Pseudomonas mutant strains that accumulate androstane and seco-androstane intermediates from bile acids

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Transposon mutant strains which were affected in bile acid catabolism were isolated from four *Pseudomonas* spp. Two of the mutant groups isolated were found to accumulate 12 α -hydroxyandrost-1,4-diene-3,17-dione as the major product from deoxycholic acid. Strains in one of these two groups were able to grow on steroids such as chenodeoxycholic acid, which lacks a 12 α -hydroxy function, whereas the one member of the second group could not. With chenodeoxycholic acid, this latter strain accumulated a yellow muconic-like derivative, tentatively identified as 3,7-dihydroxy-5,9,17-trioxo-4(5),9(10)-disecoandrost-1(10)-dien-4-oic acid. Members of two further mutant groups accumulated either 12 β -hydroxyandrost-1,4-diene-3,17-dione or 3,12 β -dihydroxy-9(10)-secoandrost-1,3,5(10)-triene-9,17-dione as the major product from deoxycholic acid. The relationship between the catabolism of *m*- and *p*-cresol, 3-ethylphenol and the bile acids was also examined.

INTRODUCTION

Bile acid catabolism by micro-organisms has been studied by a number of workers, and the results obtained have led to the proposition that there are two basic degradative pathways (Leppik, 1980, 1983*a*). The first pathway is utilized by all five Gram-positive micro-organisms studied to date, and has been elucidated by Hayakawa and co-workers (reviewed by Hayakawa, 1973, 1982), and by Severina *et al.* (1968, 1969*a,b*). While each of the five micro-organisms has its own variations of the pathway, the main point of attack for all is on the bile acid nucleus, with rupture of the B, A, C and then D rings (see 1, Fig. 1), with either no, or only partial, removal of the side-chain before the ring system is completely degraded.

In the second pathway, the side-chain is the main point of attack, with no ring rupture observed until after the side-chain is completely removed. This pathway is found in all five Gram-negative micro-organisms examined in detail to date, the results having been obtained by Mason, Owen and co-workers (Owen & Bilton, 1983, and references therein), by Park (1981, 1985), Smith & Park (1984), and by Leppik (1980, 1981, 1982, 1983*a*).

In this paper, the results of work on four *Pseudomonas* isolates are reported. These studies utilized transposon mutagenesis, and were performed in order to gain a greater understanding of this second pathway.

MATERIALS AND METHODS

Bacterial strains and media

The bacterial strains used are listed in Tables 1 and 2. The isolation of the *Pseudomonas* parent strains has been described (Leppik *et al.*, 1982). The rich medium used was LB medium, pH 7.5 (Maniatis *et al.*, 1982), and that used in plates was Difco antibiotic medium 3, solidified with 1.5% Difco Bacto-agar. The minimal medium used

was M9 medium (Maniatis *et al.*, 1982), adjusted to pH 7.4 before autoclaving. To the sterile minimal medium was added, per litre, MgSO₄ (1 ml of 1 M) and a stock salts solution (1 ml). The stock salts solution was based on that of Bauchop & Elsdon (1960), and contained CaCO₃ (4 g), FeSO₄·7H₂O (9 g), ZnSO₄·7H₂O (1.5 g), MnSO₄·4H₂O (1.2 g), CuSO₄·5H₂O (0.3 g), CoSO₄·7H₂O (0.3 g) and H₃BO₃·7H₂O (0.06 g). To these salts was added approx. 100 ml of water and 20 ml of 10 M-HCl and, when dissolved, the solution was made up to 1 litre, and filter-sterilized. The minimal medium plus salts was supplemented, where necessary, with glucose (20 mM) a bile acid (2.5 mM plates, 5 mM liquid media), 12 α -hydroxy-3-oxochola-1,4-dien-24-oic acid (1 mM), *p*-hydroxybenzoate or benzoate (10 mM), protocatechuate, *m*- or *p*-cresol, or 3-ethylphenol (2.5 mM), or L-amino acids (20 μ g/ml). Insoluble steroids (e.g. androst-1,4-diene-3,17-dione, 3 α -hydroxy-5 β -androst-17-one, etc.) were mixed with water and Triton X-100, autoclaved, then added to the minimal medium, to give a final concentration of 1.5 mM-steroid and 0.3% Triton X-100. Antibiotics were used in media at the concentrations stated in the text.

Chemicals

Many of the bile acid intermediates used as reference standards were kindly provided by Dr. Park of this laboratory. The 12 α -hydroxy-3-oxochola-1,4-dien-24-oic acid was synthesized by the method of Leppik (1983*b*), except that the intermediate 12 α -formoxy-3-oxo-5 β -cholan-24-oic acid was reacted with 2.1 equivalents of bromine, to give a 2,4-dibromo product. This, after debromination and hydrolysis of the formoxyl group, gave 12 α -hydroxy-3-oxochola-1,4-dien-24-oic acid, recrystallized from ethyl acetate, m.p. 214–216 °C, i.r._{max}. 3605 (OH), 1702 (C=O, acid), 1658 (C=O, 1,4-dien-3-one), 1614, 1598 (C=C, 1,4-dien-3-one) cm⁻¹.

Abbreviations used: secophenol, 3-hydroxy-9(10)-secoandrost-1,3,5(10)-triene-9,17-dione; secocatechol, 3,4-dihydroxy-9(10)-secoandrost-1,3,5(10)-triene-9,17-dione.

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Fig. 1. Proposed pathway for the degradation of deoxycholic acid by Gram-negative micro-organisms

R = H or OH.

Spectra, m.p., t.l.c. and column chromatography

These were performed as previously described (Leppik, 1981, 1982, 1983a). T.l.c. solvent systems used for the examination of products from deoxycholic or chenodeoxycholic acids were ethyl acetate/hexane/acetic acid (30:20:1, by vol.) and chloroform/acetone/methanol (15:4:1, by vol.). For cholic acid products, ethyl acetate/ethanol/acetic acid (48:2:1, by vol.) was used.

Tn5 mutagenesis, and screening of mutant strains

The *Escherichia coli* donor strains were patch-mated overnight at 30 °C with the *Pseudomonas* recipients on rich medium plates. The transconjugants were washed in M9, then spread on minimal medium plates containing glucose and kanamycin (100 µg/ml), and incubated at 30 °C. The kanamycin-resistant clones were spotted onto glucose plus kanamycin plates, 48 clones/plate, a

maximum of 480 clones/mating. When grown, the clones were replicated onto various test media, using a 48-pin replicator, and incubated. To isolate bile acid catabolic mutant strains, the compounds included in the test minimal medium plates were deoxycholic acid, 12 α -hydroxy-3-oxochola-1,4-dien-24-oic acid, 3 α -hydroxy-5 β -androstan-17-one and androsta-1,4-diene-3,17-dione.

Clones that appeared to be affected in bile acid catabolism were purified via single colonies, then inocula grown overnight at 25 °C from the purified colonies, in LB medium supplemented with kanamycin (50 µg/ml). Inocula (1 ml) were added to minimal medium (10 ml), containing glucose (5 mM), deoxycholic acid (5 mM), and yeast extract (0.01%), and then grown with aeration at 25 °C. At 24 and 48 h, samples (5 ml) were taken, acidified (0.5 ml of 1 M-HCl), and extracted with ethyl acetate (1 ml). A portion (5 µl) of the ethyl acetate extract was examined on t.l.c.

Isolation of deoxycholic acid products from RAL8.14

RAL8.14 was grown overnight at 25 °C with aeration, in LB medium (150 ml) containing kanamycin (50 µg/ml), and this was used to inoculate 1.5 litres of minimal medium, which contained deoxycholic acid (1, Fig. 1) (5 mM), glucose (5 mM) and yeast extract (0.01%). The mixture was dispensed to 5 × 2 litre conical flasks, and shaken (200 rev./min) at 25 °C. Growth was followed by t.l.c. examination of ethyl acetate extracts, and conversion to products was complete after 24 h. The culture was centrifuged to remove the cells, the supernatant acidified (pH 2), then extracted with chloroform. The crude material obtained from the dried chloroform extract (2.2 g) was purified by passage through a Merck Kieselgel 60 column (50 g), with a gradient of 2–20% (v/v) ethanol in chloroform. The first compound eluted (1.5 g, 66%) was recrystallized from ethanol, to give 12 α -hydroxyandrost-1,4-diene-3,17-dione (2, Fig. 1) (1.05 g, 46%), m.p. 206–208 °C [literature m.p. 206.5–207.5 °C (Leppik, 1981)], i.r. identical with that previously found (Leppik, 1981). The second compound eluted (0.38 g, 14%) was recrystallized from chloroform, to give 12 α -hydroxy-3-oxo-23,24-dinorchola-1,4-dien-22-oic acid (0.06 g, 2%), m.p. 261–263 °C (Dr. Park's sample, m.p. 249–250 °C), i.r. identical with that of Dr. Park's compound.

Isolation of deoxycholic acid product from RAL8.23

The strain growth, and product isolation, was as for RAL8.14, except that a 100 ml inoculum was added to 1 litre of minimal medium, which contained increased deoxycholic acid (1, Fig. 1) (12.5 mM). Product formation was complete in 48 h. Recrystallization from ethanol of the main compound eluted from the column gave 12 α -hydroxyandrost-1,4-diene-3,17-dione (2, Fig. 1) (2.4 g, 63%), m.p. 206–207 °C, i.r. identical with that obtained above.

Isolation of deoxycholic acid product from RAL91.11

The growth was as for RAL8.14, except that a 200 ml inoculum was added to 2 litres of minimal medium, which contained increased deoxycholic acid (1, Fig. 1) (25 mM). Product formation was complete after 53 h. The culture was centrifuged, and the pellet obtained was extracted with chloroform in a Soxhlet apparatus. The culture supernatant was also extracted with chloroform. The combined chloroform extract (9.2 g) was recrystallized from ethanol, to give 12 β -hydroxyandrost-1,4-diene-3,17-dione (3, Fig. 1) (6.4 g, 42%), m.p. 228–229 °C [literature m.p. 227–228 °C (Leppik, 1981)], i.r. identical with that previously found (Leppik, 1981).

Isolation of deoxycholic acid product from RAL1.25

The growth was as for RAL8.14, except that a 300 ml inoculum was added to 3 litres of minimal medium, which contained increased deoxycholic acid (1, Fig. 1) (15 mM). Product formation was complete after 48 h. The culture was centrifuged, and the pellet obtained was extracted with ethanol/chloroform (1:3, v/v) in a Soxhlet apparatus. The centrifugation supernatant was passed through an Amberlite XAD-2 column (200 g), then the latter was washed with water (500 ml), methanol (500 ml), then ethyl acetate/methanol (1:9, v/v), until product elution was complete. The combined product obtained (12.0 g) was purified via a Kieselgel 60 column

(110 g), which was eluted with a gradient of 20–60% (v/v) ethyl acetate in chloroform (both containing 0.25% acetic acid). The one main product obtained (7.6 g, 54%) was recrystallized from ethyl acetate, to give 12 β -hydroxysecophenol (5, Fig. 1) (7.0 g, 50%), m.p. 173–174 °C [literature m.p. 169–170 °C (Leppik, 1981)], i.r. identical with that previously found (Leppik, 1981).

RESULTS

Selection of strains for transposon mutagenesis

Seventy-eight isolates, capable of growth on one or more bile acids as sole carbon source, had been obtained from either a stale bile sample, or a soil sample, from a local abattoir (Leppik *et al.*, 1982); 70 of the isolates were found to be *Pseudomonas* spp., the remainder being actinomycetes (Leppik *et al.*, 1982). One of the *Pseudomonas* isolates, RAL8 (formerly MR108), was examined further and, from intermediates isolated and identified, a catabolic pathway for deoxycholic acid (1, Fig. 1) degradation has been proposed (Leppik, 1980, 1983a). This pathway, expanded to include results from the present paper, is shown in Fig. 1.

In order to select strains most suitable for transposon mutagenesis and subsequent work, 31 of the 70 *Pseudomonas* isolates were tested for resistance to a range of antibiotics in rich medium plates. The isolates were also tested for growth on a number of compounds in minimal medium, including *p*-cresol. This compound was included because, as the steroid A-ring degradation was analogous to the *meta*-cleavage degradation of the cresols (Bayly & Barbour, 1984), then *p*-cresol may be able to be degraded by the relevant steroid catabolic enzymes, and thus *p*-cresol may be able to be used as an analogue of 12 β -hydroxysecophenol (5, Fig. 1) [3,12 β -dihydroxy-9,10-secoandrost-1,3,5(10)-triene-9,17-dione] in subsequent mutant screenings.

Of the 31 *Pseudomonas* isolates tested, none showed significant resistance to nalidixic acid (40 µg/ml) after 24 h incubation, one was resistant to kanamycin (50 µg/ml), one to tetracycline (10 µg/ml), one to chloramphenicol (100 µg/ml), nine to piperacillin (25 µg/ml), 24 to streptomycin (50 µg/ml), and 29 to trimethoprim (50 µg/ml). Of the carbon sources tested, all strains gave significant growth after 2 days incubation on plates containing deoxycholic acid, androst-1,4-diene-3,17-dione or *p*-hydroxybenzoate. Four were unable to grow on benzoate, and seven unable to grow on *p*-cresol. From this, 15 strains were selected for a trial mutagenesis. All 15 could grow on all the carbon sources tested, and all were sensitive to all the antibiotics except trimethoprim. Some, however, were also resistant to streptomycin.

Tn5 transposon mutagenesis

For the transposon mutagenesis, the elegant system developed by Simon *et al.* (1983) was used. In this, the *recA*[−] *Escherichia coli* donor strains (SM10 or S17-1) contain an RP4-2 derivative integrated into the chromosome. The transposon-carrying plasmids are narrow host range plasmids, but contain the P-type specific recognition site for mobilization. The plasmids can thus be efficiently mobilized from the *E. coli* donor strains, but should not be able to replicate in *Pseudomonas* strains.

In the trial mutagenesis, the 15 strains selected above were each patch-mated overnight on rich medium

Table 1. *Escherichia coli* strains

Strain	Relevant phenotype*	Reference
SM10 (pSUP1011)	thi, thr, leu, recA, Cm ^r , Km ^r	Simon <i>et al.</i> (1983)
S17.1 (pSUP2021)	pro, recA, Tp ^r , Sm ^r , Ap ^r , Cm ^r , Km ^r	Simon <i>et al.</i> (1983)
S17.1 (pSUP5011)	pro, recA, Tp ^r , Sm ^r , Ap ^r , Cm ^r , Km ^r	Simon (1984)

* Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Sm, streptomycin; Tp, trimethoprim.

Table 2. *Pseudomonas* strains

Strain*	Derivation	Compound accumulated from deoxycholic acid
RAL1	Wild-type parent	—
RAL1.22	RAL1 × SM10 (pSUP1011)	12 β -Hydroxyandrosta-1,4-diene-3,17-dione
RAL1.25	RAL1 × S17.1 (pSUP2021)	12 β -Hydroxysecophenol
RAL8†	Wild-type parent	—
RAL8.14	RAL8 × SM10 (pSUP1011)	12 α -Hydroxyandrosta-1,4-diene-3,17-dione
RAL8.15	RAL8 × SM10 (pSUP1011)	12 β -Hydroxyandrosta-1,4-diene-3,17-dione
RAL8.16	RAL8 × S17.1 (pSUP2021)	12 β -Hydroxysecophenol
RAL8.23	RAL8 × S17.1 (pSUP5011)	12 α -Hydroxyandrosta-1,4-diene-3,17-dione [3,7-dihydroxy-5,9,17-trioxo-4(5),9(10)- disecoandrosta-1(10), 2-dien-4-oic acid from chenodeoxycholic acid]
RAL8.24	RAL8 × S17.1 (pSUP5011)	12 α -Hydroxyandrosta-1,4-diene-3,17-dione
RAL20	Wild-type parent	—
RAL20.5	RAL20 × S17.1 (pSUP2021)	12 α -Hydroxyandrosta-1,4-diene-3,17-dione
RAL20.8	RAL20 × S17.1 (pSUP2021)	12 β -Hydroxysecophenol
RAL91	Wild-type parent	—
RAL91.11	RAL91 × S17.1 (pSUP5011)	12 β -Hydroxyandrosta-1,4-diene-3,17-dione

* All parent strains are prototrophs. All mutant strains are kanamycin-resistant.

† A.T.C.C. 31753, formerly MR108.

plates with SM10 (pSUP1011) or S17.1 (pSUP2021) (Table 1). The transconjugants were washed, then plated on minimal medium containing glucose and kanamycin (100 μ g/ml). A variable number of resistant colonies were obtained from the various matings, so four phenotypically different *Pseudomonas* strains (RAL1, RAL8, RAL20 and RAL91) (Table 2) were selected for future work, from amongst those strains which gave a respectable number of kanamycin-resistant colonies. Of the two *E. coli* donor strains, more kanamycin-resistant colonies were obtained from matings with S17.1 (pSUP2021) so, in subsequent mutageneses, either S17.1 (pSUP2021) or S17.1 (pSUP5011) (Table 1) (Simon, 1984) were used.

For the four strains selected, a maximum of 480 kanamycin-resistant colonies/mating were spotted out onto minimal medium plates containing glucose and kanamycin, 48 colonies/plate, then replicated onto various test media. To detect bile acid mutants, the four compounds used in the plate media were deoxycholic acid (DCA), 12 α -hydroxy-3-oxochola-1,4-dien-24-oic acid (12 α OH-KDE), 3 α -hydroxy-5 β -androstan-17-one (3 α OH-AK) and androsta-1,4-diene-3,17-dione (ADD). The 12 α -hydroxy-3-oxochola-1,4-dien-24-oic acid and 3 α -hydroxy-5 β -androstan-17-one were included because of the postulation that A-ring modification and side-chain degradation could proceed independently of each

other (Fig. 1; Leppik, 1980). The ability of all four *Pseudomonas* strains to grow on both 12 α -hydroxy-3-oxochola-1,4-dien-24-oic acid and 3 α -hydroxy-5 β -androstan-17-one supported this postulation. It was thus anticipated that mutant strains blocked in side-chain degradation would be DCA⁻, 12 α OH-KDE⁻, 3 α OH-AK⁺⁺ and ADD⁺⁺, mutant strains blocked in A-ring modification would be DCA⁻ or ⁺, 12 α OH-KDE⁺⁺, 3 α OH-AK⁻ and ADD⁺⁺, and mutant strains blocked in A and B ring degradation would be DCA⁻ or ⁺, 12 α OH-KDE⁻ or ⁺, 3 α OH-AK⁻, and ADD⁻. The '— or +' designation is due to the possibility that the five carbon atoms in the side chain may support some growth in those mutant strains able to utilize it.

To date, this mutagenesis has been carried out a total of 25 times with the four *Pseudomonas* strains selected, and approximately 9000 kanamycin-resistant clones have been obtained. Plating results indicated that approx. 2% were affected in bile acid catabolism, and these clones were grown in liquid minimal medium containing glucose (5 mM) and deoxycholic acid (5 mM). Samples were taken from the culture at 24 and 48 h, acidified, extracted with ethyl acetate, and the extracts examined on t.l.c. It was found that 119 (1.3% of 9000) of the clones were affected in bile acid catabolism. The plating data for these mutant strains on the four test compounds are shown in Table 3. On the basis of the plating and t.l.c.

data, the mutant strains were divided into groups, and the four groups which appeared to accumulate androstane and secoandrosta intermediates are described in this paper.

In a number of the earlier mutageneses, the kanamycin-resistant clones were also tested for growth on benzoate, *p*-hydroxybenzoate, and *p*-cresol. The mutant strains obtained will be described later.

Mutant strains that accumulate 12 α -hydroxyandrosta-1,4-diene-3,17-dione (2, Fig. 1), and grow on androsta-1,4-diene-3,17-dione

Three of the mutant strains isolated, RAL8.14, RAL8.24 and RAL20.5 (Table 2), were found to accumulate one major compound from deoxycholate (1, Fig. 1), which had the same R_F on t.l.c. in two solvent systems, and the same colour after spraying with anisaldehyde reagent, as 12 α -hydroxyandrosta-1,4-diene-3,17-dione (2, Fig. 1). All three mutant strains accumulated a second compound in low yield, which had the same R_F and colour after spraying with anisaldehyde reagent as 12 α -hydroxy-3-oxo-23,24-dinorchola-1,4-dien-22-oic acid, a precursor of 12 α -hydroxyandrosta-1,4-diene-3,17-dione (2, Fig. 1). To confirm the identity of these two compounds, RAL8.14 was grown on a 1.5-litre scale, and the two products isolated. The m.p. and i.r. spectrum of the major compound isolated (m.p. 206–208 °C) were virtually identical with those of the 12 α -hydroxyandrosta-1,4-diene-3,17-dione (2, Fig. 1) previously isolated (m.p. 206.5–207.5 °C; Leppik, 1981). Similarly, the minor product isolated was shown to be identical with 12 α -hydroxy-3-oxo-23,24-dinorchola-1,4-dien-22-oic acid.

The three mutants were tested on a range of other steroids. All three accumulated one major product from cholic acid, which had the same R_F , and spray colour, as 7 α ,12 α -dihydroxyandrosta-1,4-diene-3,17-dione. On the other hand, all three could grow on 3 α -hydroxy-5 β -androstan-17-one, androsta-1,4-diene-3,17-dione and chenodeoxycholic acid, three steroids which lack a 12 α -hydroxy function. None could grow at a significant rate on 12 α -hydroxy-3-oxochola-1,4-dien-24-oic acid.

Mutant strain that accumulates 12 α -hydroxyandrosta-1,4-diene-3,17-dione (2, Fig. 1), but cannot utilize androsta-1,4-diene-3,17-dione

One mutant strain, RAL8.23 (Table 2), was isolated which was unable to grow on androsta-1,4-diene-3,17-dione in minimal medium plates, but which appeared to accumulate 12 α -hydroxyandrosta-1,4-diene-3,17-dione (2, Fig. 1) as a major product, and 12 α -hydroxy-3-oxo-23,24-dinorchola-1,4-diene-24-oic acid as a minor product, from deoxycholic acid, as shown by R_F and spray colour on t.l.c. The major product was isolated from a larger scale culture, and shown to have the same m.p. and i.r. spectrum as the 12 α -hydroxyandrosta-1,4-diene-3,17-dione (2, Fig. 1) above. When tested with cholic acid, RAL8.23 accumulated one major product, which had the same R_F , and spray colour, as 7 α ,12 α -dihydroxyandrosta-1,4-diene-3,17-dione. However, with chenodeoxycholic acid, the growth medium turned yellow. A portion of this medium was purified via a Waters Sep-Pak C₁₈ cartridge (Leppik *et al.*, 1982), and the yellow methanol eluate was diluted into phosphate buffer (pH 7.5). The resultant solution had a $\lambda_{\max}$. 323 and 385 nm. Addition of NH₃ initially produced a more

Table 3. Growth behaviour of the mutant strains isolated

Abbreviations: DCA, deoxycholic acid; 12 α OH-KDE, 12 α -hydroxy-3-oxochola-1,4-dien-24-oic acid; 3 α OH-AK, 3 α -hydroxy-5 β -androstan-17-one; ADD, androsta-1,4-diene-3,17-dione. Growth: +, significant growth after 48 h on minimal medium plates containing the compound stated.

Number of mutant strains	Growth on:			
	DCA	12 α OH-KDE	3 α OH-AK	ADD
52	—	—	—	—
6	—	—	—	+
41	—	—	+	+
8	—	+	—	+
1	—	+	+	+
1	+	—	—	—
1	+	—	—	+
1	+	—	+	+
7	+	+	—	+
1	+	+	—	—

intense peak at $\lambda_{\max}$. 395 nm, but, after standing overnight, this peak had disappeared, and a peak had appeared at 270 nm. This spectral data is almost identical with that given by 3-hydroxy-5,9,17-trioxo-4(5),9(10)-disecoandrosta-1(10),2-dien-4-oic acid (7, Fig. 1, R=H), formed by cell extracts of *Nocardia restrictus* from secocatechol (6, Fig. 1, R=H) [3,4-dihydroxy-9(10)-secoandrosta-1,3,5(10)-triene-9,17-dione] (Gibson *et al.*, 1966). Thus, the product from chenodeoxycholic acid could possibly be 3,7-dihydroxy-5,9,17-trioxo-4(5),9(10)-disecoandrosta-1(10),2-dien-4-oic acid. This product, and similar products formed by other mutant strains from cholic and deoxycholic acids, need further characterization.

Mutant strains that accumulate 12 β -hydroxyandrosta-1,4-diene-3,17-dione (3, Fig. 1)

Eight of the mutant strains isolated appeared to accumulate 12 β -hydroxyandrosta-1,4-diene-3,17-dione (3, Fig. 1) from deoxycholic acid (1, Fig. 1), together with variable levels of its precursors, as judged by examination of culture extracts on t.l.c. All of the strains were DCA[—], 12 α OH-KDE[—], 3 α OH-AK[—] and ADD[—]. None of the strains were completely stable, but three of them, RAL1.22, RAL8.15 and RAL91.11 (Table 2), gave only a low level of 'revertants' after 24 h growth in minimal medium containing glucose and deoxycholic acid. Eight of the 'revertants' were purified, and seven were found to be still kanamycin-resistant. All three of the more stable mutant strains accumulated only 12 β -hydroxyandrosta-1,4-diene-3,17-dione (3, Fig. 1), from deoxycholic acid (1, Fig. 1), with no other intermediates remaining after 48 h incubation. To confirm the identity of the 12 β -hydroxyandrosta-1,4-diene-3,17-dione (3, Fig. 1), RAL91.11 was grown on a larger scale, and the product, after isolation and recrystallization, was shown to have a virtually identical m.p. and i.r. spectrum to those of the 12 β -hydroxyandrosta-1,4-diene-3,17-dione (3, Fig. 1) previously reported (m.p. 228–229 °C; previously reported m.p. 227–228 °C; Leppik, 1981).

The growth of RAL1.22, RAL8.15 and RAL91.11 in

minimal medium containing glucose plus either cholic or chenodeoxycholic acids was examined, and only one product was accumulated in each case. The product from cholic acid had the same R_F and anisaldehyde spray colour as $7\alpha,12\beta$ -dihydroxyandrosta-1,4-diene-3,17-dione on t.l.c., and that from chenodeoxycholic acid had the same R_F and spray colour as 7α -hydroxyandrosta-1,4-diene-3,17-dione.

Mutant strains that accumulate 12β -hydroxysecophenol (5, Fig. 1)

Examination on t.l.c. showed that five of the mutant strains isolated accumulated one major compound from deoxycholic acid (1, Fig. 1), with the same R_F and anisaldehyde spray colour as 12β -hydroxysecophenol (5, Fig. 1). All five strains gave slight growth only on deoxycholic acid or 12α -hydroxy-3-oxochola-1,4-dien-24-oic acid in plates, and virtually no growth on 3α -hydroxy-5 β -androstane-17-one or androsta-1,4-diene-3,17-dione. All of the strains 'reverted' at a low frequency, but all the 'revertants' examined were still kanamycin-resistant. To confirm the identity of the product, the most stable of the isolates, RAL1.25 (Table 2), was grown in shake flasks with deoxycholic acid (1, Fig. 1), and the purified product was shown to have a m.p. and i.r. spectrum virtually identical with that of the 12β -hydroxysecophenol (5, Fig. 1) previously reported (m.p. found, 173–174 °C; reported, 169–170 °C; Leppik, 1981). The growth of three of the strains, RAL1.25, RAL8.16 and RAL20.8 (Table 2), with cholic or chenodeoxycholic acids was examined, and the one major product in each case was found to be $7\alpha,12\beta$ -dihydroxysecophenol from cholic acid, and 7α -hydroxysecophenol from chenodeoxycholic acid, as shown by examination of extracts on t.l.c.

Relationship between *p*-cresol catabolism and bile acid catabolism

In the earlier Tn5 mutageneses performed, the kanamycin-resistant clones were also screened on plates containing *p*-cresol, *p*-hydroxybenzoate, protocatechuate, or benzoate, as well as on the steroid substrates. Mutant strains were obtained which were unable to utilize one or more of the aromatic compounds, but none of these were affected in steroid catabolism. In addition, all the bile acid mutant strains in the four classes described in this paper were unaffected in *p*-cresol catabolism. Thus, the two pathways would appear to be separate. In fact, the mutant results obtained suggest that the *p*-cresol is catabolized via *p*-hydroxybenzoate and protocatechuate, after which this pathway joins the benzoate catabolic path, in the four *Pseudomonas* strains studied.

Because of the results obtained with *p*-cresol, *m*-cresol and 3-ethylphenol were tested. Strains RAL1, RAL20 and RAL91, but not RAL8, were able to utilize both compounds. Mutant strains from these three parent strains which were affected in either the four bile acid mutant classes described above, or were unable to use *p*-cresol, were tested on *m*-cresol and 3-ethylphenol. All mutant strains tested were able to grow on both of these compounds, showing that there is little, if any, overlap between the pathways for the degradation of *m*-cresol and 3-ethylphenol, and the pathways for the degradation of either the bile acids or *p*-cresol, in the *Pseudomonas* strains studied.

DISCUSSION

Previous studies on the degradation of deoxycholic acid (1, Fig. 1) by RAL8 resulted in a bile acid catabolic pathway being proposed (Leppik, 1980), and further results reported here have enabled that pathway to be expanded to that shown in Fig. 1. The pathway has a number of points of similarity with those proposed by other workers in the area, but three differences are worthy of comment. Firstly, as mentioned in the Introduction, this pathway is compatible with findings published for other Gram-negative micro-organisms, but not with those from studies on five Gram-positive micro-organisms. This led to the postulation that two separate pathways for bile acid degradation exist (Leppik, 1980, 1982), the pathway of Fig. 1 for the Gram-negative micro-organisms examined, and an alternative pathway for the Gram-positive micro-organisms. An interesting question is whether the pathway preference of a particular micro-organism could be altered, by mutation for example. Thus, could a Gram-negative strain, blocked in side-chain degradation, be capable of ring degradation, or a Gram-positive strain, blocked in A-ring modification, be capable of side-chain removal? A partial answer to this question will hopefully come from an extension of the mutagenesis work reported in this paper.

The second point is that, in the pathway of Fig. 1, A-ring modification and side-chain removal are shown as occurring independently of each other, an important consideration in the isolation of mutant strains in that part of the pathway. From studies on Gram-positive strains, Hayakawa (1973) had suggested that the introduction of a 4-en-3-one grouping into the A-ring was necessary before side-chain shortening can occur, by those strains capable of such shortening. Tenneson *et al.* (1979a,b) and Bilton *et al.* (1981) extended this suggestion to the Gram-negative strains, as all the compounds they isolated with shortened side-chains contained a 4-en-3-one or a 1,4-dien-3-one A-ring structure. However, Leppik (1982) subsequently isolated compounds such as $3\alpha,12\alpha$ -dihydroxy-23,24-dinor-5 β -cholan-22-oic acid and 12α -hydroxy-3-oxo-23,24-dinor-5 β -cholan-22-oic acid from a deoxycholic acid (1, Fig. 1) fermentation by RAL8. It would thus appear that, in the Gram-negative strains at least, A-ring modification and side-chain removal can occur independently of each other. This proposition has been supported by the results reported here, as the four *Pseudomonas* strains studied were all able to grow on both 12α -hydroxy-3-oxochola-1,4-dien-24-oic acid and 3α -hydroxy-5 β -androstane-17-one, the former compound having a 1,4-dien-3-one A-ring structure, but a full side-chain, and the latter compound having an unreacted 3α -hydroxy group, but no side-chain.

The third point worth commenting on concerns the epimerization of the 12α -hydroxy group to the 12β position. It had been proposed that this epimerization is a necessary prerequisite to complete side-chain removal (Bilton *et al.*, 1977; Tenneson *et al.*, 1978, 1979a). An alternative explanation was offered by Leppik (1980, 1983a), that epimerization is a necessary prelude to 9 α -hydroxylation, possibly to avoid steric hindrance of that reaction. The latter explanation is confirmed here, as three mutant strains have been isolated which are able to grow on chenodeoxycholic acid, 3α -hydroxy-5 β -andros-

tan-17-one and androsta-1,4-diene-3,17-dione, all of which lack a 12-hydroxy group, but which accumulate 12 α -hydroxyandrosta-1,4-diene-3,17-dione (2, Fig. 1) from deoxycholic acid (1, Fig. 1), and 7 α 12 α -dihydroxyandrosta-1,4-diene-3,17-dione from cholic acid, and are unable to grow on 12 α -hydroxy-3-oxochola-1,4-dien-24-oic acid.

The four classes of mutant strains described in this paper extend our understanding of the pathway proposed for the Gram-negative micro-organisms. Similar mutant strains have also been isolated from *P. putida* A.T.C.C. 31752 by Park *et al.* (1984). The first class of three mutant strains described here all accumulate 12 α -hydroxyandrosta-1,4-diene-3,17-dione (2, Fig. 1) from deoxycholic acid (1, Fig. 1), and hence would appear to be blocked in the epimerization of the 12 α -hydroxy group, as discussed above. The fact that these mutant strains are able to utilize completely chenodeoxycholic acid suggests that, if the bile acid genes are clustered into operons, there are no genes downstream of the epimerase gene which are necessary for bile acid degradation.

Evidence for some gene clustering into operons, however, is provided by the one mutant in the second class examined, RAL8.23, as the results suggest that the Tn5 transposon is inserted into, or upstream of, a gene for the utilization of the 3-hydroxy-5,9,17-trioxo-4(5),9(10)-disecoandrosta-1(10),2-dien-4-oic acid-type compounds, and that the epimerase gene is downstream of that gene. To clarify this, cell-free studies would be needed. Relevant to this is the work reported by Gibson *et al.* (1966), who showed the conversion of secocatechol (6, Fig. 1, R=H) into 3-hydroxy-5,9,17-trioxo-4(5),9(10)-disecoandrosta-1(10),2-dien-4-oic acid followed by its cleavage to form the indane acid 3 α H-4 α -(3'-propionic acid)-7 α β -methylhexahydro-1,5-indanedione (9, Fig. 1, R=H), by cell extracts of *N. restrictus*. The other fragment released was shown to be 2-hydroxyhex-2,4-dienoic acid (8, Fig. 1) by Coulter & Talalay (1968). The fact that RAL8.23 accumulates a compound which could be 3,7-dihydroxy-5,9,17-trioxo-4(5),9(10)-disecoandrosta-1(10),2-dien-4-oic acid, and that the indane lactone (10, Fig. 1) has been isolated from a fermentation of deoxycholic acid by the parent RAL8, suggests that this part of the bile acid pathway is analogous, so secocatechol (6), 2-hydroxyhex-2,4-dienoic acid (8) and the indane acid (9) are included in Fig. 1 as postulated intermediates. The point at which the 12 β -hydroxy group is lost, though, is unknown, it being present in the secophenol (5, Fig. 1), but absent from the indane lactone (10, Fig. 1).

The mutant strains in the third class accumulate 12 β -hydroxyandrosta-1,4-diene-3,17-dione (3, Fig. 1) from deoxycholic acid (1, Fig. 1), and hence would appear to be blocked in the 9 α -hydroxylation step. The mutants are phenotypically the same, but could be affected in different genes, as Strijewski (1982) has shown that this hydroxylation is catalysed by an electron-transport chain consisting of three proteins. The postulated product of this hydroxylation, 9 α ,12 β -dihydroxyandrosta-1,4-diene-3,17-dione (4, Fig. 1), could well be unstable, and spontaneously rearrange to form the 12 β -hydroxy secophenol (5, Fig. 1). The mutant

strains of the fourth and final class examined here accumulate the 12 β -hydroxysecophenol (5, Fig. 1), and hence the strains would appear to be blocked in the subsequent hydroxylation step, responsible for the conversion of the 12 β -hydroxysecophenol (5, Fig. 1) to the postulated secocatechol (6, Fig. 1, R=H or OH).

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