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

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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, $4 \times 10^6$ 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 Alk- PA- 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 Alk- 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, 1982a, b, c; Bassel & Mortimer, 1973; Bassel & Ogrydziak, 1979) or to exploit it by causing accumulation of commercially valuable intermediates (Uchio & Shio, 1972a, 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 60000 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

\[ \text{CH}_3(\text{CH}_2)_n\text{CH}_3 \]

Alkane

\[ \text{CH}_3(\text{CH}_2)_n\text{CH}_2\text{OH} \]

\[ \text{HOCH}_2(\text{CH}_2)_n\text{CH}2\text{OH} \]

\[ \text{CH}_3(\text{CH}_2)_n\text{CHO} \]

\[ \text{HOCH}_2(\text{CH}_2)_n\text{CHO} \]

\[ \text{CH}_3(\text{CH}_2)_n\text{COOH} \]

\[ \text{HOCH}_2(\text{CH}_2)_n\text{COOH} \]

\[ \text{OCH}(\text{CH}_2)_n\text{COOH} \]

\[ \text{HOOC}(\text{CH}_2)_n\text{COOH} \]

\[ \beta\text{-Oxidation} \]

Acetate

Dicarboxylic acid

Fig. 1. Pathway of alkane degradation in Candida species. In microorganisms 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 ml 0.05 M-potassium phosphate buffer pH 7.5. Cell density at this stage was about 7.5 × 108 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 inactive 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 1−1). 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 trimethylsilylchlorosilane (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 fermentor 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 vol. vol.\(^{-1}\) 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 (2:5:1). 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 ml fractions), dichloromethane (10 \(\times\) 20 ml fractions) and finally dichloromethane/methanol (99:1, v/v) (40 \(\times\) 10 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.

**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 (3–10 \(\mu\)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 (Magné, 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 OD\(_{600}\) 9-0 (about 9 \(\times\) 10\(^8\) cells ml\(^{-1}\)) the optimum amount of glucose was 2.5–5.0 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. 2. Effects of nystatin on resting cells (O) (suspended in YNB minus carbon source) and growing cells (\(\bullet\)) (preincubated for 3 h in YNB with 5 g glucose l\(^{-1}\)).](image-url)
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 (Alk\(^{-}\) PA\(^{-}\) Ac\(^{-}\)) and were deemed to be respiratory-deficient mutants. The remainder (84) utilized acetate (Alk\(^{-}\) PA\(^{-}\) Ac\(^{+}\)) and were deemed to be \(\beta\)-oxidation-deficient mutants. Eighty mutants were able to grow on palmitic acid (Alk\(^{-}\) PA\(^{+}\)) and were presumably blocked at an early step in alkane degradation.

*Properties of Alk\(^{-}\) PA\(^{-}\) 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 \(\beta\)-oxidation pathway. Eleven of these strains also produced smaller amounts of a series of shorter chain length dicarboxylic acids, indicating a partially functional \(\beta\)-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 × 10⁸ cells ml⁻¹) for the various treatments. Test cells were preincubated with hexadecane (2%, v/v) for 4 h then nystatin (7 µg 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.

<table>
<thead>
<tr>
<th>Growth phase</th>
<th>Suspension medium and additions</th>
<th>Carbon source</th>
<th>Viable cells ml⁻¹ after nystatin treatment</th>
<th>Differential kill ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stationary</td>
<td>YNB</td>
<td>None (control)</td>
<td>2.5 × 10⁷</td>
<td>92</td>
</tr>
<tr>
<td>Stationary</td>
<td>YNB</td>
<td>Hexadecane</td>
<td>2.7 × 10⁵</td>
<td></td>
</tr>
<tr>
<td>Exponential</td>
<td>YNB</td>
<td>Control</td>
<td>2.1 × 10⁶</td>
<td>54</td>
</tr>
<tr>
<td>Stationary</td>
<td>YNB + amino acids</td>
<td>Control</td>
<td>3.9 × 10⁵</td>
<td>162</td>
</tr>
<tr>
<td>Stationary</td>
<td>YNB + sophorolipid added 15 min prior to nystatin</td>
<td>Control</td>
<td>9.7 × 10⁷</td>
<td>167</td>
</tr>
<tr>
<td>Stationary</td>
<td>YNB + sophorolipid added at point of hexadecane addition</td>
<td>Hexadecane</td>
<td>8 × 10⁴</td>
<td></td>
</tr>
</tbody>
</table>

The 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 Alk⁻ 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 Alk-PA- 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, 1972a, 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 Alk− 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


Uchio, R. & Shio, I. (1972b). Tetradecane 1,4-dicarboxylic acid production from n-hexadecane by Candida cladoceae. Agricultural and Biological Chemistry 36, 1389–1397.


