***In vitro* formation of a catabolic plasmid carrying *Klebsiella pneumoniae* DNA that allows growth of *Escherichia coli* K-12 on 3-hydroxybenzoate**

Neil D. Robson,[†] Suzanne Parrott and Ronald A. Cooper

Author for correspondence: Ronald A. Cooper. Tel: +44 116 252 3455. Fax: +44 116 252 3369.

Department of Biochemistry,
University of Leicester,
Leicester LE1 7RH, UK

The four enzymes needed to convert 3-hydroxybenzoate to pyruvate and fumarate via the gentisate pathway, as well as a putative positive regulator protein, were encoded on an 8 kb *SphI* fragment of *Klebsiella pneumoniae* DNA. The five genes were clustered in the order regulator–gentisate dioxygenase–fumarylpyruvate hydrolase–3-hydroxybenzoate monooxygenase–maleylpyruvate isomerase (*mhbRDHMI*), with the catabolic genes transcribed in the dioxygenase to isomerase direction. 2-Hydroxybenzoate was found to be a non-metabolizable inducer analogue for the *mhb* genes, supporting the view that gentisate rather than maleylpyruvate was the physiological inducer. The plasmid pNDR20 encoding the full gentisate catabolic pathway endowed *Escherichia coli* with the ability to grow on 3-hydroxybenzoate but the host cell appeared to be responsible for substrate uptake.

Keywords: *Klebsiella pneumoniae*, *Escherichia coli*, gentisate pathway enzymes, gene order, cloning

INTRODUCTION

The bacterial catabolism of 3-hydroxybenzoate can involve either 3,4-dihydroxybenzoate or 2,5-dihydroxybenzoate as the ring fission intermediate, depending on the organism (Wheelis *et al.*, 1967). 3,4-Dihydroxybenzoate can be catabolized via either the β -ketoadipate pathway or the α -ketoacid pathway of aromatic degradation, the genes and enzymes of which have been investigated extensively (Harayama & Timmis, 1989). 2,5-Dihydroxybenzoate is an intermediate in the gentisate pathway of aromatic degradation (Fig. 1); although this pathway has been identified in a variety of soil bacteria (Dagley, 1975) and in the enteric bacteria *Klebsiella pneumoniae* (Jones & Cooper, 1990) and *Salmonella typhimurium* (Goetz & Harmuth, 1992) little is known about the pathway genes.

In this paper we report the cloning, partial analysis and expression in *Escherichia coli* of the *K. pneumoniae* 3-hydroxybenzoate catabolic genes, whose presence endows

E. coli with the ability to grow at the expense of 3-hydroxybenzoate.

METHODS

Plasmids, bacterial strains and growth conditions. pBR322 (Soberon *et al.*, 1980) and pUC18/19 (Norranders *et al.*, 1983) were used as vectors for DNA cloning with *E. coli* K-12 strains NM522 (Gough & Murray, 1983) and 5K (Hubacek & Glover, 1970) as hosts. The *K. pneumoniae* M5a1 strain and the details of cell culture have been described previously (Jones & Cooper, 1990).

Enzyme assays. Methods for the preparation of cell-free extracts and assay of gentisate pathway enzymes have been described (Jones & Cooper, 1990).

Purification of *K. pneumoniae* maleylpyruvate isomerase (EC 5.2.1.4). Ultracentrifuged crude extract was prepared in 20 mM Tris/HCl buffer pH 7.1 as described previously (Jones & Cooper, 1990). This crude extract (25 ml; 175 mg protein) was treated at 0 °C with protamine sulphate solution (40 mg ml⁻¹) at a ratio of 1 mg protamine sulphate for each 5 mg bacterial protein and the resulting precipitate removed by centrifugation at 25 000 *g* and 4 °C. The supernatant was treated again with the same amount of protamine sulphate and the resulting precipitate removed by centrifugation. For purification by FLPC at 22 °C the supernatant was applied to a Mono Q HR 10/10 anion-exchange column equilibrated with

[†] **Present address:** University of Cambridge, Department of Biochemistry, Tennis Court Road, Cambridge, CB2 1QW, UK.

The SWISS-PROT accession number for the N-terminal sequence reported in this paper is P80580.

Fig. 1. The gentisate pathway for 3-hydroxybenzoate catabolism. 3-Hydroxybenzoate (I) is converted to fumarate (VI) and pyruvate (VII) via 2,5-dihydroxybenzoate [gentisate] (II), maleylpyruvate [enol] (III), fumarylpyruvate [enol] (IV) and fumarylpyruvate (V) by the sequential action of 3-hydroxybenzoate monooxygenase (MhbM), 2,5-dihydroxybenzoate dioxygenase (MhbD), maleylpyruvate isomerase (MhbI), a non-enzymic (NE) keto-enol tautomerization and fumarylpyruvate hydrolase (MhbH).

20 mM Tris/HCl buffer pH 7.1. The material that did not bind to the column included the maleylpyruvate isomerase. This material was dialysed against 20 mM Tris/HCl buffer pH 7.5 and reappplied to the Mono Q HR10/10 column equilibrated with this buffer. The maleylpyruvate isomerase now bound to the column and an 80 ml gradient of 0–1 M NaCl in 20 mM Tris/HCl buffer pH 7.5, at a flow rate of 8 ml min⁻¹, was used as eluant and fractions of 8 ml collected. The maleylpyruvate isomerase was eluted at 0.06 M NaCl. The peak maleylpyruvate isomerase fraction was made 1 M with respect to ammonium sulphate and applied to a Phenyl Superose HR 5/5 column equilibrated with 50 mM potassium phosphate buffer pH 7.1 containing 1 M (NH₄)₂SO₄. The (NH₄)₂SO₄ concentration was reduced to zero over a 15 ml gradient at a flow rate of 1 ml min⁻¹ and fractions of 1 ml collected. The maleylpyruvate isomerase was eluted at 0.35 M (NH₄)₂SO₄. A sample (200 µl) of the peak fraction was applied to a Superose 12 HR 10/30 gel filtration column equilibrated against 50 mM potassium phosphate pH 7.1 containing 0.15 M NaCl, eluted at a flow rate of 0.2 ml min⁻¹ and fractions of 0.5 ml collected.

Molecular-mass estimations. Subunit molecular masses were estimated by SDS-PAGE (Laemmli, 1970). The FPLC gel-filtration step was also used to estimate the native molecular mass of the maleylpyruvate isomerase. The Superose 12 column was calibrated using bovine serum albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa) and α-lactalbumin (14.2 kDa).

N-terminal sequencing. The purified maleylpyruvate isomerase was run on a 7.5–20% SDS-polyacrylamide gel, then electroblotted onto a PVDF membrane (Matsudaira, 1987) using 50 mM glycine/50 mM Tris pH 10 and stained with Coomassie brilliant blue R-250. A portion of the stained 25 kDa protein was excised and loaded into an Applied Biosystems 470A gas-phase sequencer without polybrene.

DNA manipulations. *K. pneumoniae* chromosomal DNA was prepared by the method of Chow *et al.* (1977). Small-scale plasmid preparations were carried out as described by Sambrook *et al.* (1989). Restriction endonucleases, T4 DNA ligase, and an exonuclease III deletion kit were used according to the manufacturer's instructions. DNA fragments in low-melting-point agarose gels were isolated as described by Perbal (1988).

Transformations were carried out using MOPS-RbCl (Kushner, 1978).

Chemicals and biochemicals. Oligodeoxyribonucleotides were made with an Applied Biosystems model 380B DNA synthesizer using cyanoethyl-phosphoramidite chemistry. Restriction endonucleases were from Gibco-BRL; the kit for exonuclease III deletion was from Pharmacia. All other chemicals were of the highest purity available commercially.

RESULTS AND DISCUSSION

Maleylpyruvate isomerase N-terminal amino acid sequence

The maleylpyruvate isomerase eluted from the gel filtration column in the last step of the purification as a single symmetrical peak corresponding to a molecular mass of 30 ± 2 kDa. Analysis of this peak by SDS-PAGE (Laemmli, 1970) showed a single major band of 25 kDa that accounted for at least 90% of the protein present. The N-terminal sequence of this 25 kDa protein was found to be: Met-Lys-Leu-Tyr-Ser-Phe-Phe-Asn-Xaa-Arg-Ala. This sequence has been entered in the SWISS-PROT protein sequence database under the accession number P80580.

Isolation of a maleylpyruvate-isomerase-encoding clone

A 23-mer oligodeoxyribonucleotide corresponding to the first eight residues of the N-terminal amino acid sequence was synthesized, using inosine for the third position of the leucine codons and for the second and third positions of the serine codons. Only the first two bases for the asparagine codons were used, to keep the number of different sequence combinations to 64.

The ³²P-labelled degenerate oligodeoxyribonucleotide was used in a dot-blot to probe a size-fractionated *Hind*III total digest of *K. pneumoniae* M5a1 chromosomal DNA. A

Fig. 2. Partial restriction site map of pSP01 and subclones used to locate *mhbMI*. Only the cloned DNA is shown. The locations of *mhbMI* are shown beneath pNDR05. The direction of transcription from the *lac* promoter of pUC is indicated by the arrows. Restriction site abbreviations: B, *Bam*HI; EI, *Eco*RI; Ev, *Eco*RV; H, *Hind*III; Sa, *Sall*; Sp, *Sph*I.

fraction corresponding to approximately 10 kb fragments gave the strongest signal and this DNA was ligated into *Hind*III-digested pBR322. After transformation into *E. coli* strain 5K, ampicillin-resistant colonies were probed with the labelled oligodeoxyribonucleotide and those showing strong hybridization identified. Plasmid DNA was isolated from a selection of 12 such colonies and was analysed by digestion with *Hind*III. Each isolate carried a 9 kb *Hind*III insert. Digestion with *Eco*RI gave either two approximately 7 kb bands (four colonies) or an 11 kb band plus a 3 kb band (eight colonies). These patterns suggested that the *Hind*III fragment had been incorporated in the two possible orientations.

The 12 isolates were grown in Luria-Bertani medium containing ampicillin and 3-hydroxybenzoate. Maleylpyruvate isomerase activity was found in cell-free extracts of all 12 clones, with the amount dependent on the orientation of the *Hind*III fragment. The group of four clones showed 5–10 times higher activity than the group of eight clones. A clone from the highest activity group was selected and its plasmid designated pSP01. A partial restriction site map of pSP01 is shown in Fig. 2.

Cell-free extracts were prepared from *E. coli* 5K(pSP01) grown on glycerol minimal medium in the presence of ampicillin with or without 3-hydroxybenzoate. The maleylpyruvate isomerase activity was high and was not increased by growth in the presence of 3-hydroxybenzoate (data not shown). The extracts also showed 3-hydroxybenzoate monooxygenase activity but none of the other gentisate pathway enzymes could be detected.

A 4 kb *Sph*I–*Hind*III subclone (Fig. 2, coordinates 5–9; pNDR05) of pSP01 still expressed the two 3-hydroxybenzoate catabolic enzymes of the original plasmid but a 2.5 kb *Eco*RI–*Hind*III subclone (Fig. 2, coordinates 6.5–9.0; pNDR02) expressed only the monooxygenase activity, suggesting that the *Eco*RI site was within the maleylpyruvate isomerase gene (*mhbI*) (Fig. 2).

To determine the direction of transcription of the two *mhb* genes, pNDR05 was introduced into *E. coli* NM522 (*lacI^q*) and the cells grown on glycerol minimal medium with or without IPTG. The activity of the monooxygenase and isomerase in cell-free extracts was increased six- to sevenfold when IPTG was present (data not shown), suggesting that the genes were transcribed in the same direction as the *lac* promoter, that is *Hind*III to *Sph*I.

3-Hydroxybenzoate catabolism by *E. coli* 5K(pSP01)

Growth of 5K(pSP01) on glycerol in the presence of 3-hydroxybenzoate led to a brown coloration of the medium, which was probably due to the spontaneous oxidation of accumulated 2,5-dihydroxybenzoate (gentisate) to give melanin-like substances. The conversion of 3-hydroxybenzoate to gentisate was monitored by spectrophotometric scans of culture supernatants, and a shift from a peak absorbance at 290 nm due to 3-hydroxybenzoate to a peak absorbance at 322 nm corresponding to that of gentisate was seen. The culture supernatant could be used in place of authentic gentisate in a gentisate dioxygenase assay, supporting the suggestion that gentisate was being accumulated. 5K(pNDR02) grown under the same conditions also converted 3-hydroxybenzoate to gentisate. This showed that 3-hydroxybenzoate could enter *E. coli* cells and suggested that a clone carrying all the 3-hydroxybenzoate catabolic genes may endow *E. coli* with the novel metabolic ability of growth on 3-hydroxybenzoate.

Cloning the 3-hydroxybenzoate catabolic pathway genes

The monooxygenase and isomerase genes were located together at the *Sph*I end of the *Hind*III insert fragment of pSP01 (Fig. 2), but the other 3-hydroxybenzoate catabolic pathway genes were apparently absent. If all the genes were clustered together the DNA fragment present in pNDR05 would need to be 3–4 kb larger to ensure that, additionally, the dioxygenase, hydrolase and regulatory genes were also present. To see if a single *Sph*I fragment was large enough to include all the genes, *K. pneumoniae* DNA was digested with *Sph*I and a Southern blot probed with the 2.5 kb *Eco*RI–*Hind*III fragment that included the monooxygenase gene (Fig. 2). A single fragment of around 9 kb was detected, indicating that 5 kb of flanking DNA was present. To clone this fragment *Sph*I-digested DNA was run on an agarose gel and the region corresponding to DNA of 8–15 kb was excised and extracted. The resulting DNA was ligated into *Sph*I-digested pBR322 and ampicillin-resistant colonies isolated after transformation into *E. coli* 5K. Two Luria-Bertani-ampicillin plates with 200–300 colonies per plate were replica-plated onto 3-hydroxybenzoate minimal plates and, after overnight incubation at 30 °C, three strongly growing colonies were seen. One of these was selected

Fig. 3. Partial restriction site map of pNDR20 and subclones used to locate *mhbRDH*. pNDR30–33 were produced from pNDR22 by unidirectional exonuclease III digestion. The coordinates shown start at the *Sph*I site of pSP01. The locations of the *mhb* genes are shown beneath pNDR20. The gene boundaries are unknown but the gene letter is placed at a restriction enzyme site that is within the gene, where this is known. Other details are as for Fig. 2.

Table 1. Activities of gentisate pathway enzymes in cell-free extracts

Activities [U (mg protein)⁻¹] are representative values from at least three independent estimations. Enzymes: MhbM, 3-hydroxybenzoate monooxygenase (EC 1.14.13.24); MhbD, gentisate dioxygenase (EC 1.13.11.4); MhbI, maleylpyruvate isomerase (EC 5.2.1.4); MhbH, fumarylpyruvate hydrolase (EC 3.7.1.-).

Bacterial strain	Growth substrate	Enzyme activity			
		MhbM	MhbD	MhbI	MhbH
<i>Escherichia coli</i>					
5K(pBR322)	Glycerol	< 0.001	< 0.001	< 0.001	< 0.001
5K(pNDR20)	Glycerol	0.02	0.08	0.03	0.09
5K(pNDR20)	Glycerol + 3-hydroxybenzoate	0.14	0.64	0.36	0.80
5K(pNDR20)	3-Hydroxybenzoate	0.30	1.42	0.42	1.01
<i>K. pneumoniae</i>	3-Hydroxybenzoate	0.17	1.15	0.24	0.60

and a plasmid designated pNDR20 containing an *Sph*I insert of 8 kb was obtained (Fig. 3).

Cell-free extracts were prepared from *E. coli* 5K(pNDR20) grown on glycerol minimal medium in the presence of ampicillin with or without 3-hydroxybenzoate. As can be seen in Table 1, all four gentisate pathway enzymes were active in the glycerol-grown cells and their activities were increased 7–12 times in the presence of 3-hydroxybenzoate. Activities were undetectable in equivalent extracts prepared from *E. coli* 5K(pBR322).

Mapping of the *mhb* genes

A 4.6 kb *Bam*HI–*Sph*I subclone (Fig. 3, coordinates 8.4–13; pNDR21) of pNDR20 expressed the two gentisate pathway enzymes that were not expressed from pSP01. The 4.0 kb *Hind*III–*Sph*I subclone (Fig. 3, coordinates 9–13; pNDR22) of pNDR21 expressed only the dioxy-

genase gene, indicating that the *Hind*III site was within the fumarylpyruvate hydrolase gene (*mhbH*). The gene order deduced from pSP01 and pNDR20 was therefore *mhbDHMI*.

Although the *mhb* genes of pNDR20 were induced by 3-hydroxybenzoate, locating the regulatory gene was complicated by the fact that the actual inducer appeared to be gentisate or maleylpyruvate rather than 3-hydroxybenzoate, and that accumulated maleylpyruvate was toxic to cells (Jones & Cooper, 1990). Hence it was not possible to test the inducibility of the hydrolase and dioxygenase genes encoded on pNDR21 by addition of gentisate because the maleylpyruvate, formed by the dioxygenase, could not react further and so led to growth inhibition. To overcome this problem 2-hydroxybenzoate was used as a gratuitous inducer. When *E. coli* 5K(pNDR22) was grown on glycerol in the presence of 5 mM 2-hydroxybenzoate, dioxygenase specific activity

Table 2. Influence of host strain on gentisate dioxygenase activity expressed from various plasmids

Crude extracts were prepared from glycerol-grown cells; activities [U (mg protein)⁻¹] are representative values from a minimum of three separate estimations. Plasmids are described in Fig. 3 and the text. ND, Not determined.

Host strain	Inducer*	Gentisate dioxygenase (MhbD) activity				
		pNDR22	pNDR30	pNDR31	pNDR32	pNDR932
5K	—	0.29	7.2	11.7	15.9	ND
5K	+	2.03	5.2	8.3	17.9	ND
NM522	—	0.36	0.49	0.47	0.56	0.38
NM522	+	3.31	0.80	0.54	0.69	0.35

* 2-Hydroxybenzoate (5 mM).

increased sevenfold (Table 2). To locate the regulatory gene the insert DNA of pNDR22 was digested from the *SphI* end with exonuclease III to produce a series of deletions. Plasmid pNDR30, in which some DNA within coordinates 12–13 (Fig. 3) was deleted, with *E. coli* 5K as host, gave a very high dioxygenase specific activity during growth on glycerol which decreased slightly, rather than increased, when 2-hydroxybenzoate was included (Table 2). Deletion of DNA to coordinate 11 (pNDR33, Fig. 3) resulted in loss of dioxygenase activity, so defining the end of *mhbR* on the assumption that *mhbD* and *mhbR* are immediately adjacent genes. This loss of regulation meant that the relative order of the 3-hydroxybenzoate catabolic structural and regulatory genes was established as *mhbRDHMI*.

Mode of action of the regulatory gene

With *E. coli* 5K as host, plasmids with increasing deletions upstream of the dioxygenase gene, whose 5' end was therefore being brought closer to the pUC18 *lac* promoter, produced correspondingly greater activities of dioxygenase (Table 2). This apparent high constitutive expression in the absence of the regulator gene could indicate negative control. However, strain 5K contains the normal *lacI* gene, whose product would be unable to interact effectively with the regulatory regions of all the copies of the pUC18 plasmid, thereby leading to apparent high constitutive production of the dioxygenase due to its expression via the thus unregulated *lac* promoter. However, when the various plasmid deletions were introduced into *E. coli* NM522 expressing the *lacI^q* super-repressor gene, the amounts of dioxygenase produced were much reduced and were not affected significantly by increasing deletion (Table 2). That the dioxygenase expression in strain NM522 as host occurred mainly from the *mhb* promoter region rather than from the vector promoter was shown by the *mhbD* expression seen after re-cloning the insert fragment of pNDR32 into pUC19 to produce pNDR932. This reversed the orientation of the insert fragment with respect to the *lac* promoter of the vector such that their directions of transcription would now be

opposed. These results showed low, constitutive expression of *mhbD* in the absence of the regulator gene *mhbR*, compatible with the regulator gene encoding a positive activator of *mhb* structural gene expression.

Comparison of 3-hydroxybenzoate catabolism by *K. pneumoniae* with that of *E. coli* expressing the *mhb* genes

pNDR20 endowed *E. coli* 5K with a novel ability to grow at the expense of 3-hydroxybenzoate. The effectiveness of this was assessed by comparing 3-hydroxybenzoate catabolism of *E. coli* 5K(pNDR20) with that of *K. pneumoniae* M5a1. During exponential growth the doubling time (t_d) for 5K(pNDR20) was 118 min, which was about 50% greater than for M5a1 (t_d = 72 min). Cell-free extracts were assayed for the 3-hydroxybenzoate catabolic enzymes to seek an explanation of this slightly slower growth rate. Table 1 shows that the activities from *E. coli* 5K(pNDR20) were slightly greater than those of *K. pneumoniae* M5a1. However, in addition to the catabolic enzymes, uptake of 3-hydroxybenzoate is required for growth. Since pNDR02, which encodes the complete monooxygenase gene and parts of the isomerase and hydrolase genes, is theoretically too small also to encode a permease but nevertheless still endows *E. coli* 5K cells with the ability to convert 3-hydroxybenzoate to gentisate, it seems that uptake must be a property of the *E. coli* host. Which *E. coli* permease is involved is unknown but the general or specific aromatic amino acid uptake systems or the various systems for the uptake of the catechol siderophore enterochelin are likely contenders. Thus, the limiting factor that accounts for the somewhat slower growth on 3-hydroxybenzoate of *E. coli* 5K(pNDR20) compared to *K. pneumoniae* M5a1 may be the rate of substrate uptake.

ACKNOWLEDGEMENTS

We are indebted to Dr M. Davison for the amino acid sequencing, Mr J. Keyte for oligodeoxyribonucleotide synthesis, Mr J. M. Stringfellow for drawing the figures and Mrs

U. Gervind-Richards for producing the manuscript. N.D.R. thanks the Science and Engineering Research Council for a research studentship.

REFERENCES

- Chow, L. T., Kahmann, R. & Kemp, D. (1977).** Electron microscopic characterisation of DNAs of non-defective deletion mutants of bacteriophage Mu. *J Mol Biol* **155**, 113–121.
- Dagley, S. (1975).** A biochemical approach to some problems of environmental pollution. In *Essays in Biochemistry*, vol. 11, pp. 81–138. Edited by P. N. Campbell & W. N. Aldridge. London, New York & San Francisco: Academic Press.
- Goetz, F. E. & Harmuth, L. J. (1992).** Gentisate pathway in *Salmonella typhimurium*: metabolism of *m*-hydroxybenzoate and gentisate. *FEMS Microbiol Lett* **97**, 45–50.
- Gough, J. A. & Murray, N. E. (1983).** Sequence diversity among related genes for recognition of specific targets in DNA molecules. *J Mol Biol* **166**, 1–19.
- Harayama, S. & Timmis, K. N. (1989).** Catabolism of aromatic hydrocarbons by *Pseudomonas*. In *Genetics of Bacterial Diversity*, pp. 151–174. Edited by D. A. Hopwood & K. E. Chater. New York: Academic Press.
- Hubacek, J. & Glover, S. W. (1970).** Complementation analysis of temperature-sensitive host specificity mutations in *Escherichia coli* C. *J Mol Biol* **50**, 111–127.
- Jones, D. C. N. & Cooper, R. A. (1990).** Catabolism of 3-hydroxybenzoate by the gentisate pathway in *Klebsiella pneumoniae* M5a1. *Arch Microbiol* **154**, 489–495.
- Kushner, S. R. (1978).** An improved method for the transformation of *Escherichia coli* with ColE1-derived plasmids. In *Genetic Engineering*, pp. 17–23. Edited by H. B. Boyer & S. Nicosia. Amsterdam: Elsevier.
- Laemmli, U. K. (1970).** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Matsudaira, P. (1987).** Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J Biol Chem* **262**, 10035–10038.
- Norrande, J., Kempe, T. & Messing, J. (1983).** Construction of improved M13 vectors using oligonucleotide-directed mutagenesis. *Gene* **26**, 101–106.
- Perbal, B. (1988).** *A Practical Guide to Molecular Cloning*, pp. 368–374. Chichester: John Wiley.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Soberon, X., Covarrubias, L. & Bolivar, F. (1980).** Construction and characterisation of new cloning vehicles. IV. Deletion derivatives of pBR322 and pBR325. *Gene* **9**, 287–305.
- Wheelis, M. L., Palleroni, N. J. & Stanier, R. Y. (1967).** The metabolism of aromatic compounds by *Pseudomonas testosteroni* and *P. acidovorans*. *Arch Mikrobiol* **59**, 302–314.

Received 27 February 1996; revised 22 April 1996; accepted 24 April 1996.