesterol (10)	37	7		0
β-sitosterol (11)	37	7		0

c, complete degradation; p, partial degradation

Table 3
Percentage 'curing' of steroid degradative ability using mitomycin C

Organism	Concentration of mitomycin C (μg .ml^{-1})										
	0	5	10	15	20	25	30	35	40	45	50
Pseudomonas spp. MC 13	< 1	< 1	1	3	4	5	7	7	no growth		
Pseudomonas fluorescens MC 210	< 1	< 1	1	1	3	4	5	5	no growth		
Pseudomonas fluorescens MC 317	< 1	< 1	1	2	5	6	8	7	no growth		
Pseudomonas fluorescens MC 319	< 1	< 1	1	3	2	5	no growth				
Pseudomonas fluorescens MC 422	< 1	< 1	< 1	1	3	5	no growth				
Pseudomonas fluorescens MC 423	< 1	< 1	1	1	4	6	no growth				
Pseudomonas spp. 10590	< 1	< 1	1	2	3	7	no growth				

isolates in 'stable' plasmids. However, the possibility exists that the genetic material could have become incorporated into the main chromosome. The fact that mitomycin C gave rise to an increase in percentage loss of the steroid degradative ability in all these isolates suggests the former assumption is true. The figure of 5–7% curing compares favourably with plasmids shown to be responsible for the degradation of other substrates [1–7]. The bacteria 'cured' by mytomycin C were then incapable of degrading any of the steroid substrates studied. It is, therefore, suggested that there is a plasmid present in certain bacteria, mainly *Pseudomonas* spp., responsible for the coding of enzymes necessary for the degradation of many steroid substrates. It is probable that enzymes necessary for the deconjugation of bile salts and the various hydroxysteroid dehydrogenases are coded for on the main chromosome since these enzymes are maintained after the loss of the plasmid.

All the isolates containing a 'stable' plasmid are *Pseudomonas* spp. as are most of the bacterial strains shown to contain plasmids responsible for the degradation of other substrates [1–7]. It is possible that all the other isolates capable of steroid degradation originally obtained the relevant plasmids by genetic transfer from the isolates containing 'stable' plasmids. This is possible because all the strains of bacteria were isolated from mixed cultures on deoxycholic acid as a sole carbon source [13]. Such a genetic transfer would have to take place across generic barriers [16] and may partly explain why the plasmids exist in certain strains in an 'unstable' or non-compatible state.

The microbial degradation of bile acids is potentially of importance in the commercial production of physiologically active steroids [17] and is implicated in the aetiology of breast [18] and colon [19] cancer. If a plasmid is responsible for bile acid degrading ability then genetic transfer would be of importance. The use of genetic engineering could improve the prospects of producing specific steroid analogues from bile acids in large quantities. The transfer of plasmids from strains of bacteria either resident in the human gut or in transit [20] could increase the production of carcinogens or co-carcinogens by steroid degradation. This would be analagous to the spread of R factors through a mixed bacterial population in response to selection pressure generated by antibiotic therapy. Changes in the intake of dietary fat and fibre can lead to increased steroid secretion in the gut and thus may result in an increase in the numbers of steroid degrading strains by plasmid transfer.

Acknowledgements

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