An effective technique for enrichment and isolation of *Candida cloacae* mutants defective in alkane catabolism

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(Received 9 November 1989; revised 7 March 1990; accepted 27 March 1990)

Techniques are described which allow mutated populations of *Candida cloacae* to be enriched efficiently (up to 167-fold in one round of enrichment) for mutants deficient in the alkane degradation pathway (Alk^-). Such mutants, as well as being of scientific importance in studies of the degradation pathway, are also of commercial interest because several of the degradative intermediates are of value to the chemical industry. The Alk^- mutants were readily isolated by their inability to grow on agar plates supplied with hexadecane as sole carbon source. A total of 288 Alk^- mutants were isolated from, effectively, 4×10^6 mutagen-treated cells. They were further characterized by replica-plating using palmitic acid (PA) or acetate (Ac) as sole carbon source. Preliminary screening studies showed that of the 84 $\text{Alk}^- \text{PA}^- \text{Ac}^+$ mutants, most could accumulate dicarboxylic acids from hexadecane and palmitic acid and at least one mutant also produced 3-hydroxyhexadecanedioic acid. Of the 80 mutants characterized as $\text{Alk}^- \text{PA}^+$, 16 produced small amounts of hexadecanol.

Introduction

Over the years a number of groups have studied the alkane degradation pathway (Fig. 1) which exists in various micro-organisms, notably the yeast *Candida* (for recent reviews see Rehm & Reiff, 1981; Boulton & Ratledge, 1984). Whether the aim was to study biochemistry and genetics of the pathway (Yi & Rehm, 1982*a, b, c*; Bassel & Mortimer, 1973; Bassel & Ogrydziak, 1979) or to exploit it by causing accumulation of commercially valuable intermediates (Uchio & Shiio, 1972*a, b*; Ogata *et al.*, 1973; Furukawa *et al.*, 1986; Uemura, 1985; Taoka, 1986), generation of mutants deficient in alkane degradation (Alk^-) has played a vital role. However, none of these groups employed a mutant enrichment technique, despite the frequency of appropriate mutants being very low. For example Bassel & Mortimer (1973) reported that a mutated population of *Candida lipolytica* yielded only 5 Alk^- strains from 60 000 clones examined.

The lack of an effective enrichment technique severely restricts the efficiency of these types of studies. The objective of this work, therefore, was to develop powerful enrichment techniques to enable isolation of large numbers of Alk^- strains, thereby greatly improving the likelihood of obtaining strains blocked in each of the various steps of the pathway. Whilst powerful techniques such as penicillin enrichment are available for isolation of bacterial mutants, equivalent techniques of antibiotic

enrichment have been used only on a more limited scale in eukaryotic micro-organisms. The methods described for eukaryotes have been limited to enrichment of amino acid or adenine auxotrophs (Snow, 1966). Indeed no

Fig. 1. Pathway of alkane degradation in *Candida* species. In micro-organisms of this type, oxidation occurs from both ends of the alkane molecule.

successful method has been described for efficient enrichment of eukaryotic mutants defective in the degradation of a carbon source. The reason for this (at least for *Candida* yeast) is that the commonly held belief that antibiotics such as nystatin are toxic to growing cells, but less toxic to non-growing cells, is incorrect. Thus, attempts by Yano *et al.* (1981) to enrich for Alk^- *Candida* mutants met with very limited success.

This paper shows that the key to antibiotic sensitivity of *Candida* yeast cells is the nutrient status of the cells, particularly the nitrogen level. A procedure for efficient isolation of large numbers of Alk^- mutants of *C. cloacae* is described along with preliminary studies on the properties of these mutants.

Methods

Organisms. The wild-type organism used for the generation of mutants was *Candida cloacae* P410 (Ajinomoto reference number; Akabori *et al.*, 1972). This strain and the mutants derived from it were maintained on malt extract agar (Oxoid) slopes.

Growth of cells. Cultures were inoculated with a loopful of cells and incubated with shaking at 30 °C in 20 ml volumes of double-strength Yeast Nitrogen Base (YNB; Difco), using glucose (50 g l⁻¹) as the carbon source. Growth was generally allowed to continue until stationary phase (about 2 d). The resultant cells were carbon-limited and a biomass of about 20 g l⁻¹ was reached.

Biomass was measured either directly by dry weight determination or by optical density measurements of suitably diluted cell suspensions.

Estimation of viable cell number. This was done by serial dilution into peptone water and plating 0.1 ml volumes onto malt extract agar.

Model system used to develop the enrichment procedure. Stationary-phase cells were washed, resuspended in YNB (in some experiments YNB + amino acids was used) to give a cell density of 10⁸ cells ml⁻¹ (OD₆₀₀ 1.0) and 20 ml was placed in baffled 250 ml conical flasks. To the control flasks water (0.4 ml) was added and to the test flasks 0.4 ml of carbon substrate [glucose (60%, w/v), sodium acetate (60%, w/v) or hexadecane] was added. After 2–4 h incubation at 30 °C the test culture was beginning to divide actively whereas the control cells showed no change. In this way the control cultures, with no carbon source, simulated mutants which had lost the ability to utilize the substrate whereas the test cultures, supplied with a carbon source, represented wild-type cells having a fully functional pathway. After 4 h preincubation an appropriate volume of antibiotic solution was added and incubation continued. At suitable intervals samples were taken for estimation of viable cell numbers.

Mutagenesis. Stationary-phase cultures (20 ml) were harvested, washed and resuspended in 0.1 M-potassium phosphate buffer pH 7.5. Cell density at this stage was about 7.5 × 10⁹ cells ml⁻¹ (OD₆₀₀ 75). *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was added to the suspension at a final concentration of 0.5 mg ml⁻¹. One-third portions of the culture were removed at 10, 20 and 30 min and added to 5 vol 0.1 M-sodium citrate buffer (pH 4) to inactivate NTG. Cells were washed twice in 0.05 M-potassium phosphate buffer and the three portions were individually suspended in YNB and then mixed to give a final volume of 20 ml. Total viable cell count at this stage was about 5 × 10⁸ ml⁻¹ (about 2.5 g biomass l⁻¹). Glucose (5 g l⁻¹ final concentration) was added to the culture and incubation carried out overnight to allow fixation of the mutations. The level of the carbon

source was important with respect to the surviving cell numbers in that it would allow at least one cell division but would not allow significant replication of mutant types.

Enrichment of mutated cultures for Alk^- phenotypes. The mutated cell population was washed and resuspended in a nitrogen-free minimal salts medium (MgSO₄·7H₂O, 0.5 g l⁻¹; KH₂PO₄, 1.5 g l⁻¹; ZnSO₄·7H₂O, 10 mg l⁻¹) to an OD₆₀₀ of 9.0. Glucose (2.5 g l⁻¹ final concentration) was then added and the culture incubated overnight to allow the cells to reach the level of nitrogen limitation critical for mutant enrichment. The culture was then washed and resuspended in YNB (plus amino acids) to a cell density of 10⁸ cells ml⁻¹ (OD₆₀₀ 1.0). Several millilitres were removed for a control and the rest of the culture divided into three portions. Each portion was added to a baffled conical flask (up to 30 ml per flask) and then supplied with hexadecane at 20 g l⁻¹ final concentration. No hexadecane was supplied to the control sample. After 4 h incubation at 30 °C a small amount (10 mg) of sophorolipid was added to each flask, and also to the control culture, and incubation continued for a further 15 min. Nystatin was added to each flask, and to the control, at a final concentration of 7 µg ml⁻¹ and incubation continued for 45, 90 or 120 min. At each of these times cells were harvested from one of the flasks and the viability measured. A sample was also removed from the control at each time interval and again viable cell number measured. In this way values were obtained for the differential kill ratio between substrate-free, non-growing cells and those supplied with hexadecane. This value reflected the degree to which the mutated cultures were enriched in Alk^- mutants.

Selection. The replica-plating technique was used. Master plates were prepared on malt extract agar and these acted as templates for velvet pad replications onto YNB plates containing as carbon source either glucose (10 g l⁻¹) or hexadecane (supplied in the vapour phase by placing a filter paper soaked in hexadecane in the lid of the Petri dish). After 4–5 d growth the Alk^- mutants could be identified by comparison of the two types of plates.

Mutants were picked off with a needle and stab inoculated onto fresh malt extract plates (50 per plate). After overnight growth these new master plates were used for replication onto a range of YNB plates containing glucose, hexadecane, sodium acetate, sodium palmitate or Tween 40 as carbon source. Tween 40 acted as a readily available form of palmitic acid and in all cases growth characteristics on Tween 40 and sodium palmitate were the same.

Screening of mutants for product accumulation. Screening was carried out at a 20 ml scale in 250 ml baffled conical flasks. The medium was double-strength YNB with sorbitol (20 g l⁻¹) as carbon source, and either hexadecane (35 g l⁻¹) or sodium palmitate (20 g l⁻¹) as substrate. The flasks were inoculated with 1 ml of a starter culture (yeast extract, 0.5% w/v; glucose, 1%, w/v) of each mutant and incubated for 4 d at 30 °C.

After incubation, myristic acid (1 mg ml⁻¹ final concentration) was added and the contents of each flask adjusted to pH 2 and extracted with ethyl acetate (50 ml). These extracts were taken to dryness and dissolved in methanol (10 ml), 3 ml of which was methylated by refluxing with boron trifluoride (BF₃) in methanol (2 ml). Water (5 ml) was added and methyl esters were extracted with dichloromethane (5 ml). After evaporation to dryness, the sample was silylated by dissolving in pyridine (1 ml) and adding hexamethyldisilazane (0.5 ml) and trimethylchlorosilane (0.3 ml). Samples were then analysed using a Varian gas chromatograph fitted with a glass column packed with 3% OV-1 on 80–100 Chromosorb WHP. Where possible, compounds were characterized by comparison of chromatographic properties against authentic standards. Novel products were characterized by GC/MS and by NMR.

Large-scale incubation for production of the novel compound. Mutant 4G 2e 17 was incubated in 750 ml double-strength YNB medium

containing sorbitol (20 g l^{-1}) and hexadecane (35 g l^{-1}) in a Bioengineering fermenter for 5 d at 30°C with agitation (800 r.p.m.) and with the pH controlled at 7.0. The fermentation was carried out aerobically using an air flow rate of 150 ml min^{-1} ($0.2 \text{ vol. vol.}^{-1} \text{ min}^{-1}$). The novel compound which accumulated in the medium was characterized by NMR spectroscopy.

The medium was acidified to pH 2 and extracted with ethyl acetate (2:5 l). After evaporation to dryness, 1.6 g of material was obtained of which about 0.6 g was the unknown compound. The sample was refluxed in BF_3 in methanol (25 ml) and the esters were extracted with dichloromethane (50 ml) and applied to a column of Kieselgel 60 (Merck) slurried in dichloromethane. The column was eluted with dichloromethane/hexane (75:25, v/v) ($2 \times 50 \text{ ml}$ fractions), dichloromethane ($10 \times 20 \text{ ml}$ fractions) and finally dichloromethane/methanol (99:1, v/v) ($40 \times 10 \text{ ml}$ fractions). The fractions were analysed by TLC and visualized by spraying with 10% (w/v) phosphomolybdic acid in methanol followed by charring. The unknown compound eluted in dichloromethane/methanol (99:1, v/v). The purest fractions were pooled and a total of 217 mg of the compound was obtained. GLC analysis indicated about 90% purity. This compound was analysed by both GC/MS and NMR spectroscopy.

Reagents. Nystatin, amphotericin B and NTG were purchased from Sigma. Sophorolipid is a microbial biosurfactant (Spencer *et al.*, 1979) and was produced in this laboratory.

Results

Development of an enrichment procedure

Effect of antibiotics on growing and non-growing cells of *C. cloacae*. Stationary-phase cells (carbon-limited) of *C. cloacae* suspended in YNB and allowed to remain in the resting phase by omission of a carbon source were as sensitive to the lethal effects of nystatin ($3\text{--}10 \mu\text{g ml}^{-1}$) as were cells which were allowed to grow by addition of glucose as carbon source (Fig. 2). Similar results were obtained with amphotericin B, and when the carbon source was acetate or hexadecane. The possibility that nystatin and amphotericin B were only effective against respiratorily active cells (Moat *et al.*, 1959) was discounted because use of acetate as the carbon source did not lead to an increased rate of cell death.

Whilst microscopic examination and absorbance measurements did not detect cell growth in the absence of a carbon source, it was conceivable that the lack of differential kill between growing and resting cells was due to endogenous carbon storage compounds allowing some metabolic activity in the control cells not supplied with a carbon source. To ensure that the cells were depleted of carbon storage compounds, cell populations were starved by overnight incubation in either phosphate buffer or YNB. However, there was still no significant difference in antibiotic resistance between starved resting cells and those which were actively growing prior to antibiotic addition.

Similar experiments to the above were also carried out using 2-deoxyglucose, a compound reported to be lethal to growing cells of *Schizosaccharomyces pombe* (Megnet,

Fig. 2. Effects of nystatin on resting cells (○) (suspended in YNB minus carbon source) and growing cells (●) (preincubated for 3 h in YNB with $5 \text{ g glucose l}^{-1}$).

Table 1. Antibiotic sensitivity of nitrogen-depleted cells

Different degrees of nitrogen depletion were achieved by 6 h preincubation of stationary-phase cells ($\text{OD}_{600} 9.0$) in a nitrogen-free salts solution containing glucose at 0, 2.5, 5.0, 7.5 or 10 g l^{-1} . Cells were then washed and resuspended in YNB to a cell density of $2 \times 10^8 \text{ cells ml}^{-1}$ and 20 ml portions dispensed into 250 ml conicals. Amphotericin B ($40 \mu\text{g ml}^{-1}$) or nystatin ($7 \mu\text{g ml}^{-1}$) was added, and after incubation for 1 h (nystatin) or 3.5 h (amphotericin B), samples were removed for analysis of viable cell number.

Glucose in preincubation medium (g l^{-1})	Viable cell numbers ml^{-1}	
	Amphotericin B	Nystatin
0	7.8×10^4	4.0×10^4
2.5	2.9×10^6	2.8×10^7
5.0	2.9×10^6	2.8×10^7
7.5	2.7×10^6	2.5×10^6
10	8.0×10^5	3.6×10^5

1965). However, concentrations up to 1 g l^{-1} and contact times up to 45 h showed no lethal effect on either carbon-starved cells or cells growing on hexadecane as carbon source (results not shown).

Effect of nitrogen starvation on sensitivity to nystatin and amphotericin B. Cell preparations of differing degrees of nitrogen depletion were resuspended in YNB and their resistance to amphotericin B and nystatin was measured (Table 1). Both antibiotics exerted a severe lethal effect on nitrogen-rich cells (pre-incubated without glucose) but cells which had been nitrogen-starved, by incubation with glucose in the absence of nitrogen, were more resistant. The most dramatic differences were observed with nystatin. However, the amount of glucose used in the starvation period was critical. For a cell density of $\text{OD}_{600} 9.0$ (about $9 \times 10^8 \text{ cells ml}^{-1}$) the optimum amount of glucose was $2.5\text{--}5.0 \text{ g l}^{-1}$. Higher concentrations substantially reduced the survival abilities of the cells and therefore the degree of differential killing relative to the nitrogen-rich control cells.

Fig. 3. Effect of hexadecane on antibiotic sensitivity of nitrogen-depleted cells. The cell suspension (OD_{600} 9.0) was nitrogen-depleted by incubation in a nitrogen-free salts solution supplied with glucose (2.5 g l^{-1}) for 3 h. The cells were then washed and resuspended in YNB to OD_{600} 1.0. The culture was divided and hexadecane (2%, v/v) added to one portion. After 4 h incubation, nystatin ($7 \mu\text{g ml}^{-1}$) was added to both portions and samples were taken regularly for measurement of cell viability. Maximum differential kill ratio was 91 at 0.5 h. ○, No carbon source; ●, hexadecane added as carbon source.

Effect of hexadecane carbon source on antibiotic sensitivity of nitrogen-depleted cells. At the optimum level of nitrogen depletion, cells resuspended in YNB and then supplied with hexadecane as carbon source were more sensitive to nystatin than were cells left in the resting phase with no carbon source (Fig. 3). This experimental model simulated a mutagen-treated mixed population where the cells supplied with hexadecane represent wild-type cells and those with no carbon source represent Alk^- mutants. The duration of exposure to antibiotic was important; maximum kill of the hexadecane-supplied cells occurred at 30 min, giving a differential kill ratio of 90.

Further optimization of the differential kill ratio. Whilst nitrogen depletion of the cells was essential to achieve a high differential kill ratio, various other parameters were found to contribute to this effect. Table 2 clearly shows the importance of using stationary-phase rather than exponential-phase cultures. The low differential kill ratios obtained with exponential-phase cultures were due to the control cells (no hexadecane, i.e. simulating Alk^- mutants) not having acquired the same degree of antibiotic resistance as had stationary-phase control cultures.

The inclusion of amino acids in the YNB suspension medium increased the differential kill ratio, largely by increasing the antibiotic sensitivity of the test cells (those supplied with hexadecane, i.e. simulating wild-type cells).

Use of the surfactant sophorolipid had a marked effect on the differential kill ratio, although the stage at which this compound was added was critical. When it was added at the start of the hexadecane preincubation period, the antibiotic resistance of the test cells was increased and the differential killing effect was reduced. If, however, it was added towards the end of the hexadecane preincubation period, the resistance of the control cells was increased whilst the death rate of the test cells was unaltered, resulting in a significant increase in the differential kill ratio. An additional effect of sophorolipid was to break up cell flocs which form with an alkane carbon source, thus making sampling far easier.

Based on the results obtained with the model system a procedure was devised for enriching NTG-treated populations of *C. cloacae* in Alk^- mutants.

Isolation and properties of Alk^- mutants

Selection of phenotypes. In four separate experiments using NTG-treated cultures an average differential kill ratio of 150 was achieved. About 25000 colonies were examined by replica plating onto hexadecane plates and 288 Alk^- mutants were isolated. Phenotypes were further investigated by plating onto YNB containing either palmitic acid or sodium acetate as carbon source. The majority of the Alk^- mutants (208) were unable to grow on palmitic acid. Of these strains, 124 were also unable to grow on acetate ($\text{Alk}^- \text{ PA}^- \text{ Ac}^-$) and were deemed to be respiratory-deficient mutants. The remainder (84) utilized acetate ($\text{Alk}^- \text{ PA}^- \text{ Ac}^+$) and were deemed to be β -oxidation-deficient mutants. Eighty mutants were able to grow on palmitic acid ($\text{Alk}^- \text{ PA}^+$) and were presumably blocked at an early step in alkane degradation.

Properties of $\text{Alk}^- \text{ PA}^- \text{ Ac}^+$ mutants. The properties of these 84 strains were investigated by screening for product formation from hexadecane or palmitic acid.

The bulk (75) of these mutants produced hexadecanedioic acid (up to 7 g l^{-1}), consistent with their having a perturbed β -oxidation pathway. Eleven of these strains also produced smaller amounts of a series of shorter chain length dicarboxylic acids, indicating a partially functional β -oxidation pathway. Dicarboxylic-acid-producing strains generally gave hexadecanedioic acid from both hexadecane and palmitic acid, although some could only accumulate product from one substrate.

Time-course studies with one of these mutants showed that hexadecanedioic acid reached a maximum concentration of 7 g l^{-1} after 45 h. Another compound was apparent which continued to increase up to 70–80 h, reaching a maximum concentration of 2.5 g l^{-1} . This

Table 2. Factors influencing the differential kill ratio

Stationary-phase or early exponential-phase cultures were harvested and depleted for nitrogen as described in Methods. Cells were resuspended in YNB or YNB plus amino acids then divided into 20 ml portions (1×10^8 cells ml^{-1}) for the various treatments. Test cells were preincubated with hexadecane (2%, v/v) for 4 h then nystatin ($7 \mu\text{g ml}^{-1}$) added to both test and control cells. Where indicated sophorolipid (10 mg per flask) was added either at the point of hexadecane addition or 15 min prior to nystatin addition. Cell viabilities were estimated 1 h after the addition of nystatin.

Growth phase	Suspension medium and additions	Carbon source	Viable cells ml^{-1} after nystatin treatment	Differential kill ratio
Stationary	YNB	None (control)	2.5×10^7	92
		Hexadecane	2.7×10^5	
Exponential	YNB	Control	2.1×10^6	54
		Hexadecane	3.9×10^5	
Stationary	YNB + amino acids	Control	1.3×10^7	162
		Hexadecane	8×10^4	
Stationary	YNB; sophorolipid added 15 min prior to nystatin	Control	9.7×10^7	167
		Hexadecane	5.8×10^5	
Stationary	YNB; sophorolipid added at point of hexadecane addition	Control	7.5×10^7	12
		Hexadecane	6.1×10^6	

compound had different GLC properties from any of the metabolites in Fig. 1. Ability to form a methyl ester and a silyl ether methyl ester was consistent with this compound having carboxylic acid group(s) and hydroxyl group(s). Mass spectroscopy of the silyl ether methyl ester showed its M_r to be 402 and the major ions at m/e 175, 159 and 133 confirmed the presence of a terminal 3-hydroxymethyl carboxylate. These data are consistent with the structure being 3-hydroxyhexadecanedioic acid.

Properties of $\text{Alk}^- \text{PA}^+$ mutants. Isolates of this phenotype were presumably mutated at an early step of the alkane degradative pathway, and they were screened for product accumulation from hexadecane. Many of these isolates (64) could not metabolize the substrate and failed to accumulate any product. However, 16 of the isolates produced small amounts of hexadecanol, consistent with a loss of alcohol dehydrogenase function. Nine of these also produced a series of chain-shortened mono- and dicarboxylic acids, but seven gave a relatively pure hexadecanol product, albeit at low levels, the best being 82 mg l^{-1} .

When these strains were incubated with palmitic acid substrate it was fully utilized and gave no measurable accumulation of intermediates.

Discussion

The techniques developed in this study make it possible to isolate rapidly, in large numbers, the scientifically and commercially valuable Alk^- mutants. It is likely that

these techniques can be applied to the whole range of nystatin-sensitive micro-organisms and are valid for the isolation of mutants defective in catabolism of any carbon source. Because only one round of enrichment need be employed there is little opportunity for mutant cloning and therefore the vast majority of the isolates are generated from separate mutational events. This of course maximizes efficiency during the subsequent laborious shake flask screening work.

The experiments showed that the essence of achieving a high differential kill ratio was to starve the cells for nitrogen and then resuspend them in a nitrogen-containing medium with hexadecane as carbon source. Wild-type cells quickly degraded the alkane and with the energy released re-established their cellular nitrogen levels and became sensitive to the antibiotics. Mutants deficient in the alkane degradation pathway, however, could only re-establish their cellular nitrogen level at a much slower rate and therefore remained resistant to the antibiotics. The amount of glucose used in the starvation period was critical. If too much was used the cells were re-sensitized to the antibiotics (Table 1), possibly because carbon storage compounds were accumulated by the nitrogen-deficient cells which could then be used as an energy source on subsequent resuspension in YNB medium.

Other factors could quite dramatically influence the differential kill ratio. It was beneficial, for example, to include amino acids in the resuspension medium, presumably because this allowed a more efficient regain of cellular nitrogen levels by the wild-type cells. The

presence of sophorolipid could protect mutant cells from antibiotic attack, perhaps in a way analogous to surfactant protection of yeast cell membranes from damage by dehydration (Chen *et al.*, 1966). If, however, the sophorolipid was added prior to hexadecane addition it prevented the wild-type cells from becoming sensitized, probably because it prevented hexadecane uptake. It is known that sophorolipid disrupts the physical interaction which occurs between yeast cells and hydrocarbon droplets which may be fundamental to alkane uptake (see review by Boulton & Ratledge, 1984). Sophorolipid was chosen because it is not metabolized by *Candida* (J. Casey, unpublished observations).

A preliminary investigation into the nature of the different phenotypes showed that at least one of the $\text{Alk}^- \text{PA}^- \text{Ac}^+$ strains (presumably β -oxidation-negative mutants), when provided with hexadecane or palmitic acid, accumulated not only hexadecanedioic acid but also 3-hydroxyhexadecanedioic acid. Whilst production of dicarboxylic acids by such mutants was a common feature in this study, and indeed has been observed by several groups (Uchio & Shio, 1972*a, b*; Ogata *et al.*, 1973; Furukawa *et al.*, 1986; Uemura, 1985; Taoka, 1986), only one recent study has reported production of 3-hydroxydicarboxylic acid (Hill *et al.*, 1986).

The other phenotype was $\text{Alk}^- \text{PA}^+$, these mutants presumably being deficient at an early step of the pathway. The majority of these isolates were unable to metabolize hexadecane and were presumably unable to transport it into the cells; or perhaps they were deficient in alkane hydroxylase. Sixteen of the mutants were able to produce small amounts of hexadecanol and may have been deficient in fatty alcohol dehydrogenase. Surprisingly, in every case the level of accumulation of hexadecanol was relatively low, the highest being 82 mg l^{-1} . A low level of nonanol production from nonane was also reported to occur using alcohol dehydrogenase negative mutants of *Pseudomonas putida* (Fish *et al.*, 1982). Possibly alkanols have an inhibitory or regulatory effect, or perhaps, in these experiments, the conditions of incubation were simply inappropriate for hexadecanol production or excretion. Accumulation of hexadecanediol might have been expected but none was found.

Our future work will be directed at optimizing product accumulation by the strains already described coupled with further mutation studies, using different parental organisms, to isolate other potentially interesting strains.

References

- AKABORI, S., SHIO, I. & UCHIO, R. (1972). Japanese Patent 1300455.
 BASSEL, J. & MORTIMER, R. (1973). Genetic analysis of mating type and alkane utilisation in *Saccharomyces lipolytica*. *Journal of Bacteriology* **114**, 894–896.

- BASSEL, J. & OGRYDZIAK, D. M. (1979). Genetics of *Saccharomyces lipolytica* with emphasis on genetics of hydrocarbon utilisation. In *Genetics of Industrial Microorganisms*, pp. 160–165. Edited by O. K. Sebek & A. I. Laskin. Washington, DC: American Society for Microbiology.
 BOULTON, C. A. & RATLEDGE, C. (1984). The physiology of hydrocarbon-utilising microorganisms. In *Introduction to Topics in Enzyme and Fermentation Biotechnology*, vol. 9, pp. 11–77. Edited by A. Wiseman. Chichester: Ellis Horwood.
 CHEN, S. L., COOPER, E. J. & GUTMAN, S. (1966). Active dry yeast: protection against oxidative deterioration during storage. *Food Technology* **20**, 1585–1608.
 FISH, N. M., ALLENBY, D. J. & LILLY, M. D. (1982). Oxidation of n-alkanes: growth of *Pseudomonas putida*. *European Journal of Applied Microbiology and Biotechnology* **14**, 259–262.
 FURUKAWA, T., MATSUYOSHI, T. & KISE, S. (1986). Selection of high brassylic acid producing strains of *Torulopsis candida* by single-cell cloning and by mutation. *Journal of Fermentation Technology* **64**, 97–101.
 HILL, F. F., VENN, I. & LUKAS, K. L. (1986). Studies on the formation of long-chain dicarboxylic acids from pure n-alkanes by a mutant of *Candida tropicalis*. *Applied Microbiology and Biotechnology* **24**, 168–174.
 MEGNET, R. (1965). Screening of auxotrophic mutants of *Schizosaccharomyces pombe* with 2-deoxyglucose. *Mutation Research* **2**, 328–331.
 MOAT, A. G., PETERS, N. & SRB, A. (1959). Selection and isolation of auxotrophic yeast mutants with the aid of antibiotics. *Journal of Bacteriology* **77**, 675–677.
 OGATA, K., KANEYUKI, H., KATO, N., TANI, Y. & YAMADA, H. (1973). Accumulation of decanedioic acid from n-decane by *Torulopsis candida* no. 99. *Journal of Fermentation Technology* **51**, 227–235.
 REHM, H. J. & REIFF, J. (1981). Mechanisms and occurrence of microbial oxidation of long-chain alkanes. *Advances in Biochemical Engineering* **19**, 175–215.
 SNOW, R. (1966). An enrichment method for auxotrophic yeast mutants using the antibiotic nystatin. *Nature, London* **211**, 206–207.
 SPENCER, J. F. T., SPENCER, D. M. & TULLOCH, A. P. (1979). Extracellular glycolipids of yeast. *Economic Microbiology* **3**, 523–540.
 TAOKA, A. (1986). Production of brassylic acid by fermentation. *Bio Industry* **3**, 867–874.
 UCHIO, R. & SHIO, I. (1972*a*). Microbial production of long-chain dicarboxylic acids from n-alkanes. Part II. Production by *Candida cloacae* mutant unable to assimilate n-alkane. *Agricultural and Biological Chemistry* **36**, 1169–1175.
 UCHIO, R. & SHIO, I. (1972*b*). Tetradecane 1,14-dicarboxylic acid production from n-hexadecane by *Candida cloacae*. *Agricultural and Biological Chemistry* **36**, 1389–1397.
 UEMURA, N. (1985). Industrialisation of the production of dibasic acids from paraffins using microorganisms. *Hakko to Kogyo* **43**, 436–441.
 YANO, K., KANAMURI, M. & TAKAGI, M. (1981). Enrichment of n-alkane assimilation deficient mutants of *Candida* yeast by synergistic effect of nystatin and pyrrolnitrin. *Agricultural and Biological Chemistry* **45**, 1017–1018.
 YI, Z. H. & REHM, H. J. (1982*a*). Metabolic formation of dodecanedioic acid from n-dodecane by a mutant of *Candida tropicalis*. *European Journal of Applied Microbiology and Biotechnology* **14**, 254–258.
 YI, Z. H. & REHM, H. J. (1982*b*). A new metabolic pathway from n-dodecane to α, ω -dodecanedioic acid in a mutant of *Candida tropicalis*. *European Journal of Applied Microbiology and Biotechnology* **15**, 175–179.
 YI, Z. H. & REHM, H. J. (1982*c*). Formation of α, ω -dodecanedioic acid and α, ω -tridecanedioic acid from different substrates by immobilised cells of a mutant of *Candida tropicalis*. *European Journal of Applied Microbiology and Biotechnology* **16**, 1–4.