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Novel Screening Methods—The Key to Cloning Commercially Successful Biocatalysts

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Abstract—Providing sufficient biocatalyst to support the demands of multi tonne product supply can be problematical. Here we describe how screening for and cloning a γ-lactamase overcame biocatalyst supply issues, and greatly improved the actual biocatalytic process. The isolation of an expressing γ-lactamase clone from a gene library necessitated a combination of classical molecular biology techniques together with innovative screening methods to identify a functional clone. Once isolated the enzyme was characterised with regard to its process performance and proved to be active at 500 g L^{-1} substrate. Further development of the recombinant fermentation and downstream processing has resulted in the ability to produce sufficient biocatalyst from one 500 l fermentation to resolve 5 metric tonnes of (±)-lactam, whilst simplifying the process chemistry greatly.

Introduction

When a commercially available enzyme cannot be found for a biotransformation then the researcher has to find one from the environment, and usually this will be from a microbial source. As a biotransformation based process matures, demand for the product increases, and investment is required to find the best possible biocatalyst which is highly efficient, selective and cheap. This will most commonly emerge from a focussed programme of screening for the microbe, enzyme isolation and characterisation, then cloning of the enzyme for over-expression. In this paper we describe such a programme for the production of optically pure 2-azabicyclo[2.2.1]hept-5-en-3-one [(−)-γ-lactam] using a lactamase; highlighting this as an example of a novel method used to identify clones, and to show the benefits of a cloned enzyme on the overall process.

2-Azabicyclo[2.2.1]hept-5-en-3-one is now well established as a very versatile synthon for the production of carbocyclic nucleosides[1] (Scheme 1). Such compounds, where the ribose oxygen of the nucleoside has been replaced by a methylene, have become very valuable as chemotherapeutic agents in the fight against viral infections such as HIV or herpes.[2]

We reported a biocatalytic resolution of 2-azabicyclo[2.2.1]hept-5-en-3-one several years ago.[3] By screening several soil and sewage samples, using *N*-acetyl-L-phenylalanine as a sole source of carbon, many microbes with the ability to perform amide bond hydrolysis were isolated. Subsequent rescreening of these for hydrolysis of the γ-lactam revealed a strain of *Pseudomonas cepaecia* which was highly selective for the hydrolysis of the (+)-lactam, leaving the desired (−) enantiomer. The whole-cells gave an enantiomeric ratio (*E* value) of 94 (Scheme 1).

Several attempts were made to isolate and purify the lactamase, but it proved too unstable for the manipulations required for isolation and purification. As a result, the biotransformation process was developed using the whole-cell biocatalyst. Whilst this process allowed the development of a tonne scale (−)-lactam process, as required volumes grew further, it became more difficult to satisfy demand. The process had several drawbacks. The use of whole cells, and the subsequent lysis of these during the biotransformation (and lysis from frozen storage) complicated the isolation of the lactam. Direct solvent extraction was impossible and a complex carbon adsorption and elution cycle was needed. Whilst the volume efficiency of the biotransformation was tolerable, large volumes were encountered during the work-up. Also, the cells proved to be somewhat sensitive to the quality of racemic lactam used, which varied depending on the source. Furthermore, the quantity of cells required (a kg of cells yielded a kg of (−)-lactam)

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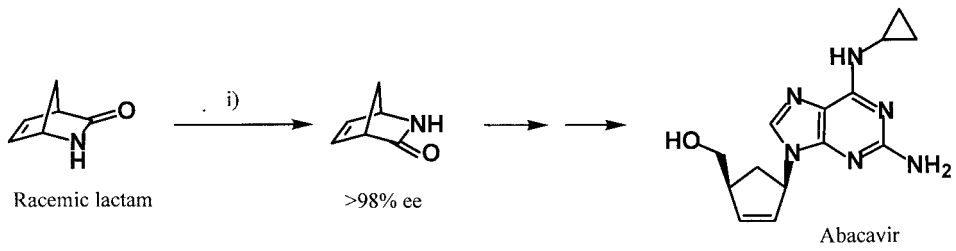


Scheme 1. Carbocyclic nucleosides from 2-azabicyclo[2.2.1]hept-5-en-3-one. (i) 50 g L^{-1} *Pseudomonas cepaecia*, 100 g L^{-1} racemic lactam, 25°C 24 h.

contributed to a significant proportion of the manufacturing cost. Thus, there was a considerable driving force to invest in the research needed to find a better biocatalyst and process. A further screen for lactamases was performed, again using *N*-acetyl-L-phenylalanine as the sole source of carbon, and from the several positives isolated, a temperature stability study was performed on the lactamase in crude cell lysates. This indicated one to be considerably more stable than the *P. cepaecia* strain,[4] and indeed retained half of its activity after incubation at 60°C for 4 h. The strain was later identified as *Comamonas acidovorans*.

Results and Discussion

The γ-lactamase was purified to homogeneity by a sequence of ammonium sulfate precipitation, HIC (butyl Sepharose), anion exchange chromatography (Q Sepharose) and gel filtration (Sephacryl S200), giving a single band by SDS-PAGE. This indicated a molecular weight of 53–55 KDa. The N-terminal sequence was then determined, as shown in Figure 1.

A random gene library of the *Comamonas acidovorans* was prepared by standard techniques. Genomic DNA was partially digested by *Sau*3A I, then DNA fragments from 2–6 kb isolated after size separation by electrophoresis. Fragments were ligated into the dephosphorylated *Bam*H1 site of pUC19, and the vector transformed into *E. coli* DH5α.

The detection of an expressing clone was attempted by the use of a radioactive oligonucleotide probe based on the N-terminal amino acid sequence. The probes used are shown in Figure 1. However, this approach was not successful due to the degeneracy of the sequence. This approach may also identify non-expressing forms of the lactamase such as truncated versions of the gene, and a direct screen for activity is more desirable, and in this case neccessary.

Conventional biocatalyst screening methods such as emulsified substrate overlay (where enzyme activity can be visualised directly in the form of a clearing zone around the biocatalyst) were not appropriate for the lactam substrate due to its high solubility (> 500 g L^{-1} in water). Furthermore, other staining methods such as ninhydrin or glutaraldehyde did not work in an agar overlay with a soluble substrate because diffusion quickly diluted any visible effects. A different approach was needed, and so a very simple screen was developed, combining the classical techniques of replica plating and ninhydrin staining. A Whatman filter-paper was dipped in a methanolic solution of (+)-lactam, then allowed to dry. This impregnated the paper with (+)-lactam substrate which was then placed directly onto the agar plate where it picked up moisture and most of the cells in each colony. The physical transfer of cells gave sufficient biomass to allow a biotransformation to occur, but the aqueous content of the paper was limited and prevented excessive diffusion of product away from the cells. The paper was incubated for several hours, dried and stained with ninhydrin. A positive clone was clearly visualised as a colony with a brown halo on the light background (Fig. 2). The paper colony pattern was then matched with the regrown cells on the agar plate, and the desired colony isolated.

DNA sequence analysis of the insert showed the fragment to incorporate an open reading frame (ORF) of 1.6 kb, shown in Figure 3. This was driven by the

NH_3 - T D T L I K V D L N R P P T D N E R V H

Underlined amino acids selected to synthesize N-terminal degenerate oligonucleotide probes.

NH_3-Asp-Thr-Leu-Ile-Lys-Val-Asp

Probe 1 5'-GAY-ACN-TTR-ATH-AAR-GTN-GA

Probe 2 5'-GAY-ACN-CTN-ATH-AAR-GTN-GA

N=A+C+T+G R=A+G Y=C+T

Figure 1. Derived N-terminal amino acid sequence of purified *Comamonas acidovorans* γ-lactamase.

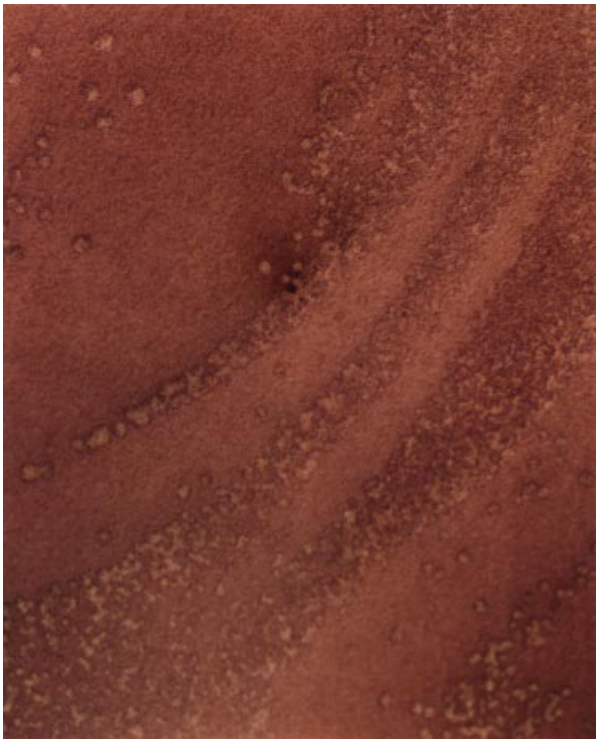


Figure 2. Ninhydrin stain of filter paper colony transfer. Genomic library of *Comamonas acidovorans* in *E. coli* screened for (+)-γ-lactamase acitivity by incubation of adsorbed colonies on (+)-lactam impregnated filter discs. Brown halo apparent around single, central colony indicating the conversion of lactam to amino acid product.

upstream *lac* promoter of pUC19, and translates to a protein of 575 residues (61 kDa). The deduced amino acid sequence of the translated ORF showed >65% homology to the acetamidase from *Mycobacterium smegmatis*[8] and *Methylophilus methylotrophus*. These enzymes have been shown to hydrolyse short chain fatty acylamides.[8]

From the cloned gene sequence the N-terminal sequence was identified. The lactamase was found to have an extra 31 amino acids upstream of the N-terminus, which arose by a fortuitous ligation of a short piece of DNA during the cloning. However, experiments showed that enzyme expression was good, and subsequent manipulation of the gene to remove these gave no added benefit. Final modification of the recombinant plasmid was the insertion of the *cer* element responsible for multimeric resolution and stable inheritance of the wild type *E. coli* plasmid ColE I.[6] The *cer* element was transferred from construct pKS492.[6] The final construct and expression vector were designated pPET1 (Fig. 4). The vector was transformed into *E. coli* MC1061 the designated recombinant lactamase host. The level of recombinant protein was significant to be visualised on Coomassie SDS-PAGE under induced growth.

The fermentation of the recombinant *E. coli* was based on a complex media/feed with glycerol as the carbon source and peptone/casein as a source of amino acids, peptides and nitrogen. Initial growth was in a batch mode after which a continuous feed containing glycerol was used. Regulation of enzyme expression was not particularly well controlled and the fermentation did not require induction. A 500 L, 4 day fermentation yielded approximately 100 g L^{-1} (wet weight) of cell paste and 3000 units g^{-1} cell paste, sufficient to resolve about 5 tonnes of racemic substrate.

Having optimised the fermentation, we focused on recovering the lactamase in a form suitable for use in the biotransformation. The goal was a simple process that gave sufficiently pure lactamase such that the protein content did not cause emulsions during isolation of the lactam. After lysis of the cells with a combination of lysozyme and Triton-X100, the bulk of nucleic acids and cell debris were removed by polyethyleneimine precipitation and centrifugation. The lactamase was precipitated from the filtrate by standard ammonium sulfate precipitation, before recovery by centrifugation. The pellet was finally redissolved in Tris–HCl buffer, sterile filtered ready for use. This was achieved in an overall yield of about 70%.

With a readily available supply of semi-purified recombinant lactamase available, a much improved process was quickly developed. The biotransformation was optimised with respect to substrate concentration, enzyme loading, pH, buffer and temperature. This highlighted 500 g L^{-1} substrate, 100 mM Tris–HCl at pH 7.5, 25°C to be optimal. The new (−)-lactam process then has been much improved (Scheme 2). Volume efficiency is much higher where the enzyme tolerates 500 g L^{-1} input of lactam, a 5-fold increase. Since a clean concentrated enzyme is used, the isolation was greatly simplified where direct dichloromethane solvent extraction of the lactam can be achieved, avoiding the carbon adsorption step, and associated problems of low volume efficiency, handling and safety issues. The final step is a recrystallisation of the product from the solvent concentrate.

Conclusion

Cloning an enzyme has the obvious benefit of reducing the cost of the biocatalyst through over-expression. However, what can be equally important is the dramatic impact that use of a cloned enzyme can have on the overall design of a process, where product recovery in particular becomes much easier. By carefully planning the screening strategy, a cloning process can be streamlined, as we have shown for the lactamase. The impregnated filter paper assay should be applicable for any soluble substrate/product where a selective staining method exists.

Experimental

Definition of units

For our purpose 1 unit is defined as the amount of biocatalyst required to produce 1 g of product per hour. This may be expressed in units mL^{-1} for fermentation broth or units g^{-1} for purified protein.

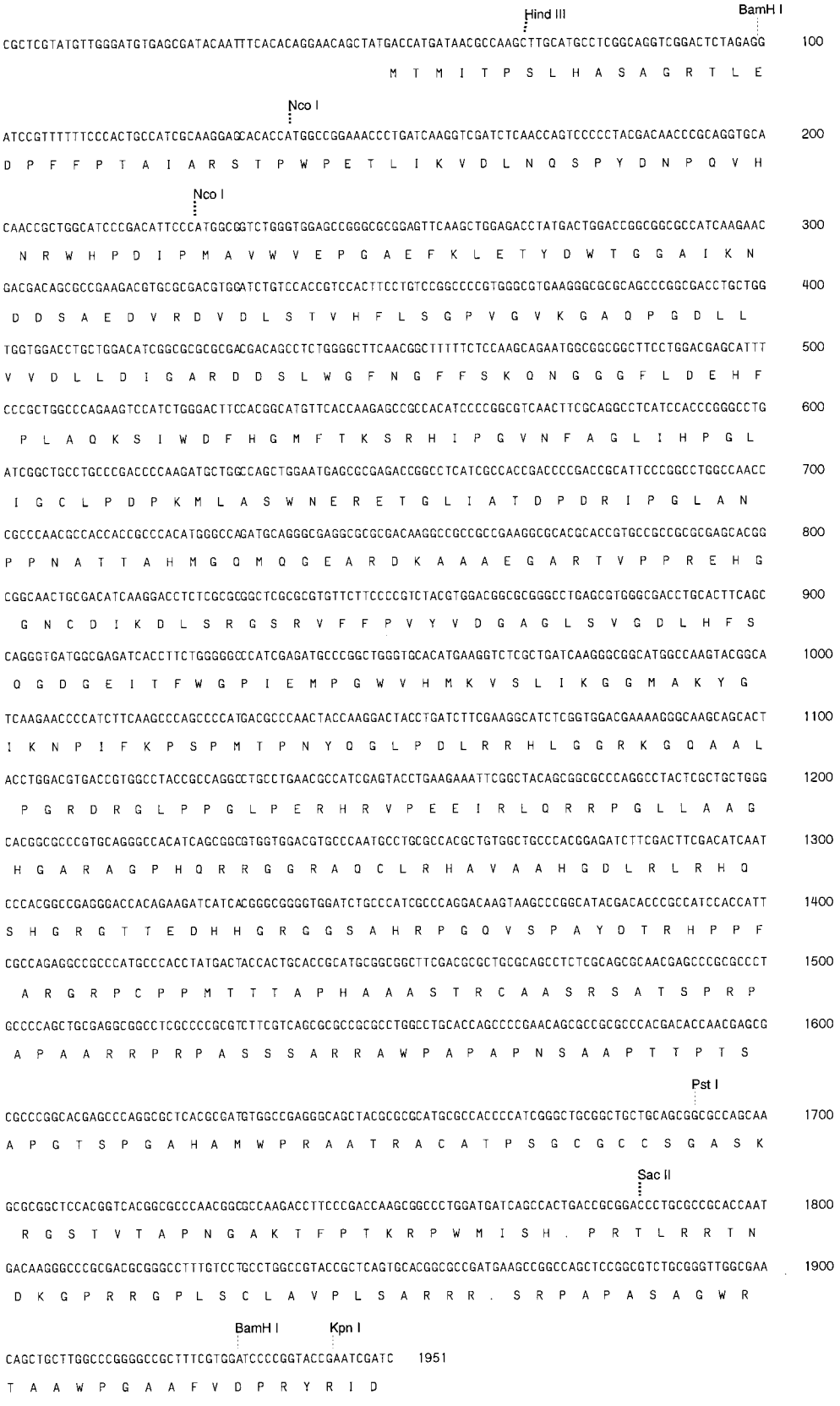


Figure 3. Complete nucleotide sequence of the (+)-γ-lactamase gene and deduced amino acid sequence from *Comamonas acidovorans* strain CMC 4093. The 1.9 kb *Sau*3A I fragment ligated in-frame with the *Bam*H I restriction sites of pUC19 backbone.

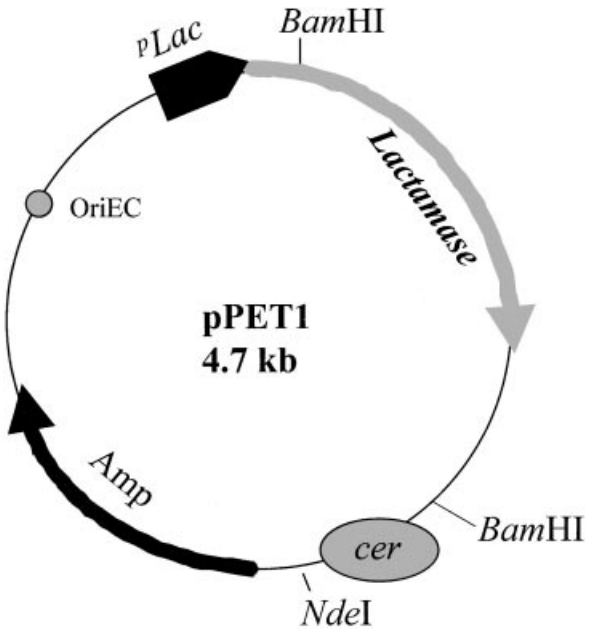


Figure 4. Schematic diagram of plasmid pPET1. *E. coli* plasmid pPET1 was derived from pUC19, which harbours a 1.9 kb *Sau*3A I genomic fragment from *Comamonas acidovorans* CMC1493, ligated into the *Bam*HI restriction site. The *cer* stability element of the wild type plasmid ColE 1 was inserted at the 3′ to the lactamase fragment via a *Bam*HI (partial) and *Nde*I restriction.

Materials

All restriction enzymes and DNA modification enzymes were purchased from NBL Gene Sciences Ltd.; Agarose was obtained from IBI Kodak Ltd.; qualitative filter papers Type 2, 125 mm ∅ (Whatman Ltd.); all other chemical reagents were acquired from Fisher Scientific UK.

Biotransformation assay

A 1 h single time point assay is routinely used. A dilution of biocatalyst is incubated in 0.1 M Tris–HCl, pH 7.5 (900 µl) in a 20 mL glass scintillation vial. The reaction is started by addition of 100 µL 100 $gL^{-1}(\pm)$ lactam. The reaction is then incubated for 1 h at 25°C in an orbital shaker of 2.5 cm throw at 250 rpm. The reaction is stopped by taking a 100 µl aliquot and dilution in to 900 µl HPLC mobile phase. The extent of the reaction is then monitored using HPLC. Column: Kromasil KR100 C8, mobile phase 50:50 (v/v) MeOH: 10mM K_2HPO_4 pH 7.0. Flow 1 mL min^{-1}. Detection by UV monitor at 225 nm.

Plasmids, bacterial strains and culture conditions

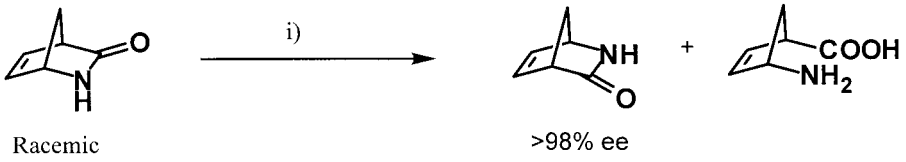
Plasmid pUC19[5] was used for the library construction, sequencing and cloning. The cloning vector was improved for stable expression by incorporation of the *cer*[6] element. *C. acidovorans* strain CMC4093, used a source of genomic DNA, was grown on BA medium (50 mM KH_2PO_4, 10 mM K_2PO_4, 4 mM $MgSO_4$. 7.5 mM $(NH_4)_2SO_4$, 1% (w/v) yeast extract, 0.1% (v/v) trace elements solution (25 mM $CaCl_2$. $2H_2O$, 25 mM ZnO, 5 mM $CuCl_2$, 10 mM $MnCl_2$, 20 mM $FeCl_2$ 10 mM $CoCl_2$, 20 mM Na_2MoO_4., 5 mM H_3BO_3, 3.3 M HCl [pH 7.0 with NaOH])). *E. coli* DH5α was used for library construction and preliminary expression of genomic fragments ligated into pUC19. *E. coli* DH5α harbouring recombinant plasmids was grown at 37°C on TSB medium (Oxoid Ltd.) with ampicillin and isopropyl β-D-thiogalactopyranoside (IPTG). Recombinant *E. coli* strain was inoculated into a 1 L baffled shake flask containing 100 ml TSB medium (Oxoid Ltd.) supplemented with ampicillin (100 µg mL^{-1}). The flask and inoculum were incubated for 16 h at 37°C, shaking at 300 rpm in an orbital shaker (25 mm throw). The seed culture was inoculated (1% v/v) into a 2.8 L laboratory bioreactor vessel containing 1.5 L TSB medium. The temperature was maintained at 25°C, pH 7, and dissolved O_2 tension at > 50%. Growth was monitored at 520nm optical density against a TSB medium blank. After 24 h growth, cells were harvested by centrifugation (5000 *g* at 4°C for 10 min) Cells were stored at −20°C until required.

Purification of lactamase from *C. acidovorans*

A 10% (w/v) cell suspension in 1 mM $NaHCO_3$, 10 mM EDTA, 0.1% (w/v) triton X-100, 1 mg mL^{-1} lysozyme was incubated overnight at 25°C. Debris was removed by the addition of 0.5% w/v PEI and centrifugation at 10,000 *g*, 10 min, 4°C.

Hydrophobic interaction chromatography

After precipitation of lactamase from the cell lysate by addition of solid $(NH_4)_2SO_4$ to 50% saturation, the pellet was resuspended in 25 mM KH_2PO_4/K_2HPO_4 + 1.5 M $(NH_4)_2SO_4$ pH 7.0. This was then applied to a butyl sepharose (Pharmacia) column (XK16×16 cm), total volume 32 mL, pre equilibrated in the same buffer. Elution was with a decreasing salt gradient over 20 column volumes at 2 mL min^{-1}. Lactamase eluted at around 0.5 M $(NH_4)_2SO_4$. Active fractions were pooled and dialysed against 100 mM Tris–HCl pH 8.0.



Racemic >98% ee

Scheme 2. Bioresolution of 2-azabicyclo[2.2.1]hept-5-en-3-one. (i) 5 mL^{-1} cloned lactamase, 500 gL^{-1} racemate, $E > 400$.

Anion exchange chromatography

The lactamase was applied to a Q sepharose (Pharmacia) column (XK10×8 cm), 6 mL bed volume, eluting with a 0.1 M NaCl increasing linear gradient in 100 mM Tris pH 8, flow 2 mL min^{-1}. Lactamase eluted at 0.25 M NaCl.

Gel filtration

Active fractions were concentrated using Amicon 30 K nmwl ultrafree microcentrifuge concentrators. Protein was applied to a Sephacryl S200HR (XK16×60 cm), 100 mM NaCl 10 mM Tris (pH 8.0), flow 1 mL min^{-1}. Protein was pure by Coomassie stained SDS-PAGE, approximate mw 53–55 kDa. Electroblotting of the lactamase from the SDS-PAGE gel onto a PVDF membrane (Biorad) allowed the N-terminal sequence to be determined. The N-terminal sequence is shown in Figure 1.

Cloning and sequencing

Total genomic DNA was prepared from *C. acidovorans* by an initial lysozyme treatment. 2 g of cell pellet was mixed with 10 mL of lysis buffer (10 mg mL^{-1} lysozyme in 50 mM glucose, 20 mM EDTA, 25 mM Tris–HCl pH 8.0), cell suspension was incubated at 37°C for 30 min, 1 mL of proteinase K (100 μg mL^{-1}) was added and the incubation was elevated to 50°C for 30 min. Cell lysis was completed by the addition of 5 mL 10% (w/v) SDS and gentle inversion at 50°C for a further 30 min. Solid cesium chloride was dissolved in the cell lysate at 1 g mL^{-1}. Finally ethidium bromide was added to the suspension at a concentration of 200 μg mL^{-1} and loaded into a 13 mL ULTRACRIMP™ (Sorvall) ultracentrifuge tube. The genomic DNA was resolved by ultracentrifugation (140,000 *g*, 20°C, 72 h), extracted with cesium chloride saturated butanol and dialysed in TE buffer pH 8.0. The DNA (250 μg) was then partially digested with *Sau*3A I (20U) restriction endonuclease for 1–10 min and electrophoresed in a 0.8% agarose TAE gel.[7] Fragments ranging from 2–6 kb were excised from the gel, electroeluted and purified by phenol: chloroform extraction and subsequent ethanol precipitation. Purified genomic fragments were ligated into the dephosphorylated *Bam*HI site of pUC19. The ligation mix was then used to transform competent *E. coli* DH5α.[5] Transformants were screened on TSB agar plates containing ampicillin (100 μg ml^{-1}) and 1 mM IPTG. The screening procedure involved adsorption of recombinant colonies onto (+)-lactam (2 mg mL^{-1} dissolved in methanol) impregnated filter paper discs (Type 2, Whatman). The replicate colonies and filter papers were incubated at 37°C for 4 h, dried and developed with 2% (w/v) ninhydrin in acetone at 80°C for 5 min. The presence of the acid product was identified as a brown halo surrounding the recombinant colony.

A single colony displaying the characteristic brown halo of activity was isolated (Fig. 2). Plasmid DNA was prepared from the lactamase expressing clone, and restriction digest analysis showed the presence of a 1.9 kb *Sau*3A I restriction fragment.

Subsequent DNA sequencing. was performed by Dye Terminator Cycle Sequencing using AmpliTaq polymerase FS (ABI) with the ABI 377 DNA Sequencer (ABI). Overlapping sequence reads were aligned into a single open reading frame using the DNAstar Inc. Lasergene software.

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