THE β-LACTAMASES OF THE MARINE GENUS *PHOTOBACTERIUM*

A THESIS PRESENTED IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
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ABSTRACT

All naturally occurring isolates of all five species of the marine genus *Photobacterium* have β-lactamase activity. In this study, the β-lactamase from the laboratory strain *P. leiognathi* 206 is fully characterised. The enzyme is constitutive and is released from the cell by osmotic shocking techniques, suggesting a periplasmic location. The enzyme is maximally active at pH 6.2 and has over 90% maximum activity at pH 7.0. The hydrolytic activity of the β-lactamase is independent of zinc ion presence. On the basis of substrate profile and inhibition studies the β-lactamase is classified as a Richmond and Sykes Class II enzyme. Crypticity tests indicated that there is no outer membrane permeability barrier to β-lactam substrates.

Comparative substrate profiles performed with the β-lactamases from three strains of each species of *Photobacterium*, indicate that the enzymes from strains of *P. leiognathi*, *P. angustum* and *P. phosphoreum* are active only on penicillins, whereas those from *P. fischeri* and *P. logei* also hydrolyze cephalosporins. This division is in agreement with an imminent taxonomic change for the latter two species. Analytical iso-electric focusing of the β-lactamases from 45 *Photobacterium* strains resulted in pI values which were not necessarily species specific and there was little correlation between the pI of the β-lactamase and its substrate profile, excepting the enzymes from *P. angustum* and *P. logei*.

Although plasmid DNA is present in many *Photobacterium* strains, conjugative transfer of β-lactamase activity from six different *Photobacterium* donors to a restrictionless *Escherichia coli* mutant was not observed. All attempts to 'cure' the bacteria of β-lactamase activity with five different curing agents, were also unsuccessful. A chromosomal location for the βla^{+} gene is postulated.

DEDICATION

I would like to dedicate this thesis to my parents, Dr and Mrs R.F. Lamb, without whose limitless financial support and unceasing encouragement this work would not have been possible.

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SECTION A

INTRODUCTION

1. THE GENUS *Photobacterium*

1.1 The Classification of *Photobacterium*

The genus *photobacterium* is composed of bacteria that are non-sporeforming Gram-negative, motile, straight or curved rods. They are restricted to but widely distributed within the marine environment, sharing a number of morphological, physiological and biochemical properties with the *Enterobacteriaceae* and *Vibronaceae*.

The taxonomy of the genus *Photobacterium* and the species assigned to the genus, remained confused until the early 1970's. Hendrie *et al* (1970) carried out a limited study on species of both luminous and non-luminous bacteria and proposed a taxonomy for *Photobacterium*. In 1973, Reichelt and Baumann performed on extensive numerical taxonomy on the phenotypic characteristics of 173 strains of marine luminous bacteria. The investigation confirmed some of the propositions of Hendrie *et al* and resulted in the retention of two species in the genus *Photobacterium*, *P. phosphoreum* and *P. mandapamensis*, and the removal of all other species previously assigned.

The species *P. mandapamensis* (Hendrie *et al*, 1970) was subsequently renamed *P. leiognathi* on the basis of priority, since Boisvert *et al* (1967) first described this species of luminous bacteria, isolated from the luminous organ of a marine-fish *Leiognathus* and Reichelt and Baumann (1975) demonstrated strains from each to be one and the same.

Reichelt and Baumann (1973) suggested that a group of five phenotypically related strains, previously characterized (Allen and Baumann, 1971), might represent another species

of *Photobacterium* although these strains were non-luminous. On the basis of DNA/DNA hybridization results, Reichelt *et al* (1976) designated the new species *P. angustum* and demonstrated it was related to *P. leiognathi* and *P. phosphoreum* by 56% and 29% homology, respectively.

Another group of strains previously assigned to *Photobacterium* and designated *P. fischeri*, was found to have intra-species relatedness (85% homology) but to be distinct from (0-11% homology) all other strains of *Photobacterium* tested. Reichelt *et al* (1976) suggested that the group of strains constituted another distinct species of *Photobacterium*.

Using DNA/ribosomal RNA (rRNA) hybridization techniques, Baumann and Baumann (1977) subdivided the genus *Photobacterium* into two groups on the basis of structural properties and DNA homologies: *P. phosphoreum* and *P. fischeri* DNA homology groups. Baumann and Baumann demonstrated that the two groups were related by rRNA homologies of over 90% and hence did not feel generic separation of the groups was justified, despite the differences between them.

In 1978, a fifth species was added to the genus, *P. logei* (Bang *et al* 1978). The strains of this species were found to be closely related to *P. fischeri* but are psychrophilic and were isolated from polar seas (Olson *et al*, 1978).

The current classification of the five species now recognised within the genus, is thus based on: -

a) the mode of flagellation
b) the mole percentage guanine and cytosine of the DNA (mol. % GC)
c) in *vitro* DNA/DNA hybridization and DNA/rRNA hybridization, and
d) nutritional and enzymatic properties.

The distinguishing properties of the species are listed in Table I. *Photobacterium* strains all require sodium ions for growth (this requirement cannot be fulfilled by potassium ions), have a GC content of 39-44 mol. % and all except *P. angustum*, exhibit luminescence in the blue-green range due to the presence of a luciferase enzyme.

All are facultative anaerobes, capable of growth at 25°C on glucose, mannose, galactose, fructose, glycerol and N-acetlyglucosamine, and most produce extracellular chitinase (Spencer, 1961). In addition, K. Smith (pers. comm.) has found that all naturally-occurring strains tested, have detectable β-lactamase activity.

Photobacterium strains are closely related to those of the genus *Beneckea*, but are less nutritionally versatile. *Photobacterium* bacteria are capable of utilizing only between 7-22 (depending on the strain) of 147 different organic compounds as the sole carbon and energy source, whereas *Beneckea* strains can utilize 28 - 45 of the compounds (Reichelt and Baumann, 1973).

Natural isolates of strains of the five species of *Photobacterium* are usually prototrophic. Ruby and Nealson (1976) demonstrated that 150 isolates taken from light organs of *Monocentris japonica* fishes grew well on defined minimal media without supplements. All 723 isolates from Leiognathid fishes, characterised as *P. leiognathi* (Reichelt *et al*, 1977) and 152 isolates of *P. phosphoreum*, taken from midwater macrourid and opisthoproctid fishes (Ruby and Morin, 1978), were shown to be prototrophs. However, naturally occurring auxotrophs of *P. phosphoreum* have been reported, with methionine being the most common supplement required Doudoroff, 1942; Reichelt and Baumann, 1973).

TABLE I: THE DISTINGUISHING PROPERTIES OF PHOTOBACTERIUM SPECIES

Photobacterium Species:	*P. leiognathi*	*P. phosphoreum*	*P. angustum*	*P. fischeri*	*P. logei*
Flagellation	1 - 3 unsheathed polar flagella, diameter 14 - 16 nu			2 - 8 sheathed polar flagella, diameter 24-30 nm	
Luminescence	+	+	-	+	+
Cell-associated pigment production	-	-	-	Pale yellow/orange pigment appears in 3-5 day old culture	
Mol. % GC content	41 - 44%			39-41%	
Growth at: 4°C 20°C 30°C	- + ++	+ ++ -	± + ++	- ++ +	+ ++ -
Symbiosis	with members of *Leiognathidae* (1)	with a variety of mid and deep water fishes (2) + (3)	only free-living forms	with monocetrid fishes (4) + (5)	with polar fishes (6)
Accumulation of PHB[a]	+	+	+	-	-
Lipase	(+)[b]	-	(±)[c]	(+)	
D-xylose	-	-	+	-	
Maltose	-	(+)	(±)	(+)	
Cellibiose	-	-	-	(+)	
D-gluconate	+	(+)	+	-	
Acetate	(+)	-	+	-	
D, L-lactate	+	(±)	+	-	
Pyruvate[7]	(+)	-	+	-	
L-glutamate	(±)	(±)	+	(±)	
L-proline	(+)	(±)	-	+	

(Footnotes) Information taken from Reichelt *et al*, 1976 and Baumann and Baumann, 1977 unless otherwise stated.

a PHB poly-B-hydroxybutyrate
b (+) more than 80% of strains tested
c (±) less than 80% of strains tested

(1) Reichelt *et al*, 1977
(2) Herring & Morin, 1978
(3) Ruby & Morin, 1978
(4) Ruby & Nealson, 1976
(5) Fitzgerald, 1977
(6) Olson *et al*, 1978
(7) Ruby & Nealson, 1977

1.2 The Ecology of *Photobacterium* Strains

Photobacterium strains are widely distributed in different marine habitats, being either free-living, commensal or symbiotic. Strains from all species have been isolated from seawater as free-living forms and it has been shown that free-living populations are dynamic and change in density with the seasons (Ruby and Nealson, 1978). Ruby *et al* (1979) examined species distribution as a function of depth and found that in parts of the Atlantic Ocean, midwater samples (100 - 1000 m) contained relatively high numbers (20 - 100 cells/litre) of *P. phosphoreum* irrespective of season, but few other species. However, in the surface waters (0 - 100m) of the same areas, *P. phosphoreum* is present in a density of less than 1 cell/litre suggesting that this species prefers decreased temperature and dissolved nutrient levels for growth. *P. phosphoreum* numbers decrease at depths greater than 1000m. Samples taken from the Arctic and Antarctic Oceans are reported to contain primarily the pschrophilic species *P. phosphoreum* and *P. logei* (Nealson and Hastings, 1979).

Quantitatively, the most important habitat of the commensal bacteria is the intestinal tracts of marine animals. It is not uncommon to find 10^6 and 10^7 colony-forming units per metre of intestine in certain marine fishes (Ruby and Morin, 1979).

The bacteria which exist as symbionts are species-specific for a particular host and inhabit either the gut or a specific light organ. The tropical and temperate water fishes harbour *P. leiognathi* and *P. fischeri*, which are mesophilic and tolerate higher temperatures. The deep sea and midwater fishes harbour mainly *P. phosphoreum* and *P. logei*.

2. β-LACTAMASE

2.1 Definition of β-lactamase

The term β-lactamase describes a group of enzymes, exclusively of bacterial origin, which hydrolyze the amide bond in the β-lactam ring of susceptible β-lactam antibiotics, namely penicillins and cephalosporins (Table IIa and b).

The action of β-lactamase on a penicillin results in the formation of a penicilloic acid (Fig. 1a) which is a stable compound, produced in stoichiometric proportions and is readily detected and assayed.

The reaction involving cephalosporins (Fig. 1b) is more complex however, due to the additional substituent at position 3 on the dihydrothiazine ring. The first product of β-lactamase hydrolysis of a cephalosporin is suggested to be a cephalosporic acid analogous to penicilloic acid. Unlike these latter compounds, the cephalosporic acids are usually very unstable and cannot be isolated as they rapidly undergo further decomposition and fragmentation (Ross and O'Callaghan, 1975).

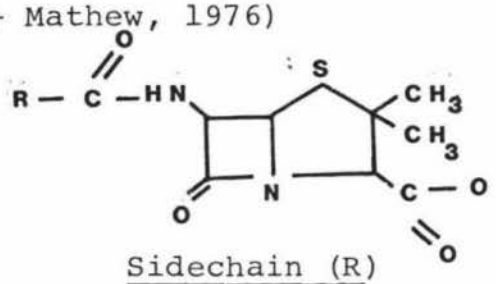
2.2 Nomenclature

The first report of a penicillin-destroying compound, detected in extracts of an *Escherichia coli* strain, described the enzyme as a 'penicillinase' (Abraham and Chain, 1940) since cephalosporins were unknown at that time. The Enzyme Commission later designated the enzyme as penicillin-amido-β-lactam-hydrolase E.C.3.5.2.6 ('Enzyme Nomenclature', 1965). Subsequently, enzymes have been found which are active on cephalosporins and some almost exclusively so, for example that of *Hafnia* species 1094E (Sykes and Matthew, 1976). In 1972, the Enzyme Commission designated 'cephalosporinase' as E.C. 3.4.2.8. and introduced the terms β-lactamase I and β-lactamase II for penicillinase and cephalosporinase

TABLE II: β-LACTAM STRUCTURES

IIa: Penicillins (Sykes + Mathew, 1976)

General structure



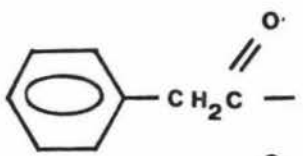
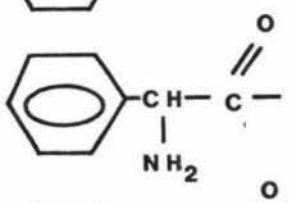
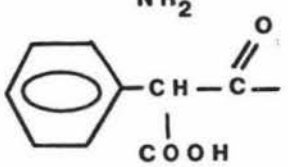
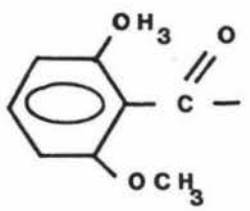
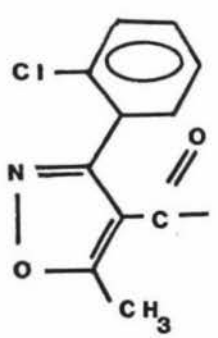
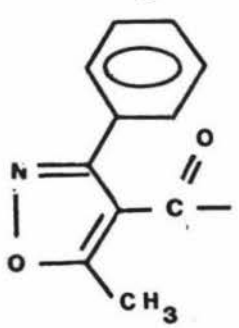
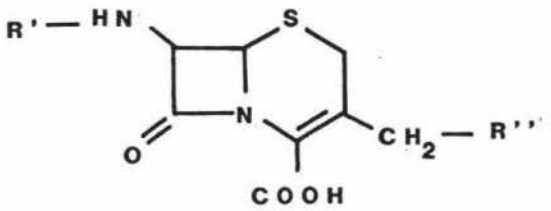
Penicillin	Sidechain (R)
6-Amino penicilloic acid	H -
Benzylpenicillin	
Ampicillin	
Carbenicillin	
Methicillin	
Cloxacillin	
Oxacillin	

TABLE IIb: Cephalosporins (Hamilton-Miller, 1979)

General structure



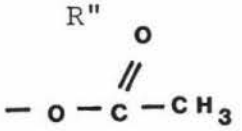
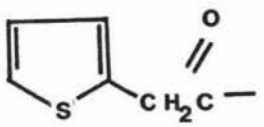
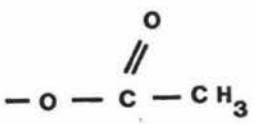
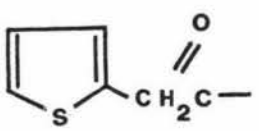
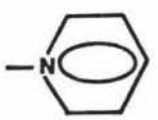
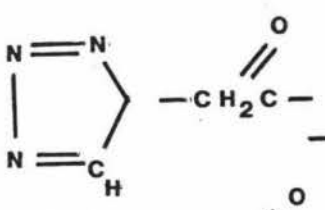
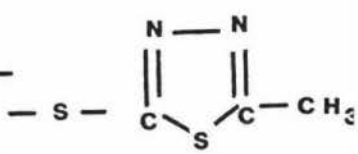
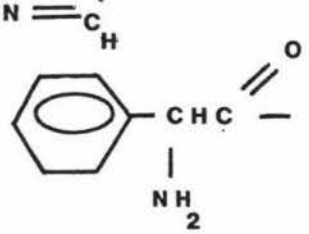
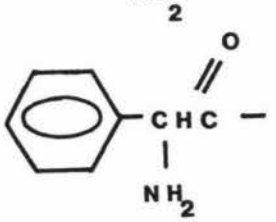
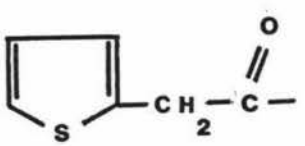
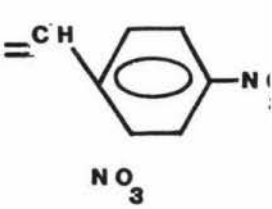
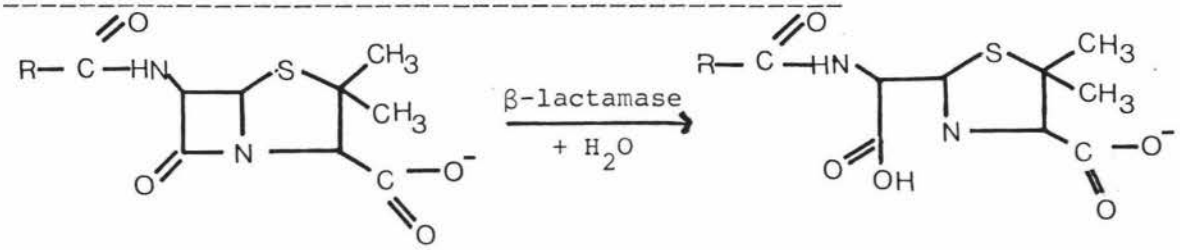
Cephalosporin	Sidechains R'	R"
7-Amino cephalosporanic acid	H -	
Cephalothin		
Cephaloridine		
Cefrazolin		
Cephalexin		
Cephradine		
Nitrocefin		

Figure 1: β-Lactamase Hydrolysis of β-lactams
(Sykes and Matthew, 1976)

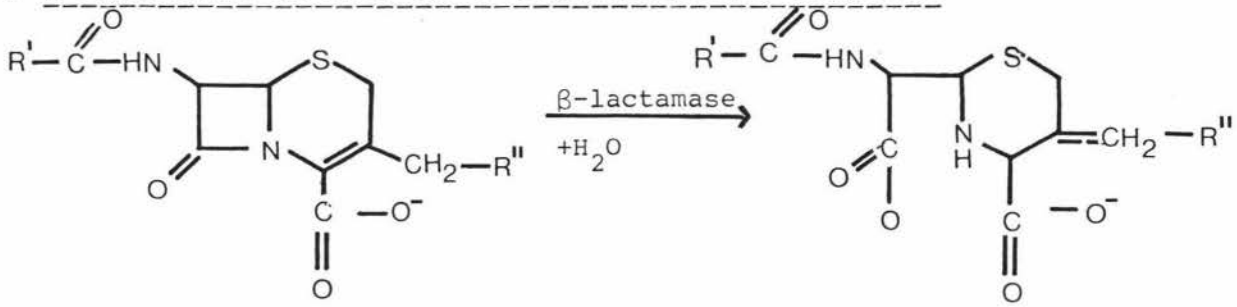
(a) β-Lactamase action on a penicillin compound



Generalised penicillin structure

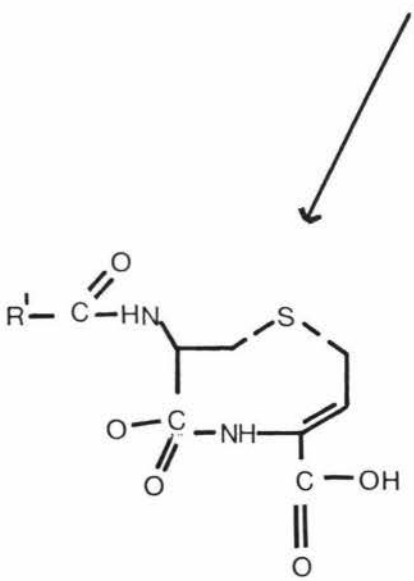
Generalised penicillin acid structure

(b) β-Lactamase action on a cephalosporin compound



Generalised cephalosporin structure

Unstable hypothetical intermediate



Breakdown fragments

respectively. The latter terms did not become popular, remaining in use only for those enzymes from *Bacillus cereus* (Kuwabara and Abraham, 1967). Confusion has arisen among workers in this field of research, because enzymes have been reported which are equally active on both penicillins and cephalosporins and therefore the officially designated enzyme numbers are not appropriate.

At the present time, submissions have been made to the Commission for Enzyme Nomenclature recommending that it subsume all β-lactamases under one heading of E.C.3.5.2.6., with the official meaning of 'β-lactamase' as opposed to 'penicilliase', in it's forth-coming revision (Hamilton-Miller, 1979).

For current usage, it is generally accepted that the term β-lactamase be used when referring to enzymes which hydrolyze both penicillins and cephalosporins; the specific terms of 'penicillinase' and 'cephalosporinase' be used only when strictly applicable.

2.3 Classification of β-Lactamses

The earliest attempt to classify β-lactamses was made by Ayliffe (1963) working on two penicillinases. He and other workers since that time, differentiated the enzymes by their substrate specificities.

The first classification systems, proposed by Jack and Richmond (1970) and Richmond *et al* (1971) were based on the substrate profiles of β-lactamases and incorporated information on inhibition of the enzyme activity by both β-lactam analogues and non-β-lactam inhibitors. The schemes originally identified fifteen types of different β-lactamases, categorized into four classes and was extended by Richmond and Sykes in 1973. The extended system proposed five classes of β-lactamase (Table III) and is the one still used when referring to enzyme types.

TABLE III: CLASSES OF β-LACTAMASE (Richmond & Sykes, 1973)

CLASS I:	Enzymes predominantly active against cephalosporins. Competitively inhibited by cloxacillin and carbenicillin. Class contains enzyme types Ia Ib Ic Id
CLASS II:	Enzymes predominantly active against penicillins. Competitively inhibted by cloxacillin. Class contains enzyme types IIa IIb IIa/b
CLASS III:	Enzymes equally active against penicillins and cephalosporins. Competitively inhibited by cloxacillin. Resistant to inhibition by *p*CMB* and other sulphydryl reagents. Class contains enzyme type IIIa
CLASS IV:	Enzymes equally active against penicillins and cephalosporins. Resistant to inhibition by cloxacillin. Inhibited by *p*CMB and other sulphydryl reagents. Class contains enzyme types IVa IVb IVc
CLASS V:	Enzymes equally active against penicillins and cephalosporins. Resistant to inhibition by cloxacillin, *p*CMB and other sulphydryl reagents. Class contains enzyme types Va Vb Vc Vd

* *p*CMB = para-chloromercuribenzoate

Fullbrook *et al* (1970) distinguished several enzymes from each other by construction of β-lactamase inhibition profiles, using methicillin, nafcillin, oxacillin, cloxacillin and dicloxacillin as inhibitors, expressing the results relative to methicillin.

Other information about β-lactamases is often considered when differentiating enzymes from one another, characteristics such as molecular weight, iso-electric point, inducibility, immunological reactions and genetic location.

The β-lactamases have molecular weights (MW) varying from 14,000 to 49,000 daltons, although a high proportion of enzymes fall in the range of 20,000 to 30,000, which is about the average size for bacterial proteins (O'Farrell, 1975). The molecular weights of purified β-lactamases have usually been determined by the gel filtration methods of Andrews (1964) but other methods, such as dodecyl-sulphate-polyacrylamide gel electrophoresis (Weber and Osborn, 1969) and equilibrium centrifugation (Yphantis, 1964), have been employed. There is often a large discrepancy of reported molecular weight values when determined by different methods for a single β-lactamase. For example, there is a 33% difference between the values obtained by Datta and Richmond (1966) for the TEM β-lactamase and the mean of all values obtained when gel filtration techniques were used.

Antisera and cross-reactions techniques have been used for comparing β-lactamases from a variety of strains (Richmond, 1975a). Cross-reaction has been reported among chromosomally-mediated β-lactamases from different strains of a single bacterial species (Ross and Boulton, 1972; Matthew *et al*, 1975) and between enzymes from different species and genera (Letarte *et al*, 1977). Only one example of plasmid-mediated β-lactamases cross-reacting has been reported, that between TEM-1 and TEM-2 (Sykes and Richmond, 1970; Matthew and Hedges, 1976). These two β-lactamases also have similar substrate profiles, hence correlation

between these two parameters for classification of β-lactamases appears possible (Sykes and Matthew, 1979). However, the conclusions concerning identity and differentiation of β-lactamases drawn from immunological studies and analytical iso-electric focusing, appear to be almost contradictory.

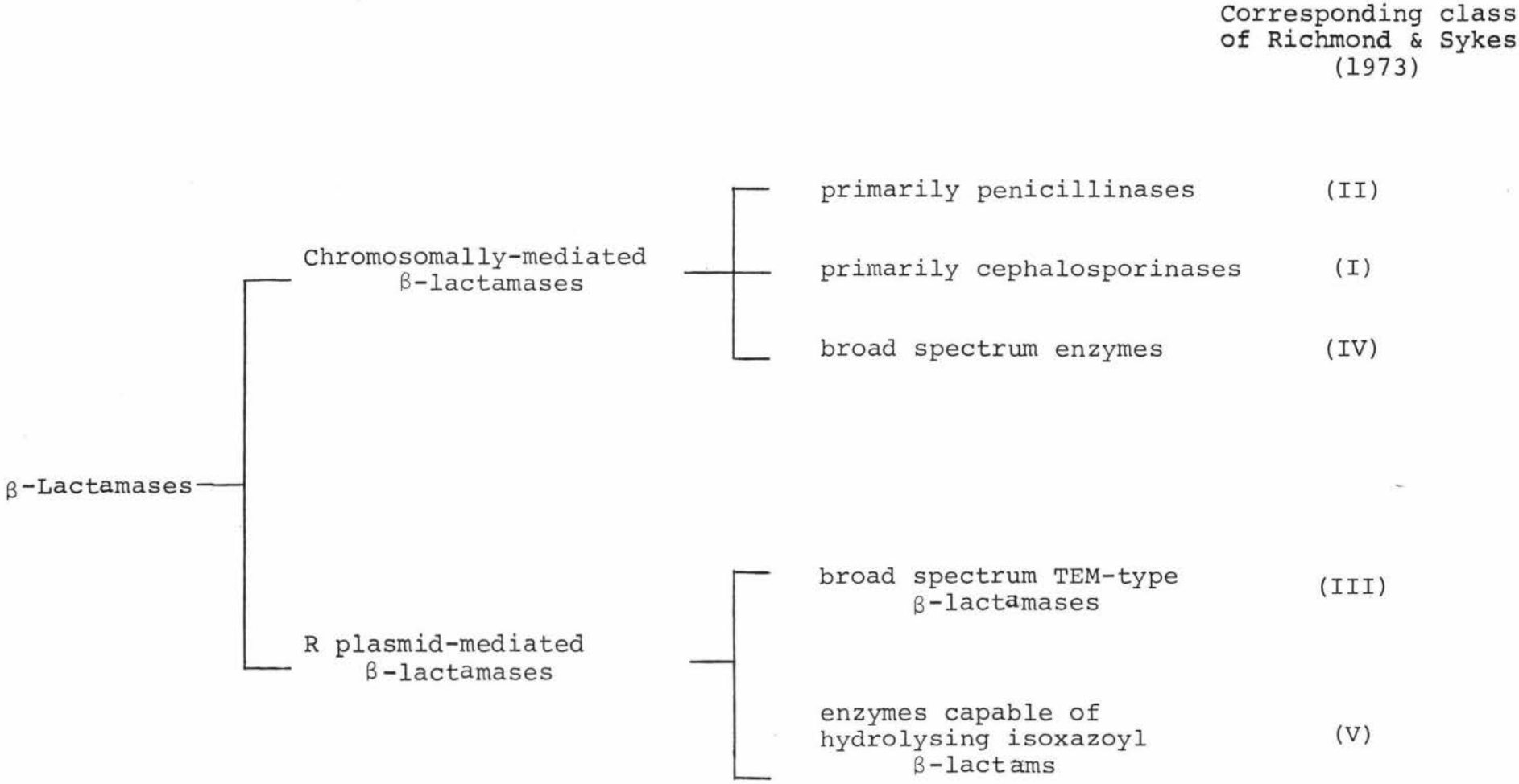
Matthew *et al.* (1975) have demonstrated the successful use of analytical iso-electric focusing (which determines the iso-electric point, pI, of the enzyme) for characterization and differentiation of β-lactamases. Knowledge of the pI of a β-lactamase alone is not sufficient for classifying the enzyme however, and is not necessarily indicative of the other properties of the enzyme. For example, the chromosomal cephalosporinase of *Bacteroides fragilis* 1604 E and the penicillinase specified by R_{GN238} have pIs less than 0.1 units apart (Sykes and Matthew, 1976).

Sykes and Matthew (1976) regrouped the classes described by Richmond and Sykes (1973) according to genetic determination of the β-lactamase (Fig. 2). In this system the enzymes are differentiated by the parameters of substrate profile, inhibition by cloxacillin and *p*CMB, inducibility, molecular weight and pI.

Amino acid composition has been determined for many Gram-positive and Gram-negative β-lactamases (Citri and Pollock, 1966). The results show a wide diversity of amino acid composition among these enzymes, with no obvious correlation between the amino acid composition and the enzyme properties. For example, the enzymes from *Enterobacter cloacae* strains 214 and 53, *E. coli* D31 and the R_{TEM} enzyme all have very similar amino acid compositions but widely different properties (Jack and Richmond, 1970).

Recently, four β-lactamases have been fully sequenced: three produced by Gram-positive bacteria, *Staphylococcus aureus* PC_1, *Bacillus cereus* β-lactamase I, *B. licheniformis* and one from the Gram-negative strain *E. coli* R_{TEM} (Ambler, 1979).

FIGURE 2: β-Lactamase Groupings Proposed by Sykes & Matthew (1976)



The β-lactamases from the two *Bacillus* species have about 60% of their enzyme sequences in common and, in fact, all four β-lactamase sequences are similar throughout their polypeptide chains with approximately 20% of the sites occupied by identical residues. Ambler (1979) suggests that these 'constant' residues probably include some, and perhaps all, of the catalytically important amino acids. He further suggests that the amount of sequence similarity between the four β-lactamases is such that a similar 3-D structure for the proteins is strongly indicated.

A β-lactamase can only be fully characterized and classified by combining information from a number of parameters, with no one characteristic alone being sufficient. Also for any classification system to be workable it is necessary to standardize the conditions for obtaining the data. Unfortunately, this has not always been done and hence information about a single β-lactamase may be variable, particularly when results from different sources are compared.

2.4 Distribution of β-lactamases among Bacterial Genera

Since 1961, the presence of β-lactamase has been reported in an ever-increasing number of genera. The list consists of widely diverse bacterial species: both Gram-positive and Gram-negative species, pathogenic and non-pathogenic species and species from genera inhabiting very different ecological niches. Kushner and Brueil (1977) have detected β-lactamase activity in blue-green 'algae' (prokaryotic cyanobacteria, Bergey (1974); Stanier *et al* (1971)).

Matthew and co-workers (1975 and 1976) using analytical isoelectric focusing techniques, detected a β-lactamase in all the bacterial species they tested (242 in total), including three β-lactamase-negative mutants and strains from *Haemophilus*, *Neisseria* and *Streptococcus* which were previously thought not to have the enzyme. They therefore suggested

that β-lactamase is of universal occurrence in bacteria. This concept is now becoming widely accepted among other authors, despite the fact that not every species of every genus has been examined for the enzyme.

2.5 Physiology and Expression of β-lactamases

(a) Location of β-lactamase in the bacterial cell

In Gram-positive bacteria β-lactamases are either cell-bound (Pollock, 1965; Lampen, 1967) or released into the surrounding medium as an extracellular enzyme (Pollock, 1962), but in Gram-negative cells, β-lactamase is almost always cell-bound. The cell envelope of Gram-negative bacteria consists of three major portions; a bilayered inner cytoplasmic membrane, a murein (peptidoglycan) layer and a bilayered outer membrane (Freer and Salton, 1971), with the region between the murein layer and the outer membrane referred to as the periplasmic space. Two different locations have been assigned for the cell associated β-lactamases, 'intracellular' or 'periplasmic'. The exact location of the enzyme within the 'periplasmic' region of the cell wall is still unknown, although it is thought to be either as free β-lactamase, attached to the outer membrane, attached to specific structures within the periplasmic space or attached loosely to the cytomembrane. The two locations for cell bound β-lactamases are differentiated by the method of releasing the enzyme from the cell. When the β-lactamase can be released by osmotic shock techniques it is termed a 'periplasmic' enzyme. The ones which are not released by shocking methods, but require disintegration of the cell, are termed 'intracellular' β-lactamases. It was originally suggested (Neu, 1968 and 1970) that the location of the β-lactamase was determined by the enzyme's genetic specification, with R-factor-mediated β-lactamases thought to be periplasmic and those considered to be of chromosomal

origin, located intracellularly. However, not all β-lactamases fitted into this hypothesis, for example the R-factor-mediated β-lactamase *Proteus mirabilis* could not be released by osmotic shocking methods (Neu and Winshell, 1970).

Smith and Wyatt (1974) showed that release or retention of β-lactamases during osmotic shock is a function of the molecular weight (MW) of the enzyme. During osmotic shock of the cells, the enzymes seem to be subjected to molecular sieving, perhaps by the petidoglycan layer of the cell wall, with those β-lactamases of MW 24,000 or less are released from the cell while β-lactamases of MW > 30,000 remain cell associated.

(b) The Permeability Barrier in Gram-negative bacteria

The outer membrane of Gram-negative cells has been shown to constitute a hydrophobic barrier, limiting both the inward and outward movement of molecules between the periplasmic space and the external environment (Payne and Gilvarg, 1968a and b; Costerton *et al*, 1974; Leive, 1974; Osborn *et al*, 1974; Nakae and Nikaido, 1975).

The permeability of the outer membrane depends on presence of matrix protein (Schnaitman, 1974; Rosenbusch, 1974), now called porin (Nakae, 1976). The findings of Benz *et al* (1977) studying isolated porin, support the suggestion that the protein forms large aqueous channels in the membrane allowing the diffusion of sugars and other water-soluble compounds up to MW 500-600, but excluding molecules of MW greater than 1000 (Nakae and Nikaido, 1975; Decad and Nikaido, 1976; Nikaido, 1976; Nikaido *et al*, 1977; Lutkenhaus, 1977; Zimmerman and Rosselet, 1977). Di Rienzo *et al*, (1978) found that porin allows transmembrane diffusion of hydrophilic substances of less than MW 650. Nakae

and Ishii (1980) reported that sugars of MW up to 800 daltons had free passage through the outer membrane of *E. coli*, which is consistent with the porin channel diameter being 0.9nm (Benz *et al*, 1977).

Evidence for the existence of the permeability barrier has come from comparisons of the cell wall structure of Gram-positive and Gram-negative bacteria, studies with spheroplasts where parts of the outer membrane have been removed (Cheng *et al*, 1970; Weiss and Fraser, 1973) and from studies of the effects of EDTA on permeability, enzyme activity and outer membrane lipopolysaccharide (Brown and Richards, 1965; Russell, 1971). Further evidence comes from the study of mutants that have defects in their outer membrane, which no longer demonstrate a permeability barrier and in which the β-lactamase is no longer cryptic (Richmond *et al*, 1976; Richmond and Curtis, 1974; Richmond and Sykes, 1973).

From experiments in these areas it appears that such a barrier may be species specific. For example, the barrier in strains of *Pseudomonas aeruginosa* excludes both penicillins and cephalosporins, while the barrier present in strains *E. coli* excludes penicillins but allows practically free passage to cephaloridine. Strains of *Haemophilus influenzae* do not exclude either penicillins or cephalosporins, therefore appear to have no barrier (Mederios and O'Brien, 1975). Recently it has been shown that strains of *Bacteroides fragilis* and *Neisseria gonorrhoeae* also produce β-lactamases that are readily accessible to β-lactam antibiotics, suggesting that they also possess no barrier to penicillins or cephalosporins (Sykes and Percival, 1978). To date, these are the only bacteria exhibiting this feature of no permeability barrier.

(c) Production of β-lactamase in the Cell

All the β-lactamases of Gram-postive bacteria are produced in large amounts (Pollock, 1962) and almost

all these enzymes are inducible (Citri and Pollock, 1966), some highly so. For example, the β-lactamases produced by *Bacillus cereus* are induced 500-fold when incubated in the presence of benzylpenicillin (Housewright and Henry, 1947).

The β-lactamases produced by Gram-negative bacteria are mostly constitutive, with the exceptions being inducible to levels usually lower than those obtained with Gram-positive β-lactamases. The first examples of inducible β-lactamases in Gram-negative strains were demonstrated by Hamilton-Miller (1963) in *Proteus*. Since this time others have reported: the Class I β-lactamases produced by strains of *Enterobacter cloacae* (Hennessey, 1967) were shown to have induction ratios greater than 100 using benzylpenicillin 500 μg/ml as inducer. The chromosomal Class Id β-lactamases synthesized by *Ps. aeruginosa* are inducible in all strains (Furth, 1979) with levels of enzyme increased in the presence of low inducer concentrations, but 2 to 5mg/ml required for maximal induction (Nordstrom and Sykes, 1974). Recently, the β-lactamases from *Yersinia enterocolitica* (Cornelis and Abraham, 1975) and *Chromobacterium violaceum* (Farrar and O'Dell, 1976) have been shown to be inducible. The enzymes from the latter species are Class Id β-lactamases very like those synthesized by *Ps. aeruginosa* and induction with benzylpenicillin causes over a 1000-fold increase in enzyme activity. The only report of inducible β-lactamse produced by *E. coli* is in strain JD41 (Dale, 1975) with the nature of the induction process similar to that described for *Enterobacter* strains studied by Hennessey.

2.6 The Genetic Determination of β-lactamases

The genes determining β-lactamases in Gram-negative bacteria are known to be located on the chromosomal DNA (Matthew and Harris, 1976) or extrachromosomally on plasmids or R-factors (Datta and Kontomichalou, 1965).

Matthew and Harris (1976) suggested that all the β-lactamases they detected in 242 strains by iso-electric focusing, were chromosomally-mediated. They concluded this because none of the bacteria were known to carry R-factors specifying β-lactamase; all attempts to transfer the enzyme-mediated resistance were unsuccessful and none of the strains produced β-lactamase with an iso-electric focusing pattern which matched that of any known R-factor-mediated β-lactamase. They also postulated that chromosomally-mediated β-lactamases are present in all bacteria. The amount of enzyme present may be very small, as in species of *Haemophilus* which produce less than 0.0012 μkat/mg dry weight bacteria (1 katal is the enzyme activity which catalyzes transformation of 1 mol. of substrate/second).

Only in two cases have β-lactamase genes (βla^+) been shown unequivocally, to be carried on the chromosome. The βla^+ genes in *E. coli* D31, a genetically modified strain of *E. coli* D1, have been investigated in detail and it is thought that at least three genes are involved in mediating β-lactamase. The structure of the enzyme is specified by a gene designated *ampC* (Burman *et al*, 1973). The expression of this gene is governed by *ampA* gene (Eriksson-Greenberg *et al*, 1965) which maps in the *E. coli* chromosome at about 81 min (Eriksson-Greenberg, 1968). The *ampB* gene modifies the expression of both chromosomal and plasmid β-lactamase genes (Boman *et al*, 1968; Nordstrom *et al*, 1968).

The second case involves the βla^+ gene carried on the *Pseudomonas* plasmid RP1, which mediates the synthesis of a TEM-2 enzyme (Ingram *et al*, 1972). The RP1 plasmid has

been transferred to *E. coli* and the βla^+ gene was found to integrate into the *E. coli* chromosome near the *leu* locus at 1 min (Richmond and Sykes, 1972).

β-lactamase genes have been found among all types of R-factors, although the number of different types of β-lactamase specified by plasmids is limited.

The first reports of βla^+ genes being carried extra-chromosomally were by Datta and Kontomichalou (1965) and Datta and Richmond (1966), who demonstrated that the Richmond and Sykes Class IIIa β-lactamase was specified by R_{TEM} in *E. coli*. This enzyme type has since been shown to be by far the most frequently occurring among R-factors (Evans *et al*, 1968; Yamagishi *et al*, 1969a; Lindqvist and Nordstrom, 1970; Sawai *et al*, 1970; Jenkins and Drabble, 1971; Dale and Smith, 1971). Kontomichalou *et al*, (1974) found that nine of ten plasmids of varying taxonomic origin mediated β-lactamases with TEM-like properties and a similarly high occurrence was reported by Yamagishi *et al* (1969b) and Ooka *et al* (1970). Matthew (1979) reported that the overall frequency of TEM-type β-lactamases was 77.4% among R-factors carried in Gram-negative bacteria.

The R_{TEM} plasmid is readily transmissible and β-lactamases specified by this R-factor have been encountered in such diverse species as *E. coli*, *P. mirabilis*, *Ps. aeruginosa*, *H. influenzae* and *N. gonorrhoeae* (Richmond, 1979).

A second type of R-factor-mediated β-lactamase is the isoxazoyl-β-lactam hydrolyzing type, Classes IV and V, which occur with an overall frequency of 15.7% of all plasmid-mediated enzymes (Evans *et al*, 1968; Ooka *et al*, 1970; Jenkins and Drabble, 1971; Rubin and Smith, 1973; Hedges *et al*, 1974; Matthew, 1979).

An R-factor-mediated penicillinase, the only one so far reported, was purified from *Salmonella typhimurium* by Neu and Winshell (1970) and later classified as a Class II enzyme by Richmond and Sykes (1973). Cephalosporinases are also rarely mediated by R-factors (Richmond and Sykes, 1973), the exception is a Class I enzyme specified by R_{22K} although this enzyme has a very different substrate profile to other cephalosporinases and a MW of 37,000, which is higher than most β-lactamases, whether plasmid or chromosomally mediated (Kontomichalou *et al*, 1974). Finally, several examples are known of a single strain producing different β-lactamases mediated by both chromosomal and plasmid genes (Matthew, 1975).

2.7 The Clinical Importance of β-Lactamases

The clinical problem caused by β-lactamases rendering β-lactam antibiotics ineffective was initially recognised in *Staphylococci* organisms. The clinically resistant *Staphylococci* caused an unacceptably high incidence of infection and mortality throughout hospitals universally, in the early 1950's and were all found to produce penicillinases (Barber, 1947; Dietz and Bondi, 1948; Sabath, 1971).

Until the early 1960's, virtually all research was focused on the β-lactamases produced by Gram-positive bacteria, namely *Staphylococcus* and *Bacillus* species (Pollock, 1961, 1963 and 1964; Citri and Pollock, 1966).

With the introduction of semi-synthetic β-lactamase-stable penicillins such as methicillin, cloxacillin and nafcillin, the problem of *Staphylococcal* infection was solved. However, a new problem was uncovered, the Gram-negative bacteria, in particular *Pseudomonas* and *E. coli*, emerged as the greatest cause of hospital infections. These Gram-negative organisms were inherently more resistant to the early penicillins such as benzylpenicillin, but the semi-synthetic β-lactam ampicillin was found effective against *E. coli*.

The introduction and wide therapeutic use of broad-spectrum β-lactams such as ampicillin, carbenicillin and cephalosporins resulted in the observation of heterogeneic Gram-negative bacterial populations and isolation of naturally-occurring resistant strains, which appeared to be selected for by the antibiotic use. It was soon found that these resistant strains produced β-lactamases (Jago *et al*, 1963; Fleming *et al*, 1963; Ayliffe, 1964; Hamilton-Miller *et al*, 1965).

Then, some β-lactamases were found to be specified by transmissible R-factors: Datta and Kontomichalou (1965) described a β-lactamase carried on R_{TEM}, an R-factor which is readily transmissible and has been encountered in widely diverse bacterial species with a high frequency (Kontomichalou *et al*, 1974). Hence the awareness of the role of β-lactamses in conferring resistance to β-lactams was expanded. However, it was realised that β-lactamase presence in Gram-negative bacteria did not necessarily account for the total resistance exhibited by the strain and that the cell wall of the bacteria was involved (Medeiros *et al*, 1974); Neu, 1974; Percival *et al*, 1963; Smith *et al*, 1969; Tsang *et al*, 1975). Therefore attention has returned to the inherent (often called 'intrinsic') resistance displayed by Gram-negative bacteria to benzylpenicillin, with recognition of the Gram-negative cell wall acting as a permeability barrier, restricting access to the antibiotic's target site (Costerton *et al*, 1974; Leive, 1974).

3. AIMS OF THIS RESEARCH

K. Smith found that β-lactamase activity was widespread among strains of *Photobacterium;* the β-lactamases from this genus had not been described, nor have those from any other marine bacterial genus.

The overall aim of this study is to characterize, describe and if possible classify the β-lactamase from the marine genus *Photobacterium.*

The naturally-occurring isolate *P. leiognathi* 206 was chosen as the laboratory strain and the β-lactamase from this strain was investigated in detail. The properties of substrate profile and inhibition of the β-lactamaseactivity were used to classify the enzyme; enzyme location within the bacterial cell, expression of the enzyme (including existence of a permeability barrier), inducibility and the genetic location of the β-lactamase gene were also investigated.

Comparative studies of the β-lactamases from a sample of *Photobacterium* strains of each species, were undertaken using substrate profile and genetic determination, with the β-lactamases from all strains compared by analytical iso-electric focusing.

SECTION B

MATERIALS

1. BACTERIAL STRAINS

All *Photobacterium* strains were obtained from Dr J.L. Reichelt, Roche Research Institute of Marine Pharmacology (RRIMP), P.O. Box 235, Dee Why, Sydney, Australia.

Table IV lists all strains used, giving their RRIMP strain numbers and any other published designations, as well as any available information on source and isolation of the organisms. For all offshore isolates the geographical location is given as the nearest landmass. In the text, the strains are referred to by their species name and the appropriate RRIMP strain number.

The *Escherichia coli* strain, used in genetic experiments, was obtained from D. Lane and P. Berquist (Department of Cell Biology, University of Auckland, Auckland). The strain has the designation *E. coli* PB1395 F^- met^- supE supF hsdR (Jamieson and Bremner, 1979). The other strains used in genetic experiments are all modifications of strain *P. Leiognathi* 206 and are listed below with their relevant genetic markers: -

strain 534 = *P. Leiognathi* 206 R68.45 βla^+ Nm^r Tc^r

strain $PqSm^r$ = *P. Leiognathi* 206 βla^- Sm^r

strain A_{43} = *P. Leiognathi* 206 βla^- leu^- Sm^r

TABLE IV: *Photobacterium* Strains

Photobacterium Species	RRIMP Strain No.	Other Designations	Origin Environmental	Origin Geographical	Date	Reference
P. Leiognathi	16	ATCC 27561 (B 480)	SW	Indonesia		Reichelt & Baumann, 1973
	80 (type strain)	ATCC 25521 (L 224)	L.O.	Gulf of Siam,	1967	Boisvert *et al*, 1967
	202	ATCC 25522 (L 225)	L.O.	Indonesia,	1975	(Dr J.W. Hastings)
	204	(B 474)	Squid	Hawaii		Reichelt & Baumann, 1973
	205	NCMB 391 (B 477)	-	India,	1958	Hendrie *et al*, 1970
	206	(L 721)	L.O.	Indonesia, (Sulu Sea)	1975	Nealson & Hastings, 1977
	345	(SVB-1)	L.O.	Tropical North East Australia	1978/9	(Dr J.R. Paxton)
	632	(SC 3a-1)	Fish	Coogee Bay, (NSW) Australia	1977	(Dr J.L. Reichelt)
	633		Fish	Coogee Bay, (NSW) Australia	1977	(Dr J.L. Reichelt)
	634	(SR-E1)	Fish	Sydney Harbour, Australia	1976	(Dr J.L. Reichelt)
	635	(SR-D1)	Fish	Sydney Harbour, Australia	1976	(Dr J.L. Reichelt)

TABLE IV: CONTINUED

Photobacterium Species	RRIMP Strain No.	Other Designations	Origin: Environmental	Origin: Geographical	Date	Reference
P. Leiognathi Cont'd.	638	($SR\text{-}C_1$)	Fish	Sydney Harbour, Australia	1976	(Dr J.L. Reichelt)
	644	($SC\ 4b\text{-}Br_1$)	Fish	Coogee Bay (NSW) Australia	1977	(Dr J.L. Reichelt)
	647	($SC_1\text{-}Br_1$)	Fish	Coogee Bay (NSW) Australia	1977	(Dr J.L. Reichelt)
	648	($SC_5\text{-}2$)	Fish	Coogee Bay (NSW) Australia	1977	(Dr J.L. Reichelt)
	771	(CHLP-2)	L.O. & I	Tropical N.E. Australia	1979	(Dr J.R. Paxton)
	806	($SV\text{-}A_3$)	L.O.	Tropical N.E. Australia	1978/9	(Dr J.R. Paxton)
P. orgustum	77	ATCC 25915	SW	Offshore Hawaii		Baumann, Baumann & Mandel, 1971
	(type strain)	(B 68)		(lat. 20° 30', long. 157° 30')	1970	"
	78	(B 69)	SW	"		"
	79	(B 70)	SW	"		"
	159	(B 67)	SW	"		"
	160	(B 71)	SW	"		"

TABLE IV: CONTINUED

Photobacterium Species	RRIMP Strain No.	Other Designations	Origin Environmental	Origin Geographical	Date	Reference
P. phosphoreum	145	(B 404)	Fish	Japan	pre-1966	Reichelt & Baumann, 1973
	146	(B 455)	Fish	Hawaii		Reichelt & Baumann, 1973
	147	(B 496)	Squid,			Nakamura & Matsuda, 1971
	164	(B 465)	Fish	Japan		Reichelt & Baumann, 1973
	174 (neotype strain)	ATCC 11040 (B 439)	(Prof. A.J. Kheyver, Delft)			Hendrie *et al*, 1970
	203	(B 444)	Fish	Hawaii		Reichelt & Baumann, 1973
	617	(CH LP - 3)	L.O. & I	Tropical N.E. Australia	1979	(Dr J.R. Paxton)
	652	(OP - 1)	Fish	Sydney	1977	(Dr J.L. Reichelt)
	661	(RH 2b-2)	Fish	Sydney	1977	(Dr J.L. Reichelt)
	671	(RH 1-8)	Fsih	Sydney	1977	(Dr J.L. Reichelt)
	672	(WI-10)	Fish	Sydney	1977	(Dr J.L. Reichelt)
	674	(H 1-5)	Fish	Sydney	1977	(Dr J.L. Reichelt)
	681	(M1 9-4)	Fish	Sydney	1977	(Dr. J.L. Reichelt)

TABLE IV: CONTINUED

Photobacterium Species	RRIMP Strain No.	Other Designations	Origin Environmental	Origin Geographical	Date	Reference
P. phosphoreum Cont'd.	808	(CHB-1)	L.O.	Tropical N.E. Australia	1979	(Dr J.R. Paxton)
P. fischeri	31	ATCC 14546 (B 395)	(Prof. A.J. Khuyver, Delft)			Spencer, 1955
	130	1004	luminous shrimp (U.S.A. collection)		pre-1975	
	148	ATCC 7744	(source unknown)			Johnson *et al*, 1943)
	(neotype strain)	(B 398)				Johnson & Shunk, 1936 (Woods Hole, Mass., U.S.A.) Hendrie *et al*, 1970
	173	ATCC 25918 (B 61)	SW	Offshore Hawaii	1970	Baumann, Baumann & Mandel, 1971
	341	(AZ 1-5)	I	Heron Island, Australia (tropical)	1978	(Dr J.R. Paxton)
	768	(AF 1-3)	I	Heron Island, Australia (tropical)	1978	(Dr J.R. Paxton)
	805	(AF 2-1)	I	Heron Island, Australia (tropical)	1978	(Dr J.R. Paxton)
P. logei	846	Scripps P_7	SW & 1	McMurdo Sound, Antarctica	1977-8	Olson *et al*, 1978
	850	Scripps P_{23}	SW & 1	McMurdo Sound, Antarctica	1977-8	Olson *et al*, 1978
	851	Scripps P_{41}	SW & 1	McMurdo Sound, Antarctica	1977-8	Olson *et al*, 1978

TABLE IV: CONTINUED

ABBREVIATIONS:

RRIMP = Roche Research Institute of Marine Pharmacology

ATCC = American Type Culture Collection

NCMB = National Collection of Marine Bacteria

Scripps = Scripps Institution of Oceanography, California, U.S.A.

(B), (L), (SV), (SC), (SR), (CHLP), (OP), (RH), (W), (H), (M), (CH), (AZ), (AF) are laboratory designations

SW = seawater

L.O. = luminous organ (fish)

I = intestine (fish)

( ) round a name means was isolated by ; no literature reference

2. REAGENTS AND MEDIA

2.1 Reagents

(a) Buffers

(i) 1.5M Tris-HCl buffer, pH 8.8

Trizma base (Sigma)	18.17 g
H_2O to	100.0 ml
12N HCl to pH 8.8	

(ii) 1M Tris-HCl buffer, pH 7.5

Trizma base (Sigma)	121.2 g
H_2O to	1.0 l
12N HCl to pH 7.5	

(iii) 0.5M Tris-HCl buffer, pH 6.8

Trizma base (Sigma)	6.06 g
H_2O to	100.0 ml
12N HCl to pH 6.8	

(iv) 0.05M Tris-HCl buffer, pH 8.0

Trizma base (Sigma)	6.06 g
H_2O to	1.0 l
12N HCl to pH 8.0	

(v) 0.05M Tris-EDTA (TE) buffer, pH 7.5

Trizma uase (Sigma)	6.06 g
EDTA	2.41 g
H_2O to	1.0 l
12N HCl to pH 7.5	

(vi) 0.025M Tris-glycine buffer, pH 8.3

Trizma base (Sigma)	6.07 g
glycine	28.20 g
H_2O to	2.0 l
12N HCl to pH 8.3	

(vii) 0.5M phosphate buffer, pH 7.0

KH_2PO_4	23.780	g
$Na_2HPO4.12H_2O$	108.775	g
H_2O to	1.0	l

(viii) 0.1M phosphate buffer, pH 7.0

KH_2PO_4	5.208	g
$Na_2HPO_4.12H_2O$	21.755	g
H_2O to	1.0	l

(ix) 2M acetate buffer, pH 4.0

Sodium acetate (anydrous)	40.0	g
H_2O to	100.0	ml
glacial acetic acid to pH	4.0	

(b) 'Shocking' solutions

(i) Washing solution:

NaCl	5.845	g
1M Tris-HCl buffer, pH 7.5	5.0	ml
H_2O (deionized) to	100.0	ml

(ii) Hypertonic solution:

NaCl	5.845	g
Sucrose	17.115	g
1M Tris-HCl buffer,pH7.5	1.0	ml
H_2O (deionized) to	100.0	ml

(iii) Hypotonic solution:

NaCl	1.461	g
$MgCl_2$	0.095	g
1M Tris-HCl buffer, pH7.5	1.0	ml
H_2O (deionized) to	100.0	ml

(c) Stock iodine reagent

I_2	40.6	g
KI	200.0	g
H_2O to	1.0	l

Dilute 1/20 in 2M acetate buffer, pH4.0 just prior to use.

(d) Electrophoresis reagents

(i) Running acrylamide:

acrylamide	30.0 g
methylene bis acrylamide	0.5 g
H_2O to	100.0 ml

(ii) Stacking acrylamide:

acrylamide	30.0 g
methylene bis acrylamide	1.6 g
H_2O to	100.0 ml

(iii) Running gel; 15%:

1.5M Tris-HCl buffer, pH 8.8	2.5 ml
running acrylamide	5.0 ml
glycerol (40%)	2.5 ml
ammonium persulphate (0.1 g/ml)	0.025 ml
N,N,N',N",-tetramethyl-ethylenediamine (TEMED) (Sigma)	0.006 ml

(iv) Stacking gel, 4%

0.5M Tris-HCl buffer, pH 6.8	2.5 ml
stacking acrylamide	1.5 ml
H_2O	6.0 ml
ammonium persulphate (0.1 g/ml)	0.03 ml
TEMED (Sigma)	0.01 ml

(v) Coomassie blue stain:

Coomassie brilliant blue	0.4 g
isopropanol	250.0 ml
glacial acetic acid	100.0 ml
H_2O to	1.0 l

(e) Transformation reagents

(i) Lysis buffer:

sucrose	25.0 g
0.05M Tris-HCl buffer, pH 8.0	100.0 ml

(ii) Ethylenediaminetetra-acetate (EDTA):

EDTA	7.45 g
0.05M Tris-HCl buffer, pH8.0	100.0 ml

(iii) Detergent:

Triton-X-100	10.0 ml
EDTA	22.4 g
0.05M Tris-HCl buffer, pH 8.0	1.0 l

(iv) 100 mM $MgCl_2$:

$MgCl_2 . 6H_2O$	20.33 g
H_2O to	1.0 l

(v) 10 mM NaCl:

NaCl	0.585 g
H_2O to	1.0 l

(vi) 30 mM $CaCl_2$:

$CaCl_2.2H_2O$	4.41 g
H_2O to	1.0 l

2.2 Marine Media

The marine media used were based on those of Reichelt and Baumann (1973) and modified by Reichelt (pers. comm.)

(a) Artificial Seawater (ASW)

ASW was made as double strength stock solutions, with all water used being first distilled and deionized.

Double strength ASW stock solution (i):

NaCl	561.6 g
KCl	24.0 g
$MgSO_4.7H_2O$	395.2 g
H_2O	4.0 l

Dissolve completely.

$CaCl_2.2H_2O$	46.4 g
H_2O	1.0 l

Dissolve completely, then combine the two solutions and add deionized water to bring volume to 16 litres.

Double strength ASW stock solution (ii):

NaCl	702.0 g
KCl	30.0 g
H_2O	4.0 l

Dissolve completely.

$MgCl_2.6H_2O$	408.0 g
H_2O	4.0 l

Dissolve completely, then combine the two solutions and add deionized water to bring volume to 16 litres.

The recipe for ASW stock solution was changed from (i) to (ii) (Reichelt, pers. comm.), during the course of carrying out this work to minimize precipitation which occurred after autoclaving the 'luminous' broth. This change had no effect on the growth rate of any *Photobacterium* strains. All ASW stock solutions were stored

in black containers to inhibit growth of algae. Single strength ASW was used ad diluent for *Photobacterium* strains.

(b) 'Luminous' Broth (LB)

yeast extract (Difco)	5.0 g
tryptone (Difco)	5.0 g
glycerol (Unilab)	3.0 ml
1M Tris-HCl buffer, pH 7.5	50.0 ml
$NH_3Fe(OH)$.citrate	1.0 ml of 2.5% solution
Na-β-glycerophosphate	1.0 ml of 15% solution
NH_4Cl	5.0 ml of 20% solution
ASW (double strength)	500.0 ml
H_2O	440.0 ml
Autoclave: 121°C, 15 lb/in^2,	30 min

(c) 'Luminous' Agar (LA)

LB	1.0 l
agar (Bacto)	15.0 g

Autoclave: 121°C, 15 lb/in^2, 30 min and dispense for plates or slants as required.

For LA + starch (LSA) plates, add 40 ml of 3% starch solution/litre of LA, resulting in a final concentration of 1.2% starch incorporated which is sufficient to act as an indicator without inhibiting the growth of *Photobacterium* strains.

(d) Marine Minimal Agar (MMA)

$NH_3Fe(OH)$.citrate	1.0 ml of 2.5% solution
Na-β-glycerophosphate	1.0 ml of 15% solution
NH_4Cl	5.0 ml of 20% solution
ASW (double strength)	500.0 ml
H_2O	473.0 ml
agar (Bacto)	15.0 g
glucose	10.0 ml of 20% solution

The glucose is added just prior to dispensing the agar and when required, amino acid supplements are added:

leucine	10.0 ml of 4 mg/ml solution

2.3 Terrestrial Media

(a) Brain Heart Infusion (BHI)

Made according to manufacturer's instructions:

BHI (Difco)	37.0 g
H_2O	1.0 l

Autoclave: 121°C, 15 lb/in^2, 30 min.

(b) BHI Agar

BHI	1.0 l
agar (Bacto)	15.0 g

Autoclave: 121°C, 15 lb/in^2, 30 min.

(c) Phosphate Buffer Saline (PBS), pH 7.2

NaCl	8.00 g
K_2HPO_4	1.21 g
KH_2PO_4	0.34 g
H_2O to	1.00 l

(d) Terrestrial Minimal Agar (TMA)

K_2HPO_4	10.5 g
KH_2PO_4	4.5 g
$(NH_4)_2SO_4$	1.0 g
Na.citrate.$2H_2O$	0.5 g
H_2O to	1.0 l
agar (Bacto)	15.0 g

Autoclave: 121°C, 15 lb/in^2, 30 min and then add following ingredients just prior to dispensing the agar:

$MgSO_4.7H_2O$	1.0 ml of 20% solution
glucose	10.0 ml of 20% solution
vitamin B_1	0.5 ml of 1.0% solution

When required, amino acid supplements were added:

methionine	10.0 ml of 4 mg/ml solution

3. ANTIBIOTICS

(a) Penicillins:

benzylpenicillin - 'Crystapen', Glaxo Laboratories
ampicillin - 'Penbritin', Beecham Research Laboratories
carbenicillin - 'Pyopen', Beecham Research Laboratories
cloxacillin - 'Orbenin', Beecham Research Laboratories
methicillin - 'Celberin', Beecham Research Laboratories
oxacillin (sodium) - Glaxo laboratories.

(b) Cephalosporins:

cephalexin - 'Cephorex', Glaxo Laboratories
cephaloridine - 'Cephoran', Glaxo Laboratories
nitrocefin - '87/312', Dr Matthew, Glaxo Laboratories, London

(c) Others:

neomycin - Sigma
streptomycin - Glaxo Laboratories
tetracycline - Sigma

SECTION C

METHODS

1. CULTIVATION AND MAINTENANCE OF BACTERIAL STRAINS

All *Photobacterium* strains were grown on luminous agar (LA), with *P. leiognathi* and *P. angustum* strains incubated at 30°C for 18 hours, *P. phosphoreum* and *P. fischeri* strains incubated at 25°C for 18 hours and *P. logei* strains incubated at 15°C for about 30 hours. Broth cultures were grown in luminous broth (LB) with shaking at the appropriate temperature and when required, optical density of the culture was measured spectrophotometrically by a Bausch-Lomb Spectronic 20 at 525 nm or by a Klett-Summerson colorimeter with a red filter. Growth was calculated as mg dry weight cells/ml by reference to a previously calibrated curve of optical density versus mg dry wt/ml (Figs. 3a and 3b, prepared by K. Smith). *Photobacterium* strains were maintained on LA plates for periods up to 8 weeks, held at 10°C in the case of *P. leiognathi*, *P. angustum* and *P. fischeri* strains and 4°C for strains of *P. phosphoreum* and *P. logei*. For more permanent storage, up to six months, the strains were kept on agar slopes in screw-capped bijoux at the temperatures mentioned above.

E. coli PB1395 was cultivated in BHI broth or on BHI agar and incubated at 37°C for 18 hours. The strain was maintained on BHI agar plates at 4°C and stored permanently in BHI broth: glycerol (5:2) at -16°C.

All overnight cultures were grown for 18 hours, unless otherwise stated and, for volumes greater than 3ml, incubation was always done with shaking. Viable counts were performed by spreading 0.1 ml samples of overnight broth cultures on appropriate agar plates. The broth cultures were diluted to give 100-200 colonies/plate which usually involved a 10^{-5} or 10^{-6} dilution.

FIGURE 3a: Spectronic 20 Optical Density at 525 nm versus mg dry weight cells
Prepared by K. Smith (1978)

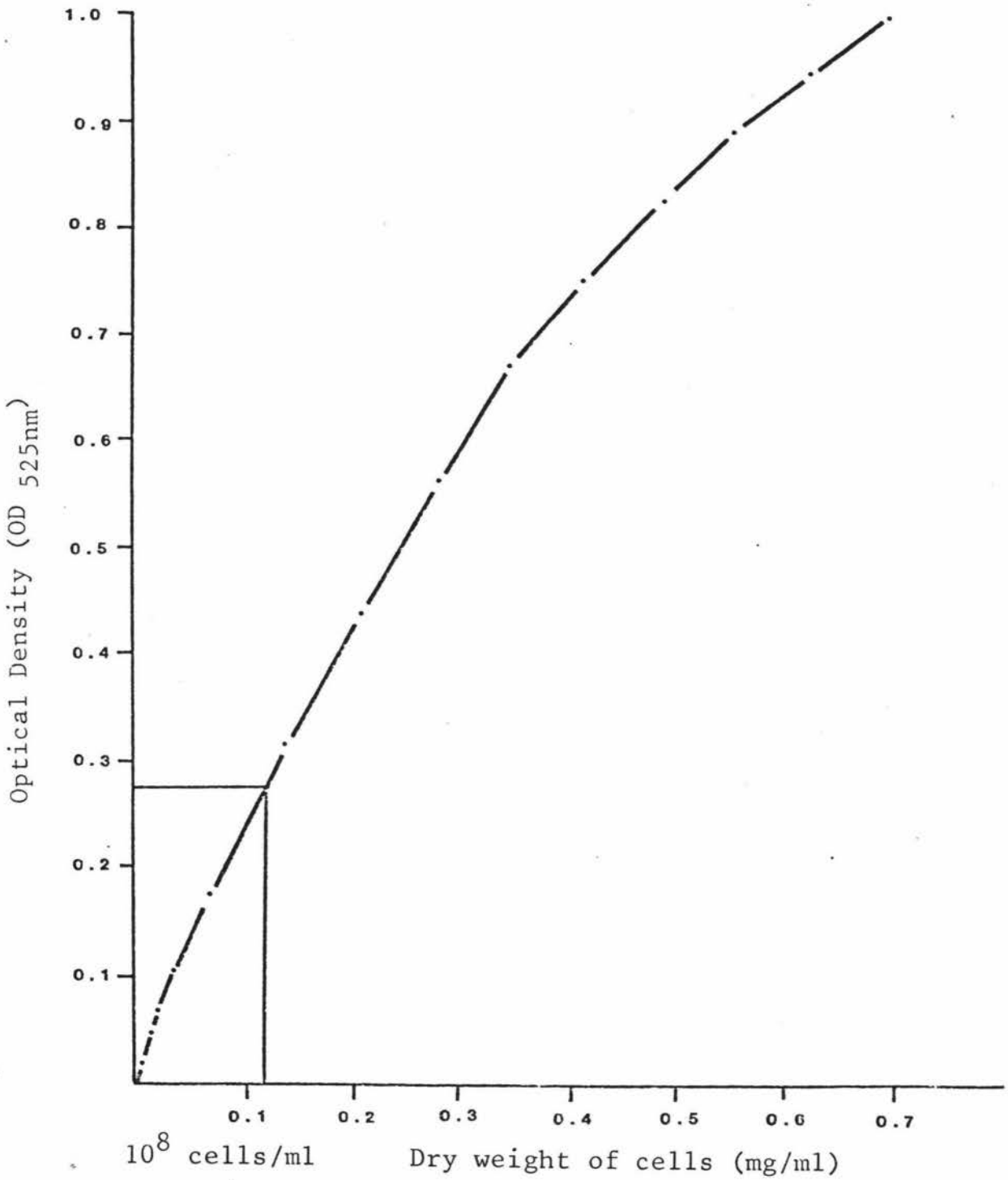
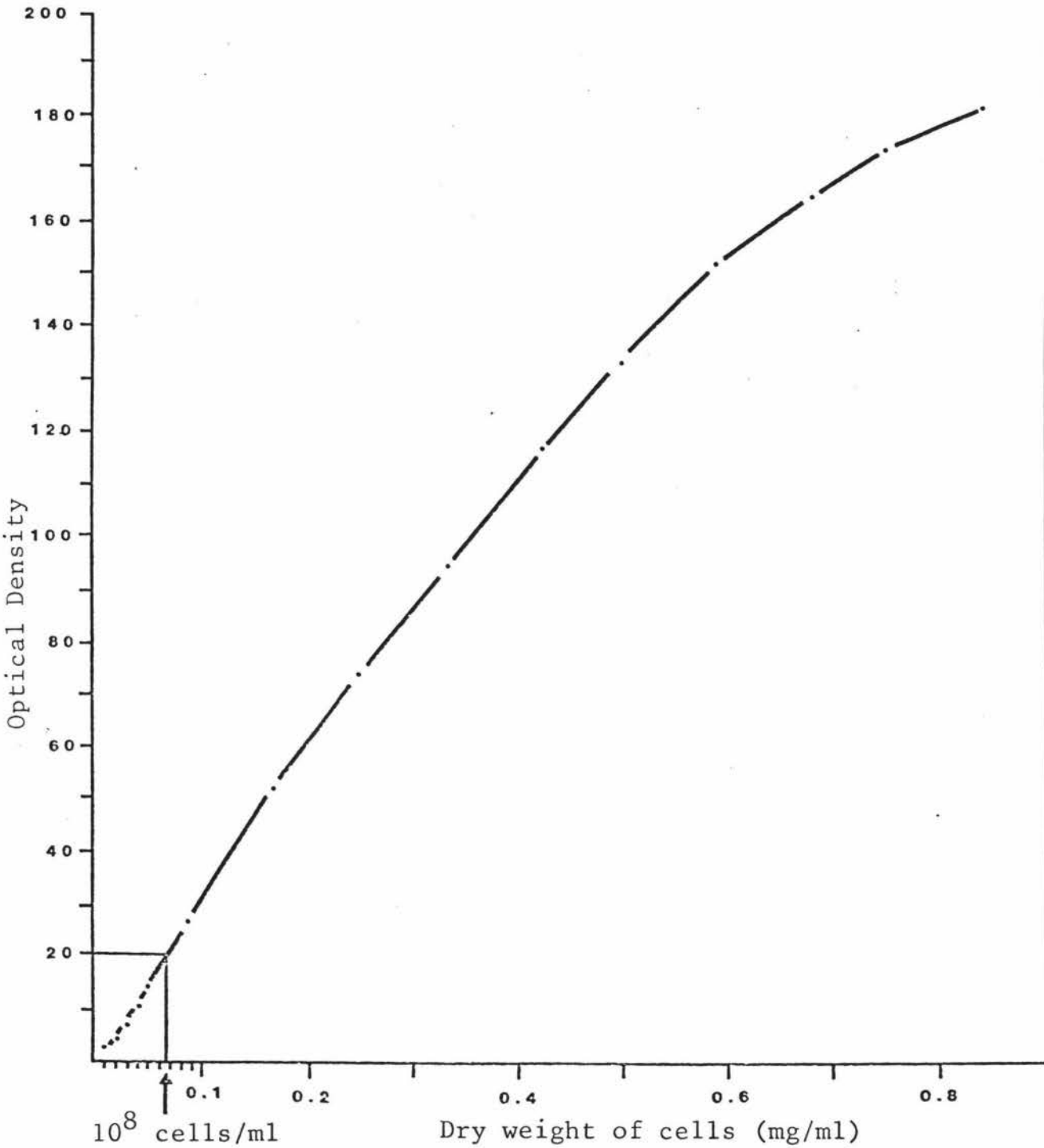


Figure 3b: Klett Optical Density versus mg dry weight cells. Prepared by K. Smith (1978) using red filter



2. CRUDE ENZYME PREPARATIONS

2.1 Sonication

When necessary, 5 ml volumes of cells held on ice were disrupted by a MSE 100 Watt Ultrasonic disintegrator, with 3 x 15 seconds bursts at maximum amplitude using a 3/4 inch diameter probe.

2.2 Osmotic Shock Treatment

The method used was that described by Neu and Chou (1967), with the modifications of Unemoto *et al*, (1974).

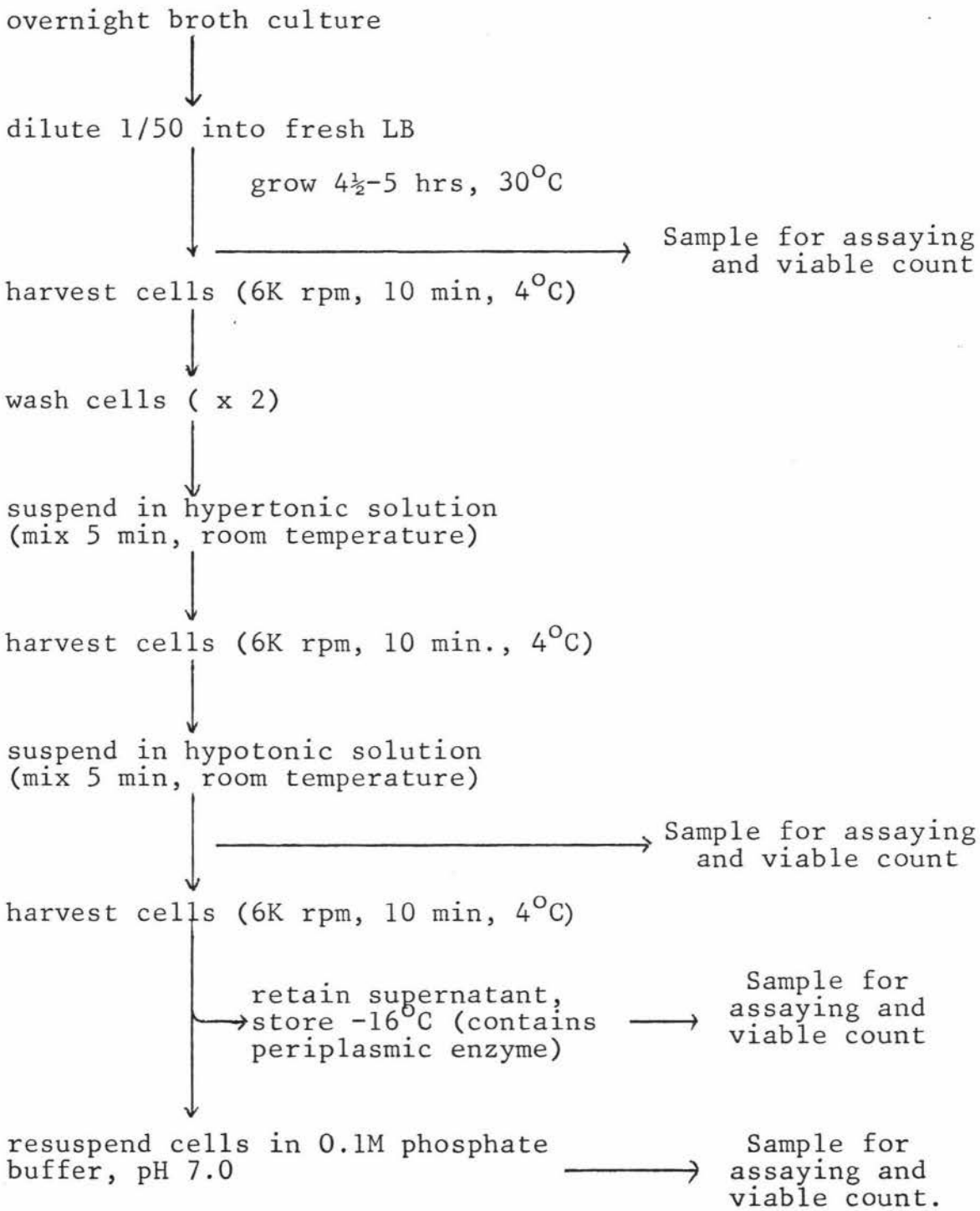
Refer to flow chart for procedure.

3. ASSAY OF β-LACTAMASE ACTIVITY

3.1 Standard Iodometric Assay

β-Lactamse activity was assayed by Perret iodometric method as described by Ross and O'Callaghan (1975). The iodometric assay relies on the stoichiometric uptake of iodine by penicilloic acid, the product of β-lactamase hydrolysis of penicillins. The extent of iodine uptake by the reaction product is the difference in free iodine between the test and the control flasks. The difference is measured by titration with sodium thiosulphate solution or by decrease in optical density at 490 nm, which is the Sargent (1968) modification of the former method and the one used in this work.

OSMOTIC SHOCK PROCEDURE



(a) Definition of enzyme activity unit

The most commonly used unit to express β-lactamase activity and the one used throughout this work, is that defined by Pollock and Torriani (1952):

1 enzyme unit (U) = 1 μmol benzylpenicillin destroyed/hour at 30°C and pH 7.0

(b) Procedure

The substrate used for assaying, was benzylpenicillin at a concentration of 10^4 units/ml of 0.1M phosphate buffer, pH 7.0, unless otherwise specified. The β-lactamase was assayed as a crude preparation from osmotically shocked cells or as present in intact cells of an overnight culture suspension, grown as in Section C, 1 then diluted into fresh medium and grown for 3½ to 5 hours, to achieve maximum enzyme production (see Section D, 2.1).

The assay was carried out at 30°C in a gently shaking waterbath, with 5 ml aliquots of substrate in 50 ml flasks, prewarmed for about 10 min prior to the start of the assay. The assay was initiated by addition of the enzyme sample and stopped after a fixed time by addition of 10 ml of iodine reagent. To allow iodine uptake, incubation was continued for a further 10 min when penicillins were used as substrate and 20 min when cephalosporins were used. Control flasks were set up and incubated in an identical fashion, with the exception of the enzyme added immediately following the addition of the iodine reagent. The optical density of the test and control mixtures were read at 490nm by a Bausch-Lomb Spectronic 20, using the test mixture as a blank with direct comparison to the control mixture.

Volume of enzyme and assay times used were adjusted prior to the start of assay, to give a final colorimeter reading of greater than 0.1 and less than 1.0 on the Spectronic 20 meter scale, to ensure accuracy of reading. Usually 0.5 ml to 1.0 ml crude enzyme preparation or intact cells assayed for 30 to 60 min was found to be satisfactory.

(c) Calculation of activity

(i) For penicillin substrates:

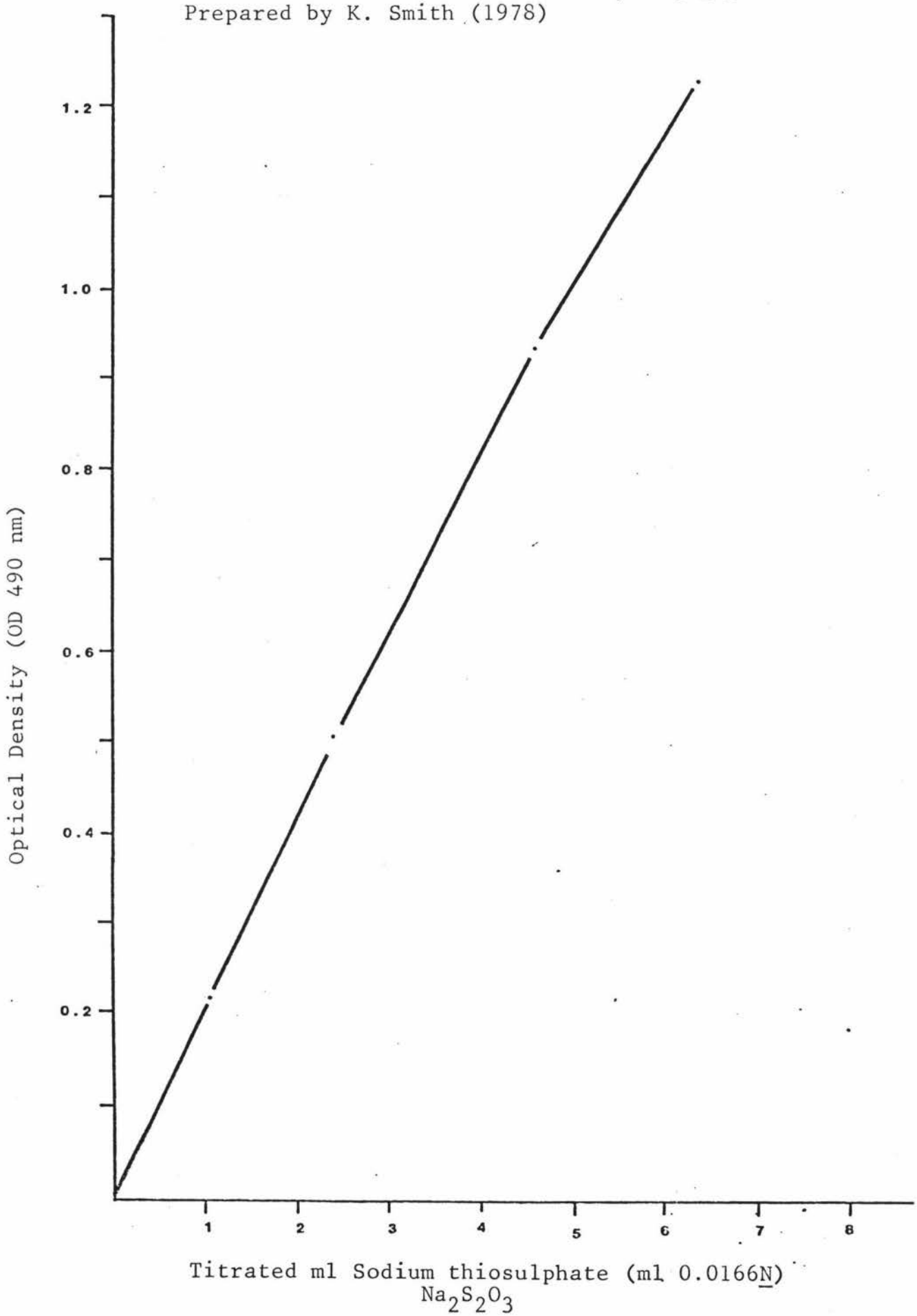
One molecule of penicilloic acid takes up 8 equivalents of iodine and the difference in optical density (OD) between the test and control mixtures was converted to volume of 0.016N sodium thiosulphate by reference to a previously calibrated standard curve of optical density versus titration volume of sodium thiosulphate (Fig. 4, prepared by K. Smith). Since 1ml 0.016N I_2 is equivalent to 1ml 0.016N sodium thiosulphate, which is equivalent to 2 µmol penicilloic acid, the enzyme activity is determined by: -

$$U = \frac{2 \times (\text{OD equivalents of mls } Na_2S_2O_3)}{a \times t/60}$$

where U = enzyme activity units/ml
a = volume assayed
t = time of assay

The specific activity of units/mg dry weight of cells is obtained by dividing U/ml by mg dry wt./ml, which is taken from the OD versus mg dry weight standard curve (Fig. 3a or 3b).

Figure 4: Optical Density at 490nm of KI/I_2 versus titrated mls sodium thiosulphate ($Na_2S_2O_3$)
Prepared by K. Smith (1978)



(ii) For cephalosporin substrates:

The stoichiometry of the reaction products with iodine following β-lactamase hydrolysis of cephalosporins is different from and more complex than, that with penicillins. Alicino (1961) found that 1 ml 0.016N $Na_2S_2O_3$ is equivalent to about 4 μmol cephalosporin destroyed and hence the equation (above) is modified to:

$$U/ml = \frac{4 \times (\text{OD equivalents of mls } Na_2S_2O_3)}{a \times t/60}$$

However the stoichiometry varies somewhat with the nature of the cephalosporin involved, hence making the assay a little less reliable when employed with cephalosporins as substrates, although for comparative studies the above formulae are considered acceptable (Ross and O'Callaghan, 1975). As the iodometric assay was used for comparative work in this thesis, the formulae presented above were used as they stand.

3.2 Detection of β-lactamase activity on solid media

Perret (1954) described a method for identifying colonies of β-lactamase-producing bacteria on solid media. A modification of this method was used as a qualitative test to demonstrate the presence or absence of β-lactamase in various strains of bacteria.

Stock iodine reagent was diluted 1/10 into 10^4 units benzylpenicillin/ml 0.10M phosphate buffer, pH 7.0. The iodine-substrate reagent was poured onto LSA plates containing 100-200 colonies/plate of 18 hour old *Photobacterium* strains. Any excess, not absorbed into the agar, was poured off. The plate was observed over the next 30 min for the appearance of white colonies which had a surrounding clear zone showing against the blue-black background, thus indicating the presence of β-lactamase. Non-β-lactamase-producing colonies

remain yellow-red (due to the iodine colour) and have no surrounding clear zone.

4. SUBSTRATE PROFILE

All substrates were prepared as 6mM solutions in 0.1M phosphate buffer, pH 7.0 (Jack and Richmond, 1970).

benzylpenicillin (PenG)	2.14 mg/ml
ampicillin (Amp)	2.09 mg/ml
carbenicillin (Carb)	2.27 mg/ml
oxacillin (Oxa)	2.41 mg/ml
methicillin (Meth)	2.41 mg/ml
cloxacillin (Clox)	2.61 mg/ml
cephalexin (Cx)	2.08 mg/ml
cephaloridine (Cd)	2.50 mg/ml

Substrate profiles were performed with crude enzyme preparations using the standard iodometric assay to determine β-lactamase activity. The β-lactamase activity on the various substrates is not given as U/ml but rather as relative rates of hydrolysis. To make this work comparable to other published reports, the rates of hydrolysis were calculated against that of benzylpenicillin, which was given the arbitrary value of 100. Thus a relative rate of, for example, 150 indicates that the β-lactamase is hydrolysing the given substrate at 1.5 times the rate of benzylpenicillin.

5. INHIBITION STUDIES

Inhibition studies were performed using the iodemetric assay to determine residual β-lactamase activity after pre-incubation of the enzyme with the inhibitor.

(a) β-Lactam Inhibitors

methicillin	6mM
cloxacillin	3, 6, 9, 12 and 18 mM

1 ml of crude enzyme was incubated with 5 ml of inhibitor solution for 15 min at 30°C, then remaining β-lactamase activity was assayed against 5ml of 6mM benzylpenicillin.

(b) Non-β-lactam inhibitors

(i) *Para*Chloromercuribenzoate (pCMB) MW: 357.0

pCMB was prepared in 0.1M phosphate buffer, pH 7.0 to a final concentration of 0.5mM (Jack and Richmond, 1970) and 5 ml aliqouts incubated with 1 ml crude enzyme preparation for 15 minutes at 30°C. Residual β-lactamase activity was assayed against 5 ml volumes of benzylpenicillin (10^4U/ml).

(ii) Phenylmethylsulfonyl (PMSF) MW: 174.2

A stock solution of 50 mM PMSF was prepared in isopropanol (Ruch and Vagelos, 1973) and 1 ml of crude enzyme preparation was incubated at 30°C with 20 µl of PMSF stock solution in 5 ml of 0.1M phosphate buffer, pH 7.0. At times 0 min, 30 min, 60 min, 2 hrs and 24 hrs, samples were assayed for β-lactamase activity using 10^4 units benzylpenicillin/ml as substrate.

6. INDUCTION OF β-LACTAMASE

The inducibility of the β-lactamase from *P. leiognathi* 206 was determined by a method based on that described by Hennessey (1967).

(a) Inducers

Benzylpenicillin, ampicillin, methicillin and cephaloridine were used as inducers, with each prepared in LB to final concentrations of 0, 50, 100, 500 and 1000 µg/ml.

(b) Procedure

An overnight culture of *P. leiognathi* 206 was diluted into fresh medium and cells were grown to exponential phase (about 90 min) at 30°C with shaking. Cells were then distributed in 40 ml volumes to flasks with appropriate concentrations of inducer added and incubation then continued. At times 0, ½, 1, 2 and 4 hours the growth of the culture was measured by optical density at 525 nm on a Spectronic 20 and mg dry weight/ml calculated as in Section C, 1. At the same time intervals, 1 ml samples were assayed to determine β-lactamase hydrolysis of benzylpenicillin (10^4 units/ml).

7. ANALYTICAL ISO-ELECTRIC FOCUSING

The technique of analytical iso-electric focusing of β-lactamases was described by Matthew *et al*, (1975). The staining techniques were modified to suit the β-lactamases from *Photobacterium* strains.

(a) Apparatus

An LKB multiphor system was used, with current applied from a LKB (biochrom) 2103 constant wattage (0 to 2.0Kv,

0 to 20mA) power pack. During iso-electric focusing (IEF) the LKB multiphor box and plate was maintained at a temperature of 7°C by connection to a cooling system. Commercially prepared Ampholine polyacrylamide gel plates (Ampholine PAG) with a range of pH 3.5 to 9.5 were used. Electrolyte solutions (anode and cathode) were made according to the manufacturer's instructions.

(b) Staining

The method of staining was by a contact gel (Brive *et al*, 1977) which relies on the diffusion of β-lactamase from the polyacrylamide gel to the staining gel.

Staining recipe:

agar (Noble)	20.0 g
starch	5.0 g
benzylpenicillin	6.68g
KI	16.6 g
I_2	0.38g
0.5M phosphate buffer, PH7.0 to 1.1	

The gel was poured to the same dimensions as the Ampholine PAG, just prior to use.

(c) Procedure

The LKB multiphor system was cooled to 7°C, prior to use. The iso-electric focusing was set up according to the instructions. Between 10 and 15 µl of crude enzyme sample was loaded onto the Ampholine PAG, by use of identical-sized filter paper pieces. Focusing was carried out at 1200 to 1500v, 40 mA and about 30 min after starting, the filter paper pieces were removed, then focusing continued for a further 60 min. Immediately after focusing was complete, 1 cm^2 sections of the ampholine PAG were cut out using a sterile

scalpel blade, corresponding to the manufacturer's template and each soaked in 2 ml sterile H_2O. The pH of the solution in each tube was measured by a Radiometer 28 pH meter, 3 to 5 hours later. The pH values were graphed to give a standard curve of the pH gradient for a particular gel.

The staining gel was laid directly onto the PAG and observed for appearance of clear bands in the blue/black gel over a period of about 2 hours, after which time bands could no longer be seen due to general decolourization of the gel.

(d) Record of Results

Permanent records were obtained by photography with a Polaroid camera model 355, using close-kit no. 543 and Polaroid type 665 positive/negative land film. To achieve good records it was necessary to have a light source underneath the gels and to take serial photographs. In addition, records were made on acetate sheets overlayed on the staining gel, by careful marking of the exact position of the bands as they first appeared, the pI for each β-lactamase determined by comparison of the band position with the plot of pH across the gel.

8. SLAB-GEL ELECTROPHORESIS

In general, the method as described by Laemmli (1970) was used, but with some modifications in accordance with the purpose.

The running gel was poured between two vertical plates (13 x 15cm) held 1 to 2 mm apart, sealed with petroleum jelly and clamped. A few mls of water was run onto the top of the gel to ensure a horizontal edge. The gel was poured 24 hours prior to use and kept at $4^{o}C$. On the day of running the electrophoresis, the stacking gel was poured on

top of the running gel and a well-making template was inserted. Once set (~30 min) the template was removed and the gel and plates were placed into the reservoir troughs so as to be in contact, top and bottom, with the buffer (0.025M Tris-glycine buffer, pH 8.3).

25 µl samples of crude enzyme preparations were applied to the wells in duplicate, with dye added to the last well to enable visual measurement of the solvent front. Current was applied, with the anode on top, from a Bio-rad Laboratories model 500, power pack. The current was begun at 10mA while the sample remained in the stacking gel, then increased to 15mA once the samples reached the running gel. Electrophoresis was complete in 5 hours, at which time the gel was removed and one half stained by overlaying a starch/iodine/PenG gel (as in the IEF method). The duplicate half was stained for protein with Coomassie blue stain. The results from the activity stain were recorded on acetate sheets and by photography. After destaining, with 10% glacial acetic acid the protein-stained gel was steam-vacuum dried for permanence.

9. GENETIC DETERMINATION OF β-LACTAMASE

9.1 Plasmid Curing

Under certain conditions or when in the presence of particular chemical agents, bacteria will grow and divide but replication of any plasmid DNA carried within the bacterium is often inhibited. This results in a percentage of daughter cells no longer carrying the plasmid DNA and which therefore, no longer demonstrate the characteristics specified by the plasmid genes.

P. leiognathi 206 is known to carry plasmid DNA (B. Dymock and K. SMith, this laboratory) and attempts were made to cure the cells with the following agents: -

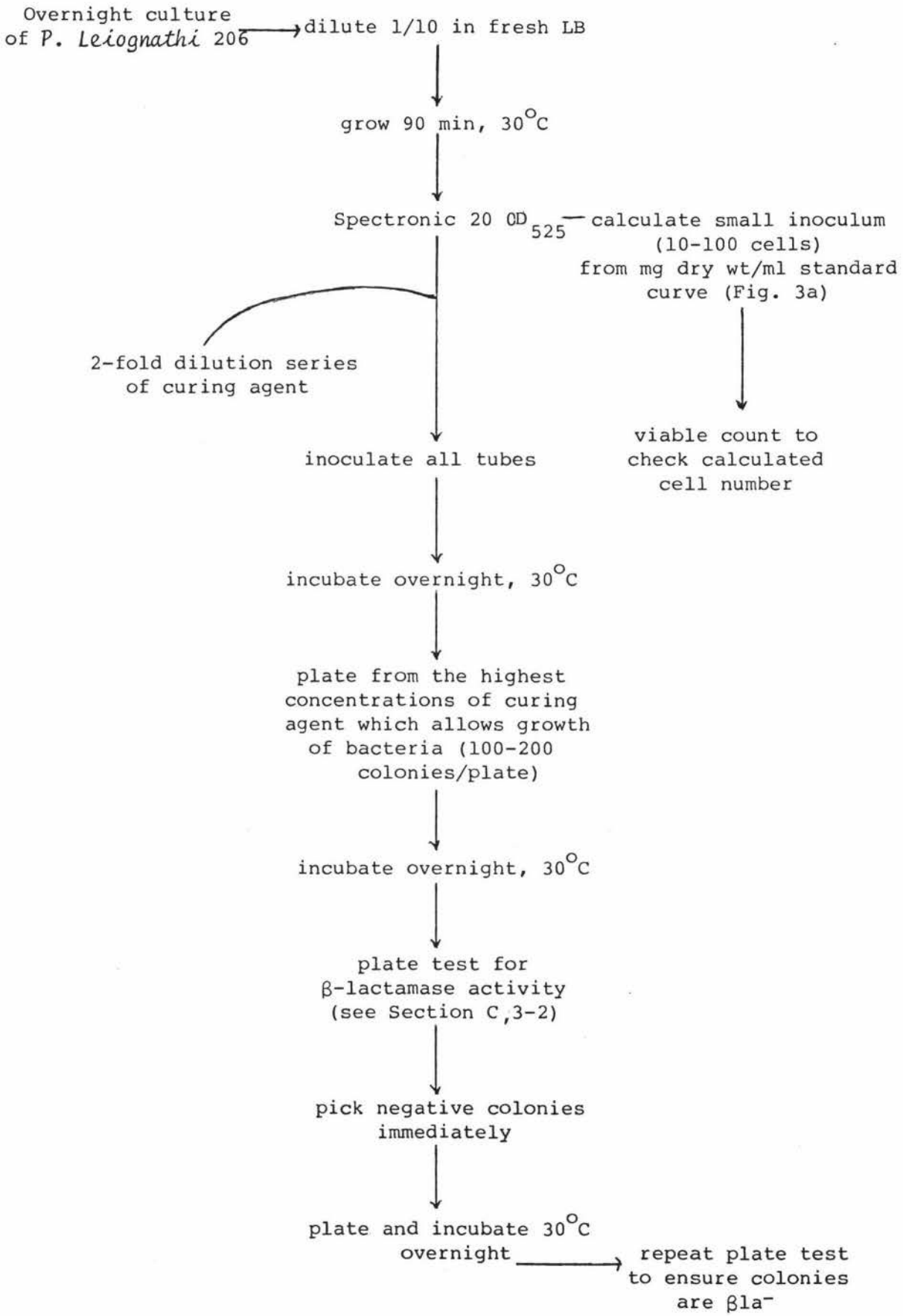
Ethidium bromide (EB):	0.7 - 200 µg/ml
Acridine orange (AO):	0.7 - 200 µg/ml
Acriflavine (AF):	0.7 - 200 µg/ml
Mitomycin C (MC):	0.03 - 10 µg/ml
Sodium dodecyl sulphate (SDS):	0.003 - 1.0% solution

All agents were prepared in sterile LB as 2-fold dilution series, with a control containing no 'curing' agent.

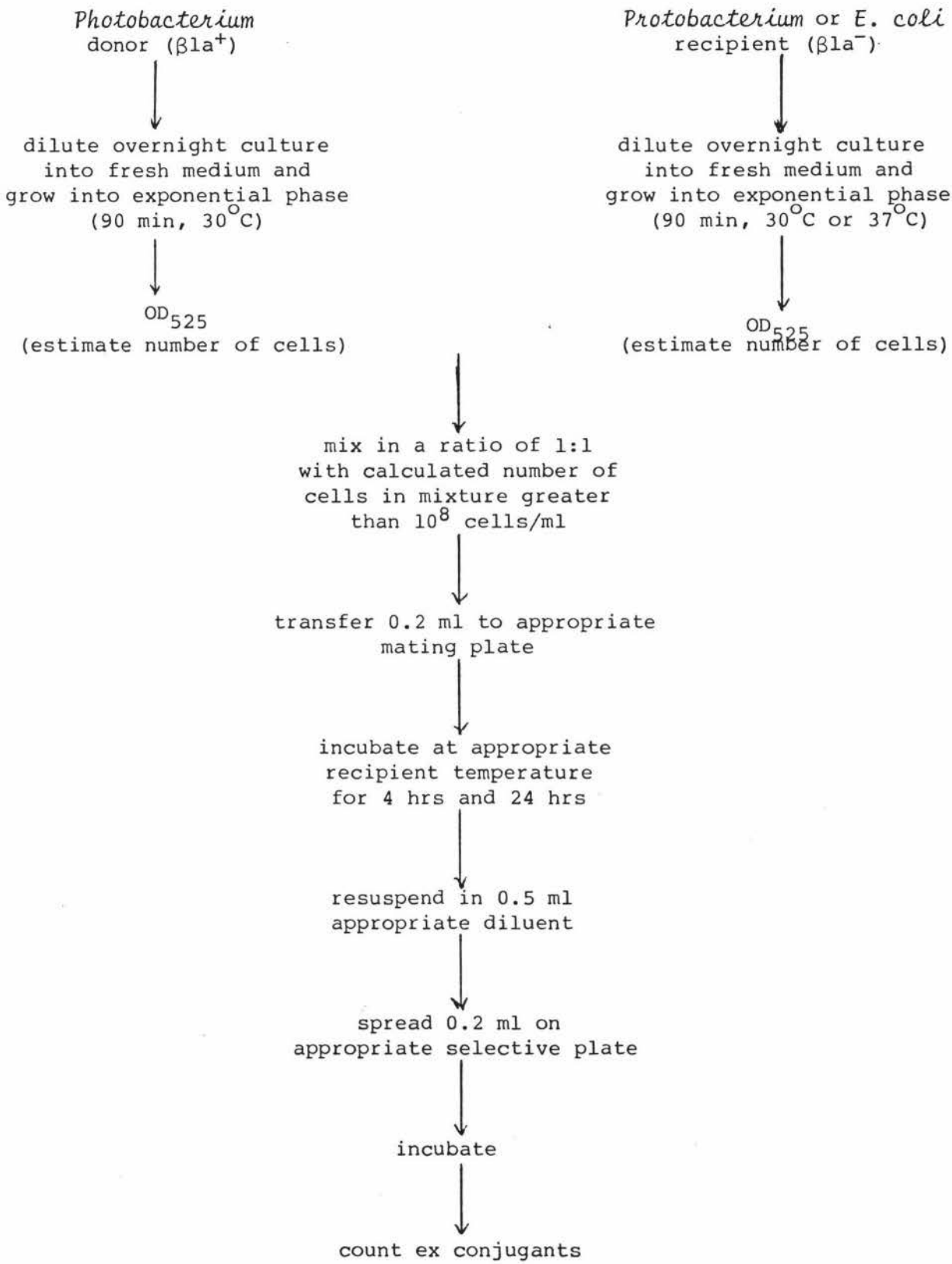
9.2 Conjugation

Plasmid DNA can be transferred from one bacterium to another by conjugation and hence, conjugation experiments were performed to transfer the βla^+ genes from strains of *Photobacterium* to new hosts in an attempt to demonstrate that the β-lactamase activity was plasmid-mediated.

PLASMID CURING PROCEDURES



(c) Conjugation Procedure



9.3 Transformation

Transformation involves the uptake of naked donor DNA by competent recipient cells. When the donor bacterium contains a plasmid, the extracted DNA is a mixture of both chromosomal and plasmid DNA, hence transformation experiments were attempted with *P. Leiognathi* 206 DNA, to try to mobilize the βla^+ genes.

(a) DNA Extraction Methods

(i) Crude SDS lysis:

overnight culture of *P. Leiognathi* 206

↓

harvested (6K rpm, 4°C, 10 min)

↓

wash (x2) in equal volume TE buffer

↓

resuspend in 1/20 volume TE buffer + 0.1% SDS

↓

hold in water bath at 60°C, 5 to 60 min until lysed

(ii) Lysozyme/SDS lysis:

overnight culture of donor strain

↓

harvest (6K rpm, 4°C, 10 min)

↓

wash (x2) in equal volume TE buffer

↓

resuspend in 1/20 volume lysozyme (2mg/ml)

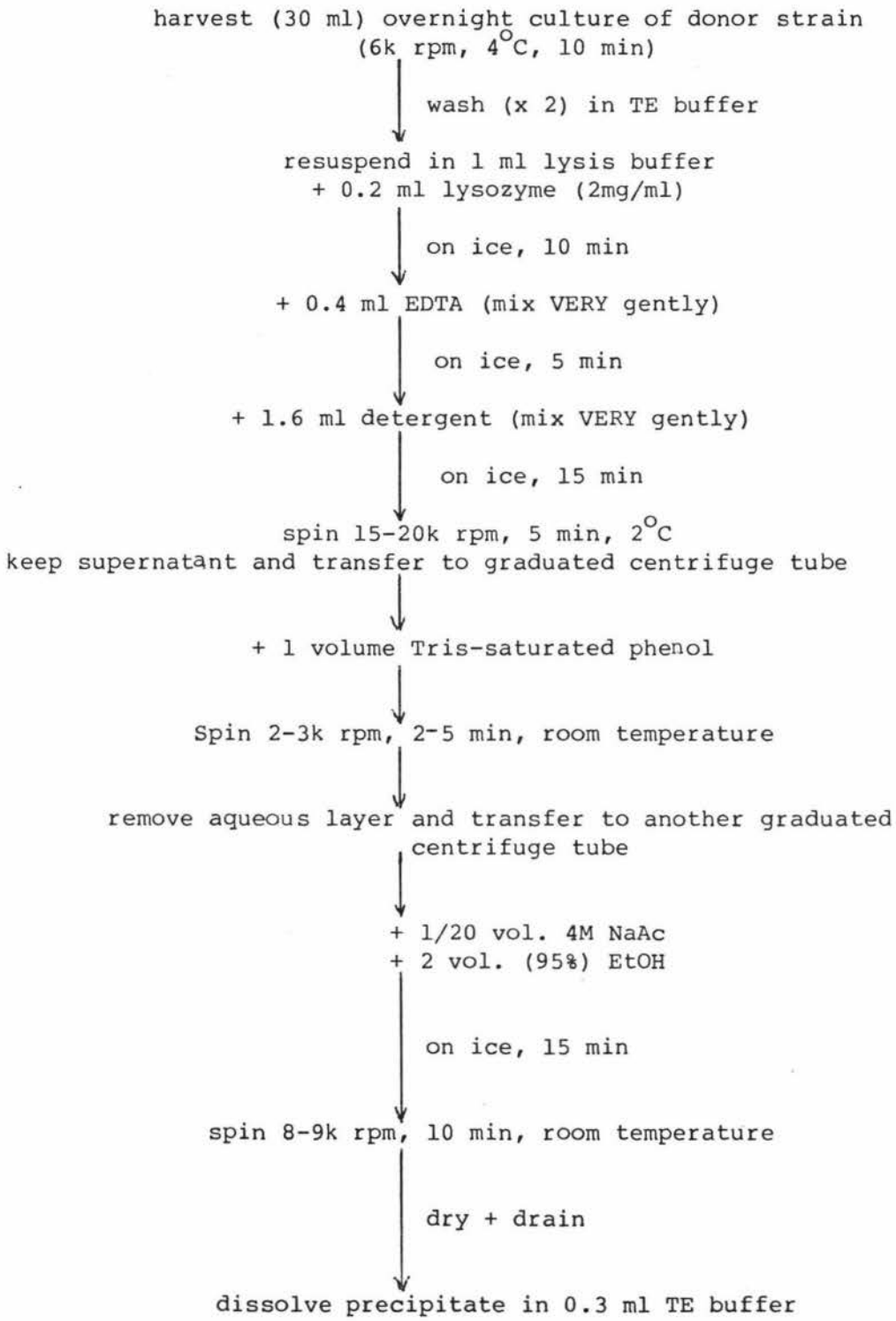
↓

add equal volume of TE buffer + 0.1% SDS

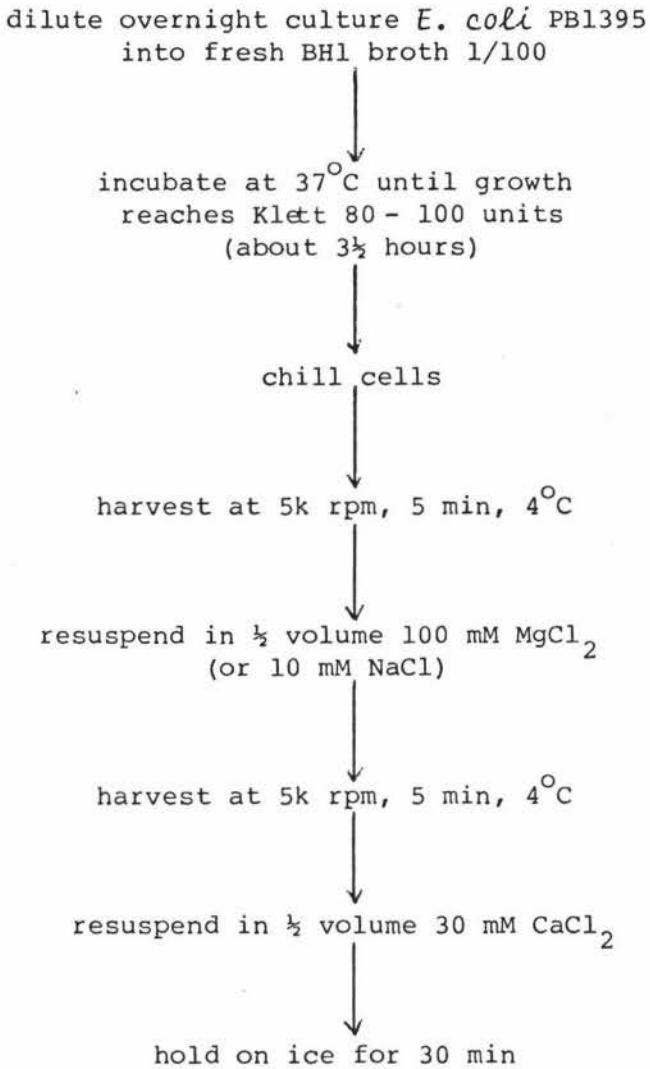
↓

hold in water bath at 60°C
5 to 60 min until lysed

(iii) Partially purified preparation

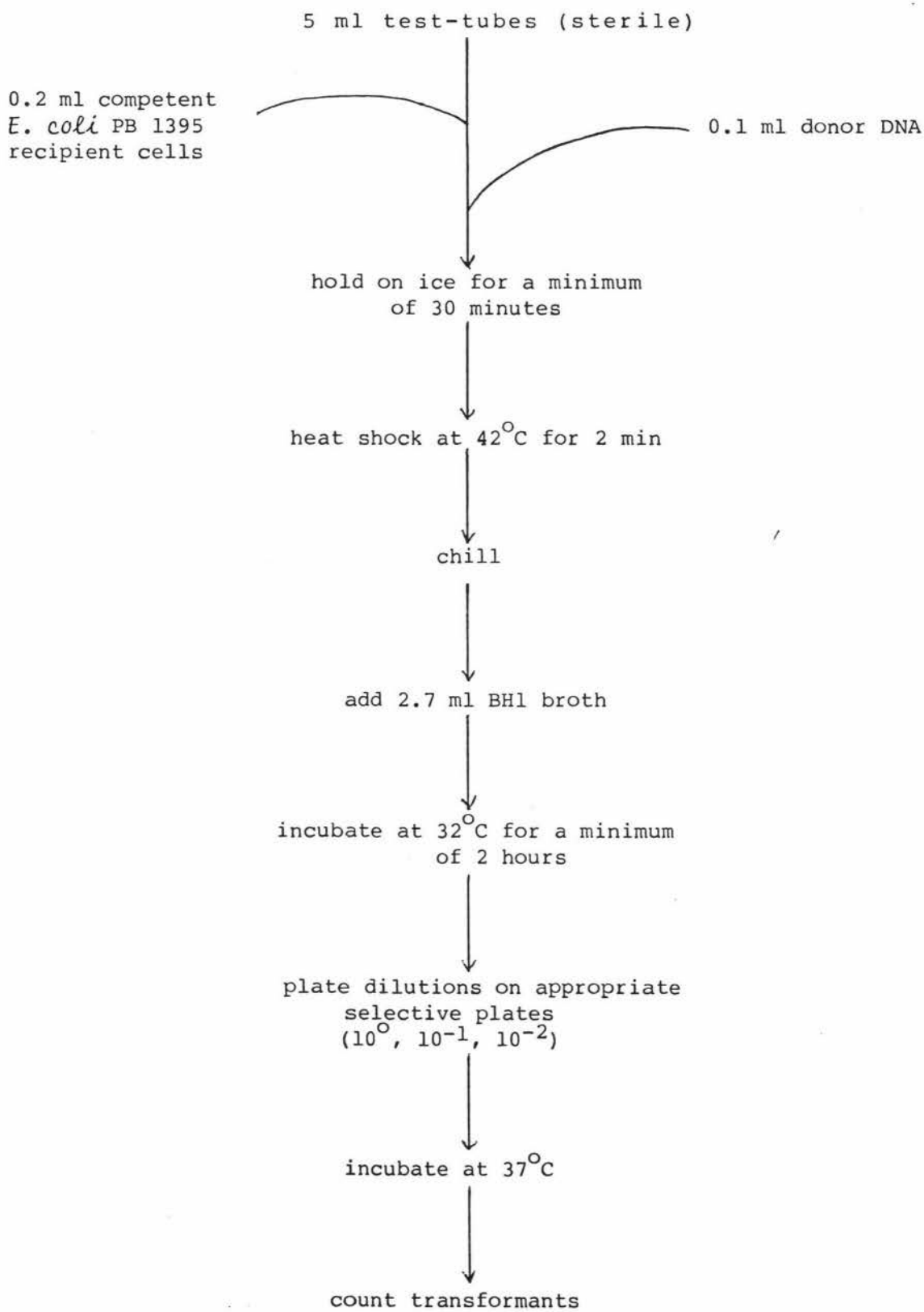


(b) Competence method



For storage, the cells were reharvested and suspended in 30 mM $CaCl_2$ + 15% glycerol. The addition of the glycerol allows the calcium-treated cells to be kept at -20°C and such cells can be thawed and refrozen many times without loss of competence or viability.

(c) Transformation procedure



SECTION D
RESULTS

1. FACTORS AFFECTING β-LACTAMASE ACTIVITY AND ASSAY

1.1 Factors affecting iodometric assay

The unit of activity for β-lactamases has been defined (Pollock and Torriani, 1953) for precise conditions of pH and temperature. In order to establish whether the standard assay conditions were suitable for the β-lactamase from *P. leiognathi* 206, the following data was obtained.

(a) Effect of temperature

The standard temperature for assay of β-lactamase activity is 30°C, although 25°C and 37°C have often been used. Temperature sensitivity of the enzyme from *P. leiognathi* 206 was tested, with both cell-bound enzyme and crude preparation of cell-free enzyme (obtained by osmotic shock methods, see Section C, 2.2), at temperatures of 30°C, 40°C and 50°C.

Results are given in Table V and show that 30°C is an acceptable temperature for assay of both forms of enzyme. It is seen that when the temperature of assay is raised by 10°C there is a slight decrease (2 to 4%) in β-lactamase activity and at 50°C, the cell-bound enzyme is protected to a greater extent than the cell-free enzyme, which is practically inactivated at this temperature.

(b) Effect of pH

The standard pH used for definition of the β-lactamase unit of activity is pH 7.0. Crude enzyme preparation from *P. leiognathi* 206 was assayed against benzylpenicillin when in a series of 0.1M phosphate buffers (Dawson, *et al*, 1979) over the pH range of 5.8 to 8.0 in 0.2 pH unit steps. Enzyme activity at each pH is shown graphically in Figure 5 and it is seen that pH 6.2 is optimal for

TABLE V:

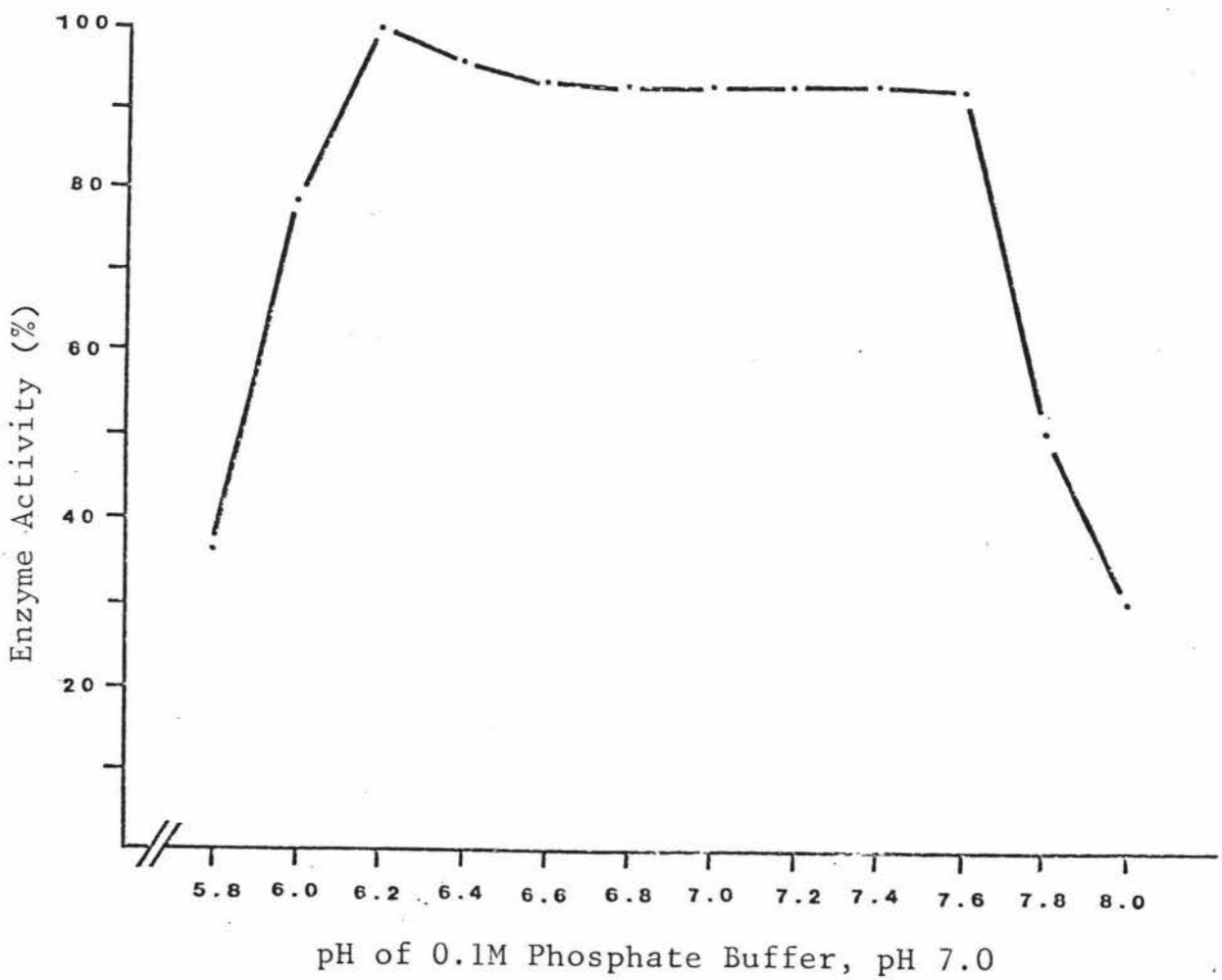
β-LACTAMASE ACTIVITY PRESENT AT DIFFERENT TEMPERATURES OF ASSAY

Cell-bound enzyme is that present in intact cells and cell-free enzyme was prepared by osmotic shock techniques (see Section C, 2.2). β-Lactamase activity was determined by the iodometric assay (see Section C, 3.1) and results given are the mean of 3 assays.

Temperature °C	Cell-bound enzyme U/ml	%	Cell-free enzyme u/ml	%
30	9.30	100.0	9.30	100.0
40	9.08	97.6	8.91	95.8
50	6.35	68.2	0.13	1.3

Figure 5: Effect of pH on the Activity of β-Lactamases From *P. Leiognathi* 206

The activity of crude preparation (see Section C, 2.2) of the β-lactamase from *P. leiognathi* 206 at different pH, was determined by the iodometric assay and results, calculated as a percentage of the maximum activity demonstrated, given as the mean of 2 experiments.



maximum β-lactamase activity, with near maximal activity (92%) over the pH range 6.2 to 7.2, hence assaying at pH 7.0 was satisfactory.

For gel-electrophoresis purposes (see Sections C, 8 and D, 4.2d) it was necessary to establish whether activity of the β-lactamase was retained after incubation at pH 8.3. Samples of crude enzyme preparation were incubated at pH 8.3 under the conditions of gel-electrophoresis (5 hours, room temperature), then diluted 1:5 into 0.1M phosphate buffer, pH 7.0 and incubated at room temperature for a minimum of 30 min. It was found that no β-lactamase activity remained after incubation at pH 8.3, while the activity present in the control enzyme samples for incubation at pH 7.0 for identical periods of time, was the expected amount.

1.2 Reproducibility of the iodometric assay

To determine the error incurred when using the standard assay technique 30 identically prepared flasks, each containing 1ml of crude enzyme preparation were assayed for 30 mins. The results were subjected to statistical analysis and the standard error obtained on 9.64 U enzyme activity / ml was ± 0.13 U/ml.

Assays were also performed on enzyme diluted appropriately to give a range of spectrophotometric readings, in an attempt to establish to what degree of accuracy low levels of β-lactamase were measured. The readings obtained with various levels of enzyme activity are given in Table VI. The percentage error incorporates assay error, spectrophotometric reading error and inaccuracies arising from conversion of OD_{490} to thiosulphate ml equivalents. The data clearly show that error is over 10-fold greater when low enzyme levels are being measured.

TABLE VI:

REPRODUCIBILITY OF THE ASSAY WITH DIFFERENT LEVELS OF β-LACTAMASE ACTIVITY

β-Lactamase activity was measured by the iodemetric assay with the results given as the mean of 3 assays.

Enzyme activity u/ml	O.D. reading at 490 nm	I_2 concentration in $Na_2S_3O_3$ ml equivalents ± SD (% S.D.).
1.10	0.051	0.227 ± 0.030 (13 %)
2.20	0.119	0.527 ± 0.035 (6.6%)
3.75	0.195	0.853 ± 0.025 (3 %)
5.50	0.312	1.363 ± 0.025 (1.7%)
11.00	0.505	2.187 ± 0.006 (0.2%)

1.3 Stability of the β-lactamase

(a) Effect of centrifugation temperature and resuspending media

An experiment was carried out to determine whether the temperature of centrifugation or the resuspending media had any effect on the β-lactamase activity which was recoverable from *P. leiognathi* 206. A five-hour old culture of strain 206 was harvested by spinning the cells at a speed of 6K rpm for 10 min either at room temperature (RT) in a bench top centrifuge, or at 4°C in a Sorvall RC-2B centrifuge. The cell pellets were resuspended in either 0.1M phosphate buffer pH 7.0, artificial seawater (ASW) or buffered-ASW pH 7.5, then assayed for β-lactamase activity. Samples of the culture itself without treatment of any kind were used as controls and assayed to determine the total β-lactamase activity present.

Table VII gives the cell-associated β-lactamase activity recovered in several independent experiments as a percentage of that originally present and it can be seen that although loss of β-lactamase activity does occur, it appears to be largely independent of the resuspending media. Activity consistently proved to be slightly higher when 0.1M phosphate buffer was used compared to ASW or buffered-ASW and hence, was used as the resuspending media in future assays. A large difference in recoverable enzyme activity occurred when the cells were spun at the two different temperatures. With cells harvested at 4°C, about 90% of the total β-lactamase activity was recovered but only 50 to 70% was recovered after the cells were centrifuged at room temperature. For all future work, cells were harvested at 4°C.

TABLE VII:

EFFECT OF TEMPERATURE OF CENTRIFUGATION OF BACTERIAL CELLS AND RESUSPENSION OF THE CELLS IN DIFFERENT MEDIA, ON CELL-ASSOCIATED β-LACTAMASE ACTIVITY.

Whole culture was spun at RT or 4°C at 6K rpm for 10 min and the cells resuspended in different media, samples of which were assayed for β-lactamase activity by the iodometric assay.

		% of total β-lactamase activity present			
		Experiment 1	Experiment 2	Experiment 3	Experiment 4
Whole culture		100	100	100	100
Supernatant		5	2	2	4
Resuspended in	Centrifugation Temperature				
0.1M phosphate buffer pH7.0	R.T.	59.3	50.7	70.0	72.2
	4°C	91.3	91.5	97.7	87.2
ASW	R.T.	ND	44.6	60.8	55.5
	4°C		84.6	84.6	67.7
Buffered ASW pH7.5	R.T.	ND	ND	67.7	61.1
	4°C			70.0	75.0

ND = not determined

(b) Effect of zinc ions on β-lactamase activity

Sabath and Abraham (1966) reported that the β-lactamase II from Gram-positive *Bacillus cereus* bacteria was dependent on zinc ions for maximum activity of the enzyme. The requirement for this divalent ion by the β-lactamase from *P. leiognathi* 206 was tested. Assays were conducted with crude enzyme of 10.2 U activity/ml in the presence of a range of molarities of zinc sulphate.

From the results (Table 8) it appears that zinc ion present in a concentration of 10^{-5} M increases the hydrolytic activity of the β-lactamase by about 13%. For the Zn^{++}-dependent β-lactamase II, Sabath and Abraham (1966) state that "the value of V_{max} for benzylpenicillin fell from about 80% of its maximum at 10 μM - $ZnSO_4$ to a very low value with 1 μM added $ZnSO_4$". From Table VIII it can be seen that no decrease in β-lactamase activity occurred at any zinc ion concentration.

2. PHYSIOLOGY AND EXPRESSION OF β-LACTAMASE

2.1 β-Lactamase production during bacterial growth

For the chromosomal penicillinase from *E. coli*, the amount of β-lactamase in *amp*A (penicillin-resistant) mutants and wild-type cells is directly proportional to the growth rate of the cells (Lindstrom *et al*, 1970). Kontomichalou *et al* (1974) also observed a fluctuation of specific activity of β-lactamases depending on the age of the culture, with R-factor mediated enzymes.

To determine the specific activity of a *P. leiognathi* 206 culture, growth of the cells was measured spectrophotometrically (see cultivation, C 1) and samples taken at progressive time intervals to determine the amount of β-lactamase present. The results plotted in Figure 6,

TABLE VIII:

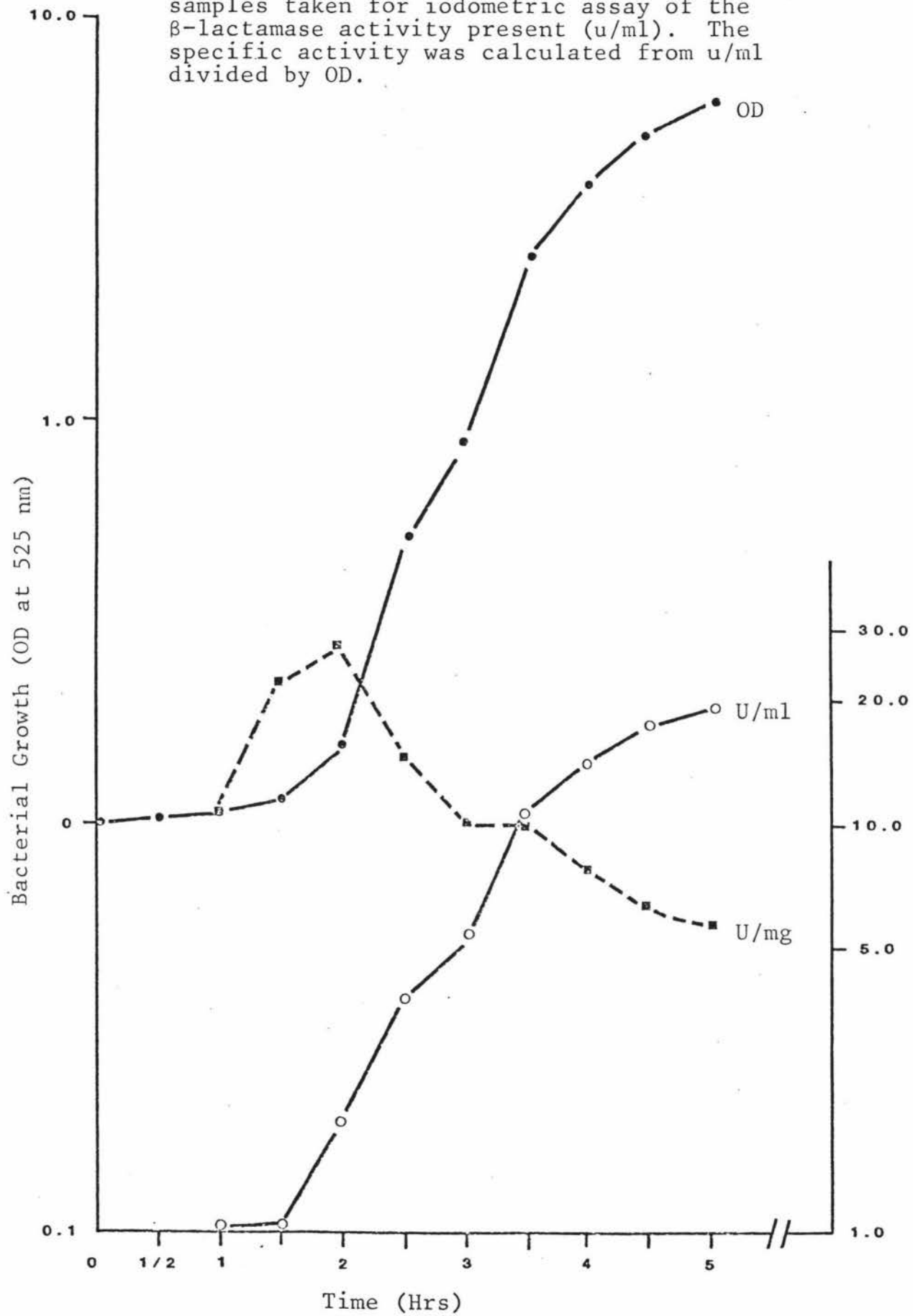
β-LACTAMASE ACTIVITY IN THE PRESENCE OF ZINC IONS

The β-lactamase activity of enzyme with 10.2 u activity/ml, was assayed by the iodometric assay in the presence of various concentrations of zinc ion. Enzyme activity is presented as relative rates of that in the absence of Zn^{++}, with each result the mean of 3 assay.

Zn^{++} concentration (M)	β-lactamase activity (relative rate ± 1.6)
0	100
10^{-3}	99.5
10^{-4}	98.5
10^{-5}	112.9
10^{-6}	98.7
10^{-7}	99.3

Figure 6: β-Lactamase Production During Bacterial Growth

P. leiognathi 206 was grown in LB at 30°C with shaking and samples taken for iodometric assay of the β-lactamase activity present (u/ml). The specific activity was calculated from u/ml divided by OD.



At times of 30, 60, 75, 90 and 120 min, samples of the culture were stained and observed microscopically to estimate the relative size of the cells during growth phases. From the β-lactamase activity (units/ml) curve in Figure 6 it can be seen that late exponential phase (3½ to 5 hour) culture produces sufficient β-lactamase for practical working purposes. The specific activity curve (units/mg dry weight) shows that β-lactamase production is largely independent of bacterial growth phase, apart from a short 'burst' of activity in late lag phase and very early exponential phase. The same results were obtained in separate experiments, implying that the anomalous peak of activity is real. At each sample time, 125 cells were measured with an eyepiece micrometer and all found to be of equivalent size.

2.2 Inducibility of the β-lactamase from *P. leiognathi* 206

Most β-lactamase produced by Gram-negative bacteria are constitutive in that their rate of formation is not influenced specifically by presence of penicillins or cephalosporins in the growth medium. This is in direct contrast to those produced by Gram-positive bacteria which are almost always inducible.

To determine whether the β-lactamase from *P. leiognathi* 206 was inducible or constitutive, exponential phase cells were grown in the presence of benzylpenicillin, ampicillin, methicillin and cephaloridine at concentrations ranging from 0 to 1000 μg/ml. Optical density of the culture was measured over a period of 4 hours and at the same time, samples were assayed for β-lactamase activity. The efficiency of β-lactamase induction may be expressed numerically as the ratio of the enzyme activity present in the culture with inducer (β-lactam) to that of the control culture, as measured at identical stages in the growth cycle. However, the specific activity (U/mg dry weight cells) of the β-lactamase could only be calculated for the controls in the experiment, since the standard curve of OD versus mg dry wt

cells was prepared with cells growing in the absence of β-lactams. For specific activity to be calculated in all other cases, mg dry wt cells/ml of culture curves need to be plotted at every concentration. For practical reasons this was not done since the β-lactamase activity curves (U/ml), Figs. 8a to 8d, seem to follow the growth curves obtained for the bacteria (Figs. 7a to 7d). From Figs. 8a to 8d it can be seen that the β-lactamase production is never greater in the presence of any of the β-lactams used as inducers than in the control culture, meaning the induction ratio for the β-lactamase is less than 1 in all cases. It was therefore concluded that the β-lactamase from *P. leiognathi* 206 was not inducible.

When comparing the growth of the bacteria and the β-lactamase production by the culture in the presence of the β-lactams, it is seen that at high concentrations of β-lactam, the growth of the culture is often markedly inhibited (for example, growth in the presence of 500 and 1000 μg ampicillin/ml) and yet at such concentrations the β-lactamase production in the culture is only a little less than that produced by the culture growing in the absence of the β-lactam. Since β-lactam antibiotics interrupt the synthesis of the bacterial cell wall by inhibiting either cell elongation or cell division (Holtje and Schwarz, 1974) it was thought possible that in cases where the bacterial growth was inhibited, the cells were elongating but not dividing completely. The optical density of the culture therefore, was not increasing as expected although the amount of β-lactamase present per ml of culture is about the same as that in the control culture.

To test this hypothesis, stained preparations of exponential phase cells, incubated in the presence of growth-inhibiting concentrations of β-lactam for 3 hours were observed microscopically and a random sample of 100 cells were measured with an eyepiece micrometer at 400x magnification. It was found (Table IX) that the cells incubated with methicillin 1000 μg/ml and ampicillin, 500 and 1000 μg/ml were respectively

Figure 7: Growth of *P. leiognathi*206 in the presence of β-lactams.

Exponential phase cells were grown in LB with shaking at 30°C, Growth was measured by a Klett colorimeter.

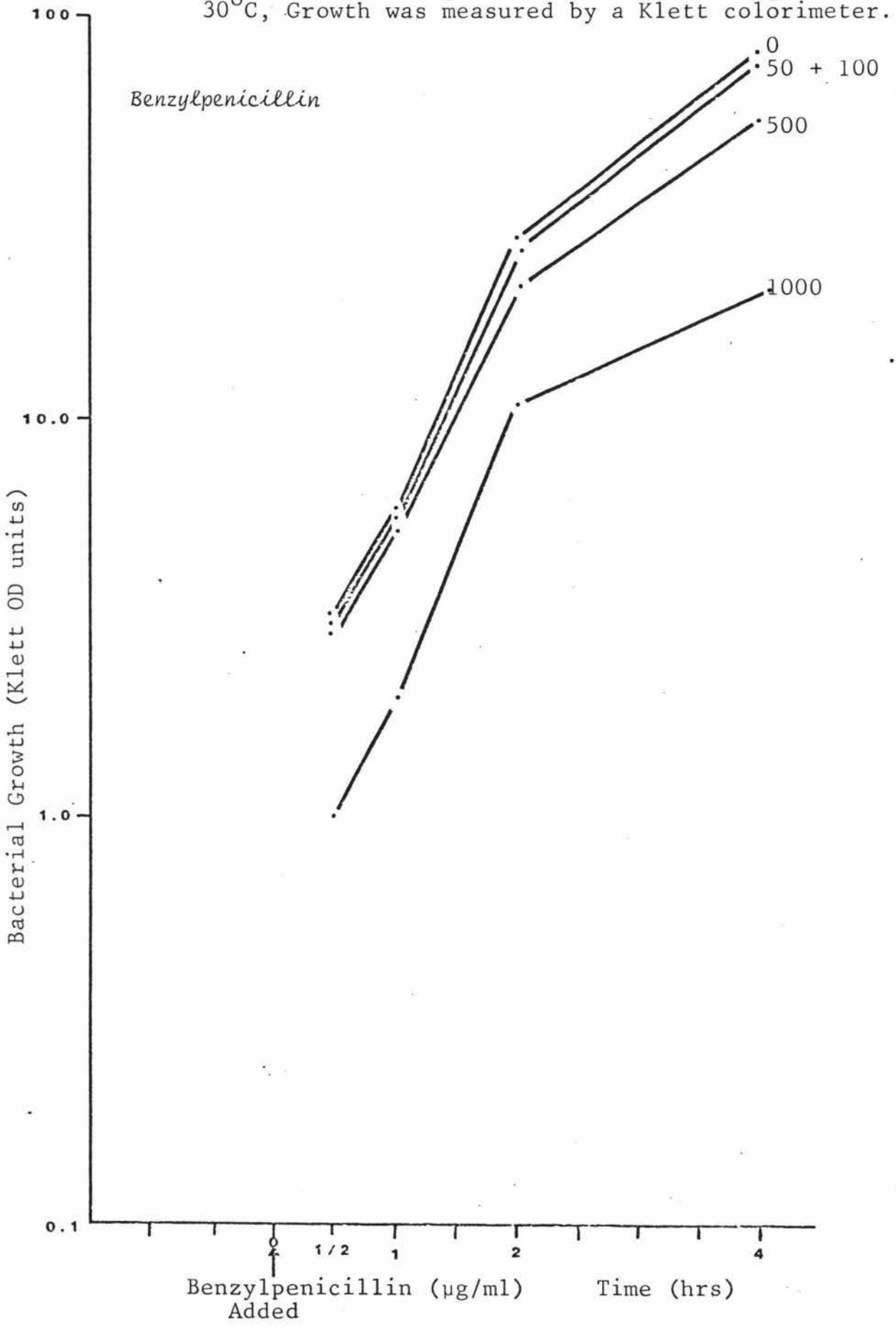


Figure 7b: Growth of *P. leiognathi* 206 in the presence of β-lactams.

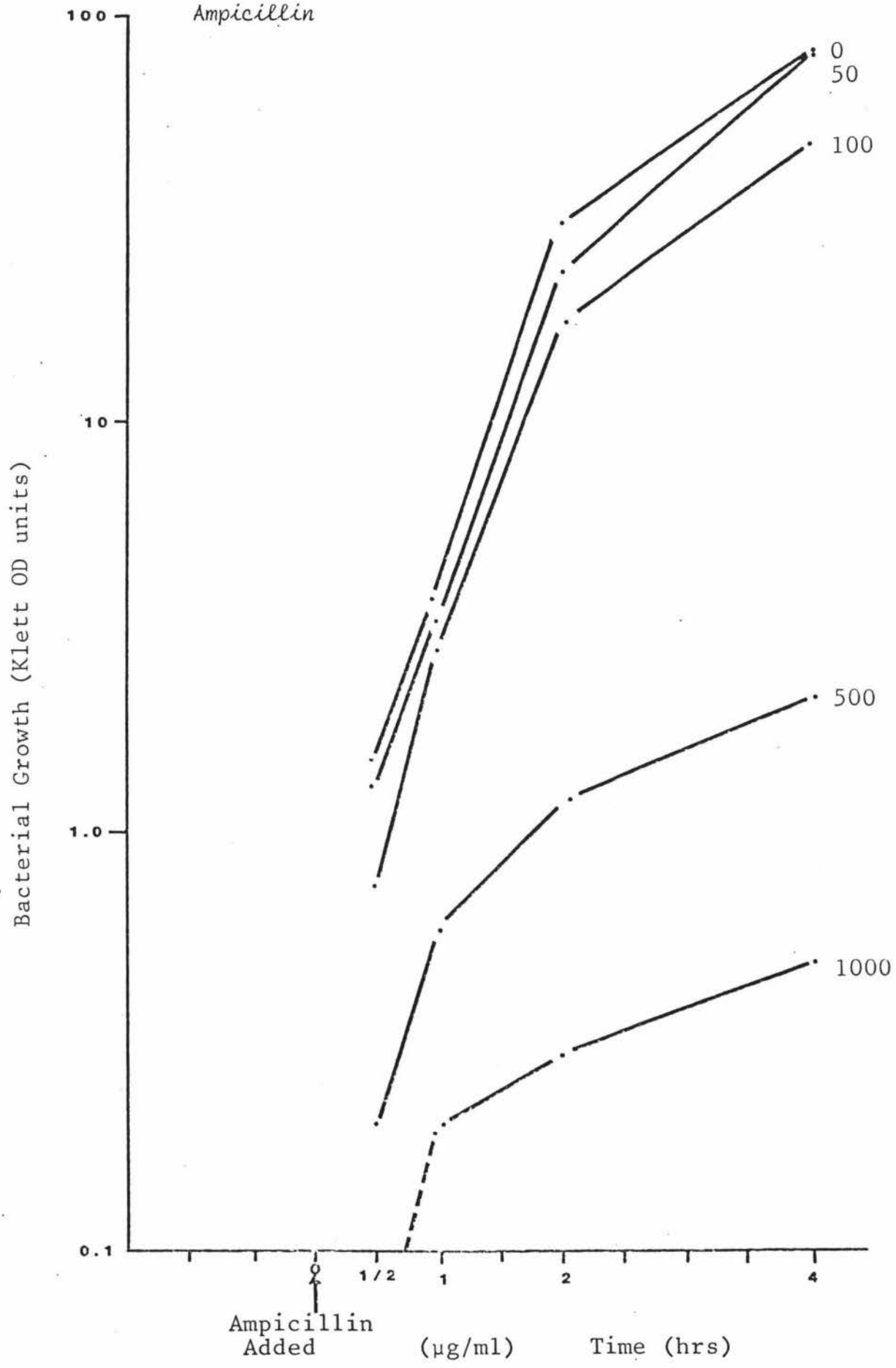


Figure 7c: Growth of *P. leiognathi* 206 in the presence of β-lactams.

Figure 7d: Growth of *P. leiognathi* 206 in the presence of β-lactams

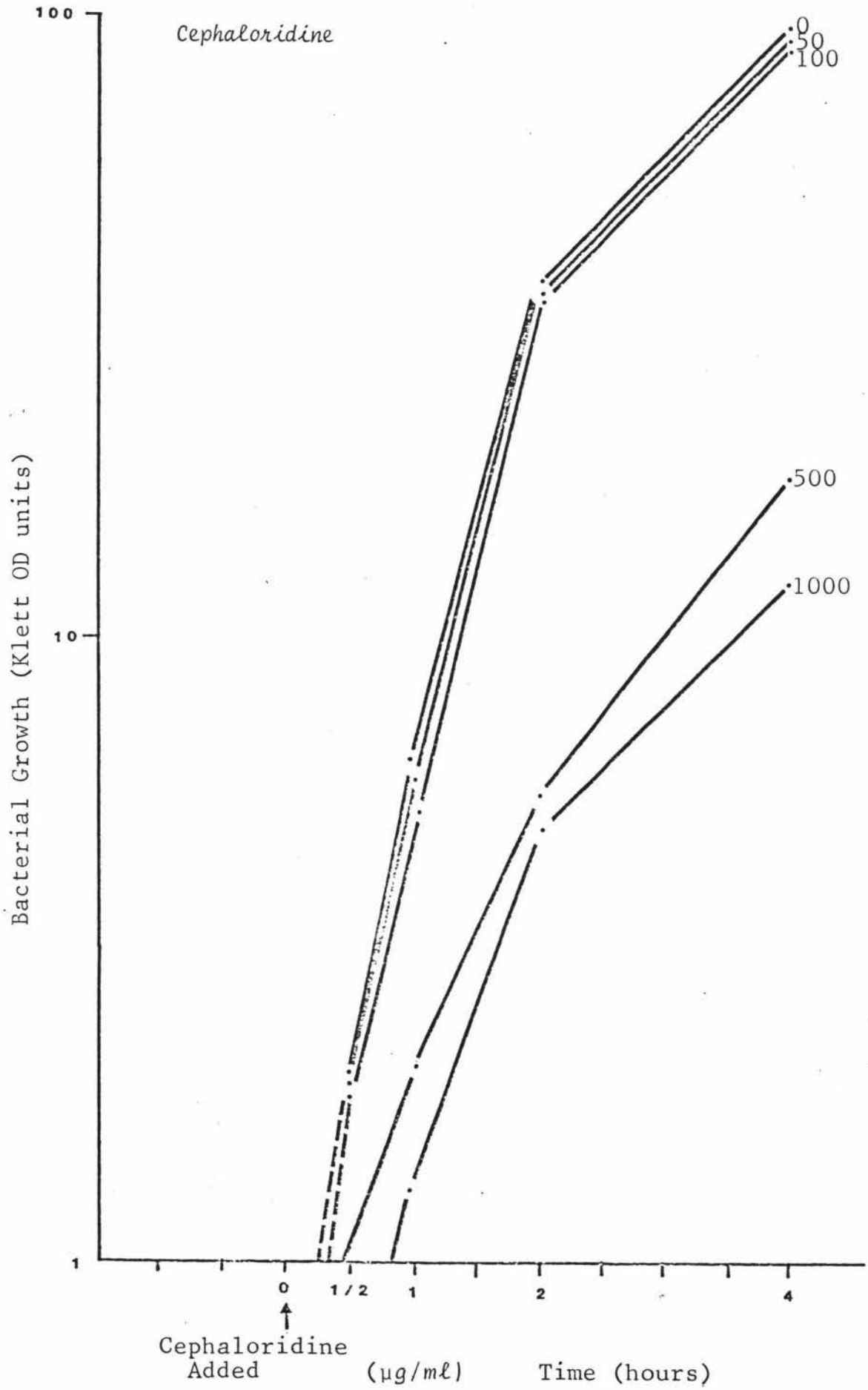


Figure 8a: *Benzylpenicillin*

β-Lactamase production by *P. leiognathi* 206 in the presence of β-lactams

Enzyme activity was measured by the iodometric assay.

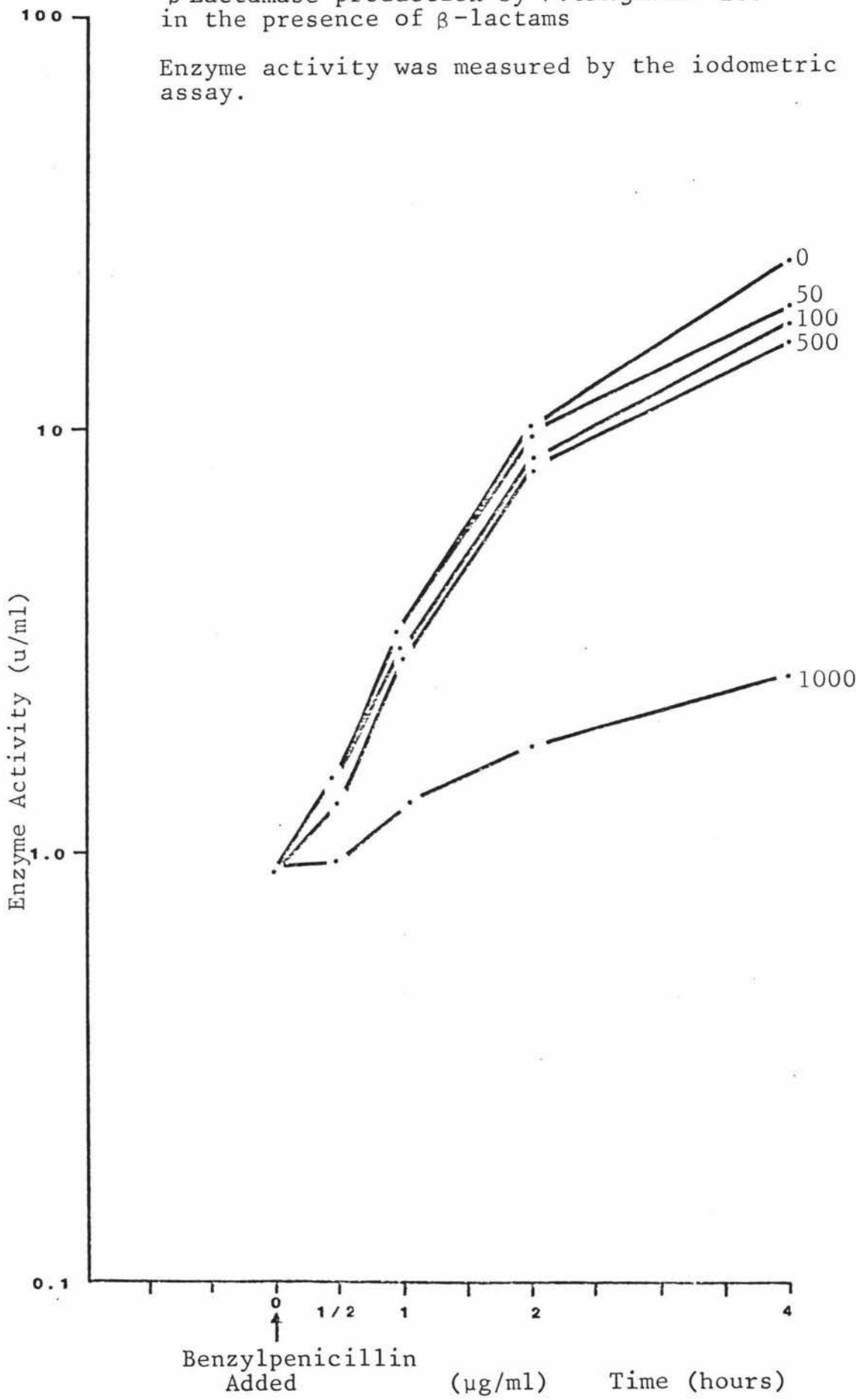


FIGURE 8b: *Ampicillin*

β-Lactamase production by *P. leiognathi* 206 in the presence of β-lactams.

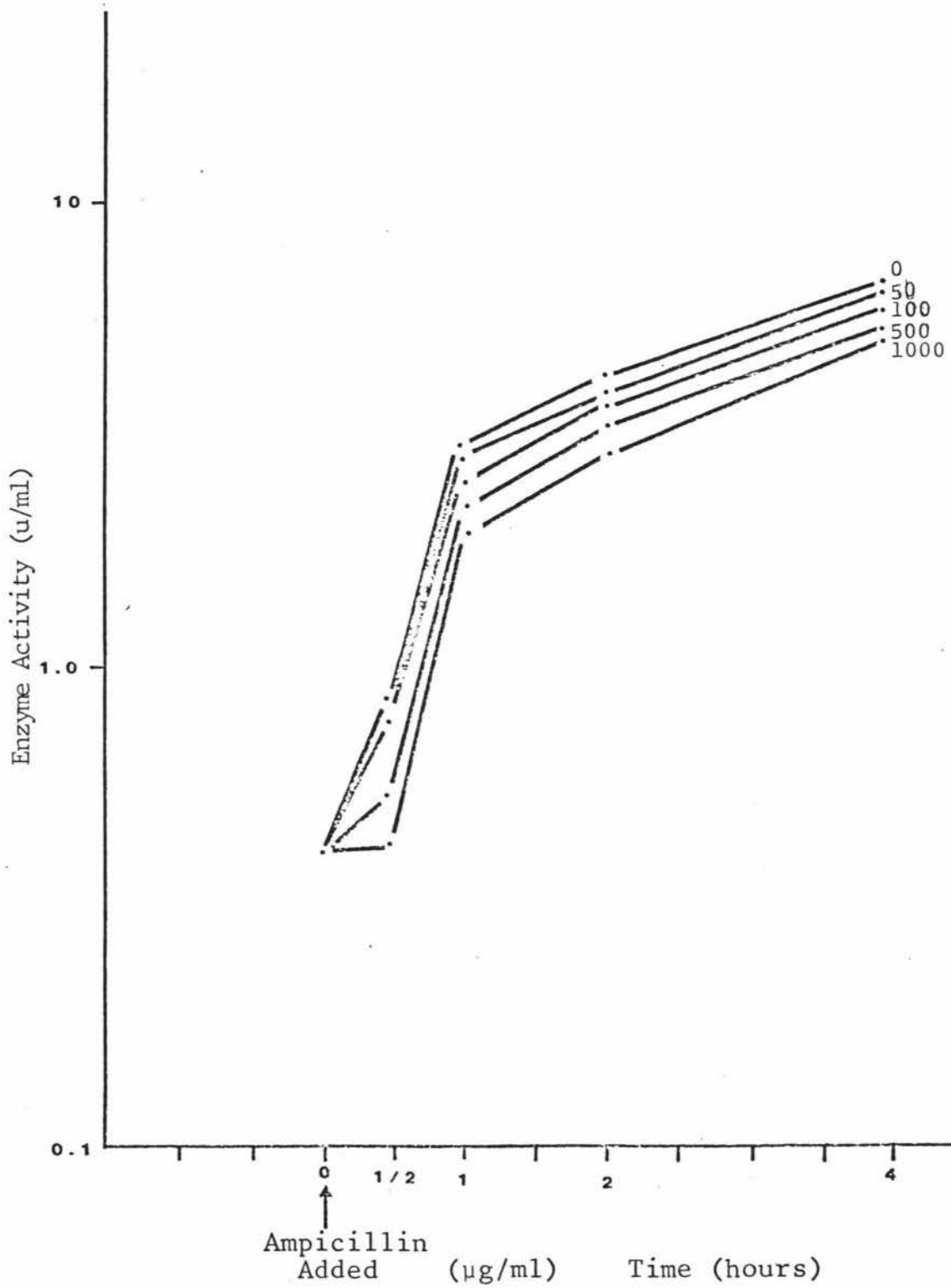


Figure 8c: *Methicillin*

β-Lactamase production by *P. leiognathi* 206 in the presence of β-lactams

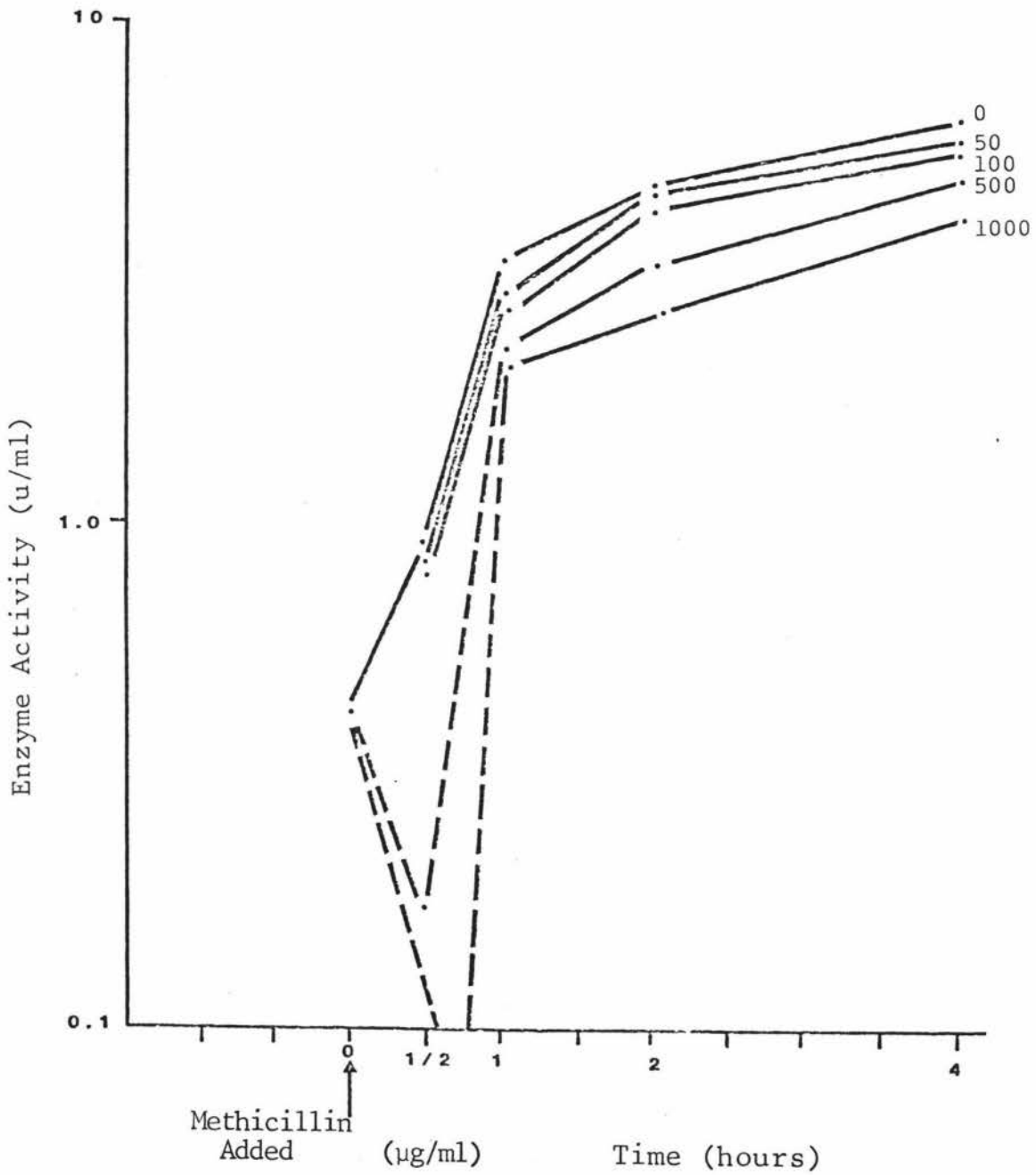


Figure 8d: *Cephaloridine*

β-Lactamase production by *P. leiognathi* 206 in the presence of β-lactams

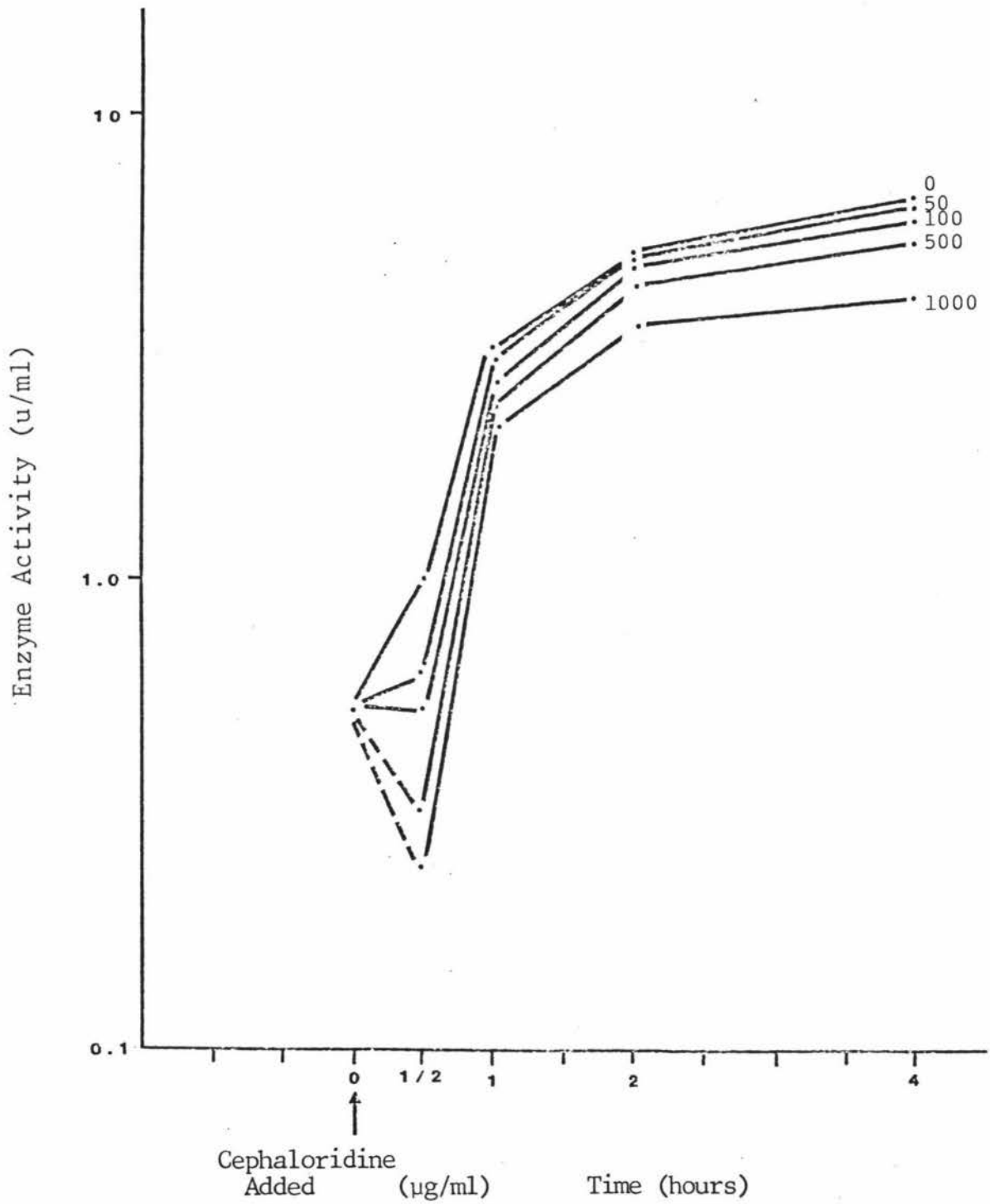


TABLE IX:

EFFECT OF GROWTH-INHIBITORY β-LACTAM CONCENTRATIONS ON THE GROWTH OF *P. leiognathi* 206 CELLS

Results are the mean length of 100 cells, expressed in micrometer divisions as measured at 400 x magnification.

Additions to medium (µg/ml)	Average length of cells (micrometer divisions ± S.D.
0	0.5 ± 0.1
Methicillin 1000	1.7 ± 0.1
Ampicillin 500	2.6 ± 0.1
Ampicillin 1000	2.8 ± 0.1

about 3x and 5x greater in average length than cells grown in the absence of the β-lactam. When the OD growth of the cells at these β-lactam concentrations is multiplied by the average cell length, the amended OD curve obtained correlates very well with the β-lactamase production curve.

2.3 Cellular location of β-lactamase

The β-lactamases from Gram-positive bacteria are usually liberated into the external environment as an extracellular enzyme (Pollock, 1962). The β-lactamases produced by Gram-negative bacteria are almost always cell-bound, located as either intracellular or periplasmic enzyme.

(a) Test for extracellular enzyme

To establish whether the β-lactamase from *P. leiognathi* 206 is released as an extracellular enzyme or remains cell-bound, a late exponential phase culture was centrifuged and the supernatant removed from the sedimented bacteria which were then resuspended in an equal volume of 0.1M phosphate buffer pH 7.0. Equal volumes of the whole culture, the supernatant and the resuspended cells were assayed, with the results given in Table X.

It is seen that the major proportion of the total β-lactamase activity present remains cell-associated. The small percentage of activity which appears in the external medium is not very significant as it could result from lysis of a small proportion of the cells allowing release of enzyme into the medium; from cells still present in the supernatant ($<10^6$ cells/ml could account for about 4% of the total activity) and because, for such low enzyme levels, the limits of reliable detection are approached.

TABLE X:

EXTRACELLULAR β-LACTAMASE FROM *P. leiognathi* 206

The β-lactamase activity present in the supernatant and resuspended cells after centrifugation at 6K rpm, for 10 min at 4°C, was measured by iodometric assay, and given as a percentage of that present in the initial whole culture, with data presented as mean of 3 separate experiments.

	β-lactamase activity (u/ml)	% of total activity ± std error
Whole culture	11.5	100 ± 2 %
Supernatant	0.8	7 ± 25%
Resuspended cells	10.2	89 ± 2 %

(b) Cell-bound β-lactamase

Cell-bound β-lactamases are located within the periplasmic region, between the inner cytoplasmic membrane and the outer membrane of the cell but their exact location in this region is still unknown. Liberation of 'periplasmic' enzymes (Neu and Heppel, 1964) from cells of most of the enteric bacteria is achieved using osmotic shock techniques developed by Neu and Chou (1967) to release such enzymes as cyclic phosphodiesterase, 5'-nucleotidase and acid phenylphosphotase, which were known to be located in the periplasmic region in *E. coli*. Later, Neu (1968) achieved 100% release of R-factor mediated β-lactamase from *E. coli* and *Salmonella typhimurium*, using the same methods. Enzyme which is not liberated by osmotic shock treatment of the cells, but remains cell-associated, is termed 'intracellular' enzyme for which crude preparations are obtained by ultrasonic disintegration of the cells.

Osmotic shock techniques cause temporary damage to the outer membrane of the cells but leave the cytoplasmic membrane intact and do not impair cell viability. It is now thought that liberation of enzymes by osmotic shock methods may be a function of the molecular weight of the protein (Smith and Wyatt, 1974) and β-lactamases of molecular weight greater than 30,000 may be retained within the 'shocked' cells.

(i) Modifications to the osmotic shock method

The method described by Neu and Chou (1967) was for terrestial bacteria, but strains of the genus *Photobacterium* exist in a marine environment (hence, high ionic strength) and so may have altered outer membrane structure and permeability. It was therefore, considered necessary to determine whether the technique was suitable for *Photobacterium* strains.

Neu and Chou (1967), and most other workers since, included 1mM ethylenediaminetetra-acetate (EDTA) in the solutions used for osmotic shock treatments. However, Unemoto *et al* (1974) when attempting to release periplasmic enzymes from marine strains of *Vibrio*, found that the presence of EDTA caused the cells to lyse. As the genus *Photobacterium* is taxonomically related to *Vibrio*, it was possible that EDTA would also cause lysis of *Photobacterium* cells, which effect must be avoided since successful release of periplasmic enzyme by osmotic shock methods require that only the outer membrane be damaged, leaving the inner membrane intact and the cells viable. The effect of EDTA on *Photobacterium* cells was tested by following the viability of *P. leiognathi* 206 during incubation with 1mM EDTA in LB at room temperature. Samples were removed and plated for viable counts (see Section C,) with results given in Figure 9. It is seen that presence of EDTA causes a sharp drop in cell viability during the first minute of incubation, with continued slight loss after this time. From these results, it was decided that solutions which omit the EDTA, should be used for osmotic shocking of *Photobacterium* cells.

Another modification was the substitution of ASW for the washing and hypotonic solutions described by Unemoto *et al* (1974). Table XI presents the results of the osmotic shock procedure performed with both series of 'shocking' solutions, from which it can be seen that with both solutions there is a slight loss of viable cells, but as Unemoto's solutions appeared to yield a better enzyme recovery, they were the ones used in future osmotic shock methods.

(ii) β-Lactamase released by osmotic shock methods

Osmotic shock techniques released about 75% of the total β-lactamase activity from *P. leiognathi* 206 cells and hence the enzyme appears to be periplasmic (Table XIIa).

Figure 9: Effect of 1mM EDTA on Viability of *P.leiognathi* 206 Cells

Viable counts obtained after 24 hours incubation at 30°C with each count being the mean of 5 plates.

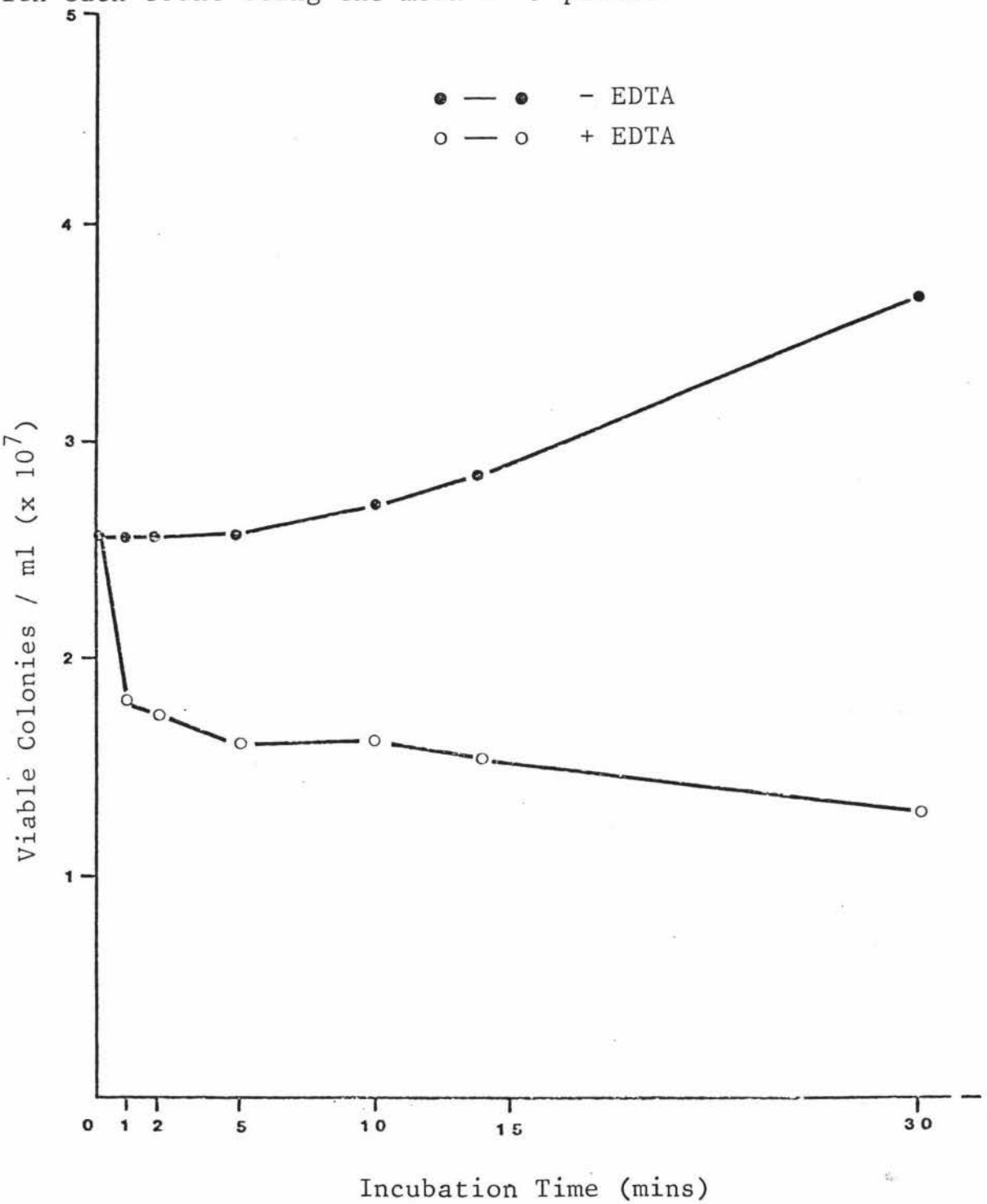


TABLE XI:

RELEASE OF β-LACTAMASE DURING OSMOTIC SHOCK AND COMPARISON AND 'SHOCKING' SOLUTIONS

The β-lactamase activity was determined by the iodometric assay in the whole culture prior to treatment, in the hypotonic supernatant after osmotic shocking of the cells and that remaining in the cells after treatment. Viable counts were performed on the cells before and after treatment to check that no major cell damage occurred. The results are the mean of 3 experiments.

	Unemoto's solutions[a]		ASW substituion	
	β-lactamase activity[b] u/ml	Viable count[c] $x10^8$/ml	β-lactamase activity[b] u/ml	viable count[c] $x10^8$/ml
Whole culture	13.9	3.5	13.9	3.5
Cell-free enzyme	11.1		10.4	
Cell-associated enzyme	1.5	2.3	1.7	2.3

(a) Unemoto *et al* (1974)

(b) mean of 3 assays

(c) mean of 3 plates

TABLE XII:

RECOVERY OF β-LACTAMASE ACTIVITY AFTER OSMOTIC SHOCK TREATMENT OF *P. leiognathi* 206 CELLS

β-Lactamase activity was measured by the iodometric assay.

(a) β-lactamase activity released by the osmotic shock method

	Initial enzyme in whole culture	Released cell-free enzyme	Remaining cell-bound enzyme	Total % recovered
u/ml[(a)]	11.2	8.4	0.8	
%	100	75	7	82

(a) mean of 6 experiments

(b) β-lactamase activity and viable count of *P. leiognathi* 206 cells following each stage of the osmotic shock procedure

Procedural step	β-lactamase activity[a]			Viable count[b]	
	u/ml	%	% lost at each step	$x10^8$/ml	lost at each step ($x10^8$/ml)
Whole culture	13.9	100		3.5	
after washings (x2)	13.3	95.7	4.3	3.3	0.2
after hypertonic suspension	13.0	93.5	2.2	3.2	0.1
hypotonic supernatant	11.1	79.8))90.6	2.9	<0.1))3.1	0.1
resuspended pellet	1.5	10.8		3.1	0.1

a mean of 3 assays

b mean of 3 plates

After performing the procedure several times it was noticed that the amount of activity released plus that which remained cell-associated, never equalled the total β-lactamase activity initially present. It was found (Table XIIb) that a small amount of activity was lost at each step and that this loss paralleled a small loss in viable cells, suggesting that cells and hence β-lactamase activity, were being lost during the procedure, perhaps in the discarded supernatant. This was not investigated further, since the amount of β-lactamase which was recovered was certainly sufficient for use in experiments with cell-free enzyme.

The crude enzume preparation was stored frozen at -16°C. The loss of β-lactamase activity incurred by freezing and thawing five samples was 1.0% ± 0.5%.

(iii) Concentration of cells to increase β-lactamase yield

Experiments were carried out in an attempt to increase the β-lactamase yield by concentrating the cells during the osmotic shock procedure. *P. leiognathi* 206 was used as the β-lactamase source and the cells were concentrated at the hypotonic suspension stage of the procedure by altering the resuspension volume. β-lactamase activity present in the initial whole culture, an unconcentrated preparation and preparations concentrated two, five and ten-fold, was measured, with the relative activities of each given in Table 12c. The data show that β-lactamase yield by osmotic-shock does increase with cell concentration, although not proportionally.

(c) Permeability barrier of the cell wall to β-lactams

The outer membrane of Gram-negative cells acts as a permeability barrier limiting the entry of β-lactams into the periplasmic region, with the degree of permeability being a function of the bacterial species

TABLE XII: CONTINUED

(c) Effect of concentrating cells during the Osmoitic Shock Procedure on enzyme yields from *P. leiognathi* 206.

Enzyme Preparation	β-lactamase activity[b] u/ml ± SD	%
Initial whole culture	12.6 ± 0.2	100
Concentrated [a] x 1	10.6 ± 0.1	84.1
x 2	19.3 ± 0.2	76.6
x 5	39.5 ± 0.2	62.7
x 10	53.3 ± 0.2	42.3

(a) cells were concentrated at the hypotonic suspension stage of the osmotic shock procedure.

(b) results are the mean of 3 experiments

and the β-lactam substrate involved. The barrier is demonstrated by measuring the β-lactamase activity of intact cells and comparing it to the activity present after the cells have been disrupted. The term 'crypticity' is used to describe the permeability barrier effect (Richmond and Sykes, 1973); a cryptic enzyme demonstrates greater activity on a substrate after cell disruption than when the cell wall is intact. Crypticity can be expressed numerically: -

$$\text{Crypticity} = \frac{\text{specific enzymic activity of broken cells}}{\text{specific enzymic activity of whole cells}}$$

and the value obtained is often called the crypticity factor, defined for the β-lactamase of a particular species on a particular substrate.

The degree of crypticity of the β-lactamase from *P. leiognathi* 206 was determined in a late exponential phase culture by assaying enzyme activity against benzylpenicillin before and after sonication (see Section C, 2.1), and it was found that the β-lactamase was not cryptic as no increase in activity was observed after cell disruption. In order to establish whether the lack of a permeability barrier was characteristic of the bacteria in the genus *Photobacterium* and if it applied to all β-lactam substrates, eleven sample strains of each *Photobacterium* species were assayed before and after sonic disruption and activity against four different substrates measured. The strains were chosen on the basis of their high β-lactamase activity, with only one strain each from *P. fischeri* and *P. logei*, as a taxonomic change for these two species is imminent (see Section F). The β-lactam substrates tested are those most commonly referred to by other workers and which are hydrolysed by the strains used (see substrate profiles, Section D 4.1). The results in Table XIII are given as crypticity factors and show that there is no permeability barrier to benzylpenicillin or cephaloridine in the strains tested. The enzymes also appear to be non-cryptic to

ampicillin but slightly cryptic to carbenicillin, although the greatest crypticity factor obtained was 3 with *P. phosphoreum* 674. From Table XIII it is seen that in some strains β-lactamase activity has decreased significantly after cell disruption with the loss of activity not uniform for all substrates. Possible explanations and comments for this effect are presented in the Discussion (Section E5).

3. CLASSIFICATION OF β-LACTAMASES

The main parameters by which β-lactamases are classified are substrate profile and inhibition studies (Richmond and Sykes, 1973; Sykes and Matthew, 1976). The substrate profile of a β-lactamase shows which β-lactams are hydrolyzed by the enzyme and is stated in terms of rates relative to the hydrolysis of benzylpenicillan, which is given the arbitrary value of 100. All the substrate profiles were carried out with crude enzyme extracts, prepared by the osmotic shock method (see Section C 2.2). The substrates tested are those most commonly referred to by others working with β-lactamases and which are available in New Zealand. Inhibition of β-lactamase activity is used as a parameter to distinguish between enzyme types (Richmond and Sykes, 1973), differentiating the β-lactamases by which compound inhibits a particular enzyme, the degree and duration of the inhibition and by the response of the β-lactamase to the inhibitor (O'Callaghan and Morris, 1972). For classification purposes it is sufficient to establish whether a β-lactamase is sensitive or resistant to inhibition by a particular compound without investigating the kinetics of the enzyme. To classify the β-lactamase from *P. leiognathi* 206 detailed studies of both substrate profile and inhibition sensitivities were performed.

TABLE XIII:

THE β-LACTAMASE CRYPTICITY FACTORS OF SEVERAL INDEPENDENT ISOLATES OF *Photobacterium* SPECIES

β-lactamase activity was determined by iodometric assay, before and after sonic disruption of the cells.

$$\text{Crypticity factor} = \frac{\text{activity present in disrupted cells}}{\text{activity present in intact cells}}$$

All results are the mean of 3 assays with an error of C.F. $\pm$ 0.04.

STRAIN		SUBSTRATE			
		PenG	Amp	Carb	Col
P. leiognathi	206	0.93	1.01	2.61	ND
	648	0.77	0.99	1.45	ND
	632	0.92	1.04	1.17	ND
P. angustum	77	0.76	1.10	1.00	ND
	79	0.32	1.10	1.35	ND
	159	0.31	0.75	1.14	ND
P. phosphoreum	617	1.04	1.14	1.57	ND
	145	0.97	0.96	1.48	ND
	674	1.00	0.64	2.90	ND
P. fischeri	31	0.99	0.95	2.12	0.97
P. logei	846	0.83	0.46	1.01	1.01

ND = not determined

3.1 Substrate Profile of the β-lactamase from *P. leiognathi* 206

The substrate profile for the β-lactamase from *P. leiognathi* 206 is given in Table XIV, from which it can be seen that the enzyme hydrolyses bezylpenicillin, ampicillin and carbenicillin but has relatively little activity on oxacillin. The β-lactamase does not hydrolyse methicillin or cloxacillin, nor either of the cephalosporins tested. Hence, based on this limited selection of antibiotics at least, the β-lactamase has exclusive activity on penicillins and appears to be a Class II enzyme as described by Richmond and Sykes (1973) in their classification system.

3.2 Inhibition studies with the β-lactamase from *P. leiognathi* 206

Penicillins and cephalosporins which are hydrolysed slowly or not at all, by β-lactamases often act as inhibitors of that enzyme (Hamilton-Miller and Smith, 1964; O'Callaghan *et al*, 1962; Cole *et al*, 1972). Methicillin was the first β-lactam shown to be an inhibitor of a β-lactamase (Rolinson *et al*, 1960) and it has been shown to competitively inhibit some β-lactamases from Gram-negative bacteria (Bach *et al*, 1967; Cole *et al*, 1972; Fujii-Kuriyana *et al*, 1977). Since the inhibitory effect of methicillin was reported, other β-lactam analogues such as cloxacillin (Hamilton-Miller *et al*, 1965; O'Callaghan and Muggleton, 1967; Labia *et al*, 1967), carbenicillin (Zemelman and Olivari, 1972; Olsson *et al*, 1976), oxacillin (Farrar and Newsome, 1973) and cephalosporins (Olsson *et al*, 1976; Mahoney *et al*, 1976; Petrocheilou *et al*, 1977) have been used successfully to inhibit β-lactamases.

Non-β-lactam compounds which inhibit β-lactamases include organic cations and anions (Olsson *et al*, 1976; Yamagishi *et al*, 1969; Sawai *et al*, 1970; Hedges *et al*, 1974), proteolytic enzymes (Hesselwood and Smith, 1974) and antisera (Richmond, 1975). The compound *para*chloromercuribenzoate (*p*CMP), was first observed to inhibit β-lactamase activity

TABLE XIV:

SUBSTRATE PROFILE OF THE β-LACTAMASE FROM *P. leiognathi* 206

β-lactamase activity against 6mM β-lactam substrates, was measured by the iodometric assay, with the rates of hydrolysis given relative to that of benzypenicillin. The results are the average of 13 experiments.

Penicillins						Cephalosporins	
Benzylpenicillin	Ampicillin	Carbenicillin	Oxacillin	Cloxacillin	Methicillin	Cephaloridine	Cephalexin
100	135 ± 3	91 ± 2	10 ± 3	0.7 ±0.7	0.3 ±0.5	0.04 ± 0.1	0.3 ± 0.5

by Smith (1963) who achieved 50% inhibition of the β-lactamase from *Aerogenes cloacae* with 2 μM *p*CMB and Olsson *et al* (1976) showed that the enzyme from *Bacteroides fragilis* was completely inhibited by 0.5mM *p*CMB. The sulphydryl reagent will react with any free thiol groups available and hence inhibition of a β-lactamase by *p*CMB implies that cysteine residue(s) occupy a key position in the molecule. Phenylmethylsulfonyl flouride (PMSF) reacts with available serine residues (Joshi and Wakil, 1971; Ruch and Vagelos, 1973) and since a strong similarity between transpeptidases (which enzymes have a serine residue at the active site) and β-lactamases has been proposed (see Section D 2.2), PMSF can be used to determine whether a serine residue is present in the β-lactamase molecule. Conditions for the use of PMSF are usually 25°C and pH 7.0 and under these conditions the compound has a half-life of 110 min, while at higher temperature and pH, PMSF is inactivated (James, 1978).

Inhibition by both cloxacillin and *p*CMB are used to separate β-lactamases into the classes described by Richmond and Sykes (1973) and therefore, the *P. leiognathi* 206 enzyme was tested with these agents to assist in its classification. Since methicillin was not hydrolysed by the β-lactamase (see substrate profile, Section D 3.1) it was also tested for inhibitory activity, with the presence or absence of serine residues tested by sensitivity of the enzyme to PMSF. All inhibition experiments were performed by preincubating the β-lactamase with the inhibitor, followed by an assay of the remaining enzyme activity. The details of the methods used are given in Section C 5 and the results are given in Table XV.

The inhibition studies showed that, under the experimental conditions, 6mM methicillin almost completely inhibited the *P. leiognathi* 206 β-lactamase. After incubation in the presence of cloxacillin the enzyme activity was found to decrease linearly as the cloxacillin concentration was increased to 12mM, with 18mM cloxacillin totally inhibiting the β-lactamase. In order to establish whether cloxacillin was

TABLE XV:

INHIBITION OF THE β-LACTAMASE FROM *P. leiognathi* 206 BY FOUR COMPOUNDS

β-lactamase was preincubated with the inhibitor at 30°C, then activity measured by the iodometric assay. Details of methods used are given in Section C5.

Inhibition conditions			β-lactamase activity after incubation with inhibitor %
Inhibitor	Concentration	time of preincubation	
Methicillin	6mM	15 min	1.6 ± 0.8
Cloxacillin	6mM	15 min	47.7 ± 1.9
	12mM	15 min	7.5 ± 0.5
	18mM	15 min	0 ± 0.5
pCMB	0.5mM	15 min	100.0 ± 0.7
PMSF	1mM	60 min	71.0 ± 0.9
		120 min	71.0 ± 0.9

competitively inhibiting the β-lactamase, Michelas Menten constants (K_I) would need to be determined. Incubation with 0.5mM *p*CMB had no effect on the activity of the enzyme and hence, it was concluded that the *P. leiognathi* 206 β-lactamase is resistant to this inhibitor (Jack and Richmond, 1970). When incubated with 1mM PMSF in 0.1M phosphate buffer pH 7.0 at 30°C, the β-lactamase activity decreased linearly to 71% of the initial activity in a 60 min period and the enzyme did not appear to be further inhibited after this time. Since it is known that PMSF is inactivated (James, 1978) it is suspected that failure to observe further inhibition of the β-lactamase was due to inactivation of PMSF at 30°C and hence, the results do not indicate whether the *P. leiognathi* 206 enzyme is 100% sensitive to inhibition by PMSF, or 30%. This could be checked by addition of further PMSF after 60 mins incubation. However as the β-lactamase is not completely resistant, it seems that serine residues are present in a key position in the molecule.

4. THE β-LACTAMASES PRODUCED BY OTHER *Photobacterium* STRAINS

Since β-lactamase activity has been demonstrated in all strains of the five species of *Photobacterium* (K. Smith, pers. comm.), it was of interest to establish whether the enzyme was identical in all the strains. The β-lactamases were compared by substrate profile and analytical iso-electric focusing techniques.

4.1 Substrate profiles of 16 independent isolates of *Photobacterium*

Three strains from each species of *Photobacterium* were chosen on the basis of being independent isolates and having high levels of β-lactamase activity. The substrate profiles of the β-lactamases were determined as in Section C4, with the results given in Table XVI. Also included in the Table are the results for the breakdown of the chromogenic

TABLE XVI:

SUBSTRATE PROFILES OF THE β-LACTAMASES FROM INDEPENDENT ISOLATES OF *Photobacterium* SPECIES

Substrate profiles were determined by the iodometric assay with crude enzyme preparation as in Section C_4.

Nitrocetin (C87/312) was assessed visually (see Table 18, Section 4.2b)

Each result is the mean of 3 assays

		Pen	Amp	Carb	Oxa	Clax	Meth	Cx	Col	C87/312
P.angustum	77	100	154.5	117.8	7.5	0	0	0	0	-
	79	100	173.1	90.2	0	0	0	0	0	±
	160	100	165.7	124.2	0	0	0	0	0	±
P.leiognathi	206	100	135.0	94.4	9.8	0	0	0	0	-
	80	100	95.4	60.6	34.4	0	3.0	0	0	-
	205	100	144.7	115.7	14.0	12.4	5.3	0	0	-
	632	100	104.0	79.3	0	0	0	0	0	±
P.phosphoreum	174	100	100	5.3	3.9	0	0	0	0	++
	617	100	100.6	51.0	21.5	0	0	0	0	±
	652	100	257.0	0	0	0	0	0	0	-
P. fischeri	31	100	65.0	24.7	7.2	4.0	5.5	0	30.4	+++
	173	100	70.7	55.2	0	0	0	0	83.0	+++
	768	100	60.4	9.5	0	0	0	0	54.8	+++
P. logei	846	100	60.8	20.3	79.7	14.6	0	11.3	27.3	++
	850	100	55.5	34.3	6.5	15.0	0	12.0	0	++
	851	100	95.7	125.4	62.2	0	0	0	10.6	++

cephalosporin, nitrocefin (taken from Table 18, Section D 4.2b) and the substrate profile of the β-lactamase from *P. leiognathi* 206 (Table 14, Section D 3.1). The results show that the β-lactamases from the species *P. leiognathi*, *P. angustum* and *P. phosphoreum* are all predominantly active on penicllin, with activity against cephalosporins extremely limited. This suggests that they are probably all Richmond and Sykes Class II enzymes (refer Table 3). The substrate profiles of β-lactamases from strains of *P. fischeri* and *P. logei* indicate that the enzymes are active on both penicillins and cephalosporins. It appears therefore, that the β-lactamases from these two species belong in Classes IV or V of the Richmond and Sykes classification system, but inhibition by cloxacillin and *p*CMB data is needed for verification. It is unlikely that they are Class III or I enzymes as β-lactamases of these two classes demonstrate very rapid hydrolysis of cephalosporins.

4.2 Analytical Iso-electric Focusing of β-Lactamases

Iso-electric focusing (Haglund, 1967) is a method in which proteins separate as sharp bands in a pH gradient produced electrophoretically in poly-acrylamide gel, and which allows direct visual comparison of β-lactamases by examination of the resultant enzyme band patterns (Thomas and Broadbridge, 1972; Matthew, 1975; Matthew *et al*, 1975). The technique is extremely sensitive, requiring only small amounts of enzyme, and a high degree of resolution is obtained as the proteins are concentrated during separation since focusing is caused by forces that act against diffusion. Iso-electric focusing of β-lactamases often results in a 'pattern' of bands, consisting of one main band and one or more satellite bands (Matthew *et al*, 1975; Matthew and Harris, 1976; Brive *et al*, 1977; Olsson *et al*, 1977; Labia *et al*, 1979). Some β-lactamases not previously differentiated either biochemically or immunologically, can be distinguished by analytical iso-electric focusing techniques. For example, the enzymes mediated by R_{TEM} and RP1 appear to have exactly the same

biochemical properties, yet show as different bands when focused with this technique (Matthew *et al*, 1975). Matthew *et al*, (1975) also found that chromosomal and plamid-mediated β-lactamases produced in a single strain of bacteria are clearly separated as distinct entities in an iso-electric focusing pattern.

The β-lactamases from independent isolates of all five species of *Photobacterium* available from the RRIMP culture collection (refer to Table IV, Section B1), were compared by iso-electric focusing methods.

(a) Enzyme preparations

Purification of a crude intracellular enzyme preparation is not necessary for iso-electric focusing (Matthew *et al*, 1975, Matthew, pers. comm.) and even though the β-lactamases were in a moderately ionic medium, it was found unnecessary to dialyze the preparations. Where possible the crude enzyme preparations of the *Photobacterium* β- lactamases were prepared by the osmotic shock method, concentrating the cells 5-fold (see Section D 2.3b). For the strains, *P. leiognathi* 634 and 638 and *P. phosphoreum* 147, 661 and 808, it was found necessary to concentrate the cells 15-fold and disrupt the cell wall by ultrasonic disintegration to achieve adequate concentration of the very low activity β-lactamase present in these strains. The resultant cellular debris was removed by centrifugation. The results of enzyme preparations from all strains are given in Table XVII. Since β-lactamase activity varied greatly between strains the enzyme preparations were adjusted where necessary, to about 5U/ml. This was done for iodometric staining purposes (see below).

(b) Staining of iso-electric focusing gels for β-lactamase activity

The use of the chromogenic cephalosporin, nitrocefin, for detection of β-lactamase is considered to give

TABLE XVII:

LEVELS OF β-LACTAMASE ACTIVITY PRESENT IN CRUDE ENZYME PREPARATIONS

The β-lactamase was released by osmotic shock methods with the cells concentrated 5-fold. Strains marked with * required sonic disruption of the cell to release the enzyme and in these cases the cells were concentrated 15-fold. β-lactamase activity was measured by the iodometric assay.

SPECIES	RRIMP #	E u/ml	SPECIES	RRIMP #	E u/ml
Ph. leiognathi	16	15.03	*Ph. phosphoreum*	145	3.28
	80	6.91		146	6.10
	202	12.42		147*	2.73
	204	15.92		164	3.86
	205	17.28		174	61.77
	206	39.55		203	5.83
	345	31.06		617	28.21
	632	16.15		652	4.28
	633	11.74		661*	3.54
	634*	3.41		671	7.00
	638*	2.09		672	3.54
	644	3.00		674	7.00
	647	19.93		681	6.42
	648	42.11		808*	3.88
	771	32.90			
	806	3.84	*Ph. fischeri*	31	46.60
Ph. angustum	77	22.61		130	3.75
	78	17.36		148	6.31
	79	29.82		173	19.66
	159	33.65		341	7.30
	160	10.77		768	20.39
Ph. logei	846	8.19		805	7.16
	850	8.96			
	851	5.64			

particularly reliable results as it is very sensitive (Matthew *et al*, 1975) and O'Callaghan *et al* (1972b) found a complete absence of false negative results using the compound. Nitrocefin possesses a highly reactive β-lactam ring and is sufficiently sensitive to indicate β-lactamase presence in strains previously thought not to produce the enzyme. Nitrocefin is a yellow compound in its natural state but giving an immediate colour change to pink/red following disruption of the β-lactam ring. The red colouring is comparatively stable in aquaeous solution, with a half-life of approximately 18 hours. Matthew (pers. comm.) recommended the use of nitrocefin to stain for β-lactamase activity after iso-electric focusing of the enzymes. Hydrolysis of nitrocefin by the β-lactamases from *Photobacterium* strains was tested with samples of full strength crude enzyme preparations spotted on to nitrocefin-soaked filter paper as described by Matthew (pers. comm.) and reactivity assessed by visible colour change, over a total of 2 hours, with results shown in Table XVIII.

As can be seen from the results, the R-factor-mediated β-lactamases in *E. coli* are very active on nitrocefin. All strains of the two species *P. fischeri* and *P. logei* are capable of hydrolyzing this chromogenic cephalosporin, but of all the other *Photobacterium* strains only *P. phosphoreum* 174 demonstrated this ability to a significant extent. It is obvious from the results shown in Table XVIII therefore, that the use of nitrocefin for staining the iso-electric focusing gel is not suitable for *Photobacterium* strains.

An alternative method described by Brive *et al* (1977), is an agar-overlay technique which relies on the diffusion of the β-lactamase into a starch-iodine stained gel, which in in direct contrast with the ampholine poly-acrylamide gel. To determine whether this technique was suitable for *Photobacterium* β-lactamases a preliminary trial iso-electric focusing experiment was run with 20 µl aliquots of β-lactamases

TABLE XVIII:

HYDROLYSIS OF THE CHROMOGENIC CEPHALOSPORIN, NITROCEFIN, BY THE β-LACTAMASES FROM *Photobacterium*

Colour change of the chromogenic cephalospoin from yellow to red was assessed visually over a total period of 2 hours.

KEY: +++ red colour (fast) fast = < 1 min
slow = ~30 min
++ redish colour
+ faint pink
± very faint

STRAIN		APPEARANCE OF RED COLOURING	STRAIN		APPEARANCE OF RED COLOURING
P. leiognathi	16	-	*P. phosphoreum*	145	-
	80	-		146	-
	202	-		147	-
	204	-		164	-
	205	-		174	++
	206	-		203	-
	345	-		617	+ (slow)
	632	+ (slow)		652	-
	633	-		661	-
	634	-		671	-
	638	-		672	-
	644	-		674	± (?)
	647	-		681	+ (slow)
	648	± (?)		808	-
	771	+			
	806	-	*P. fischeri*	31	+++
				130	+
P. angustum	77	-		148	+
	78	-		173	+
	79	+ (slow)		341	++
	159	-		768	+++
	160	+ (slow)		805	+++
P. logei	846	++	CONTROLS		
	850	++	*E. coli*	R 6K	+++
	851	+	*E. coli*	PBR322	+++

of varying activity levels. For each concentration of enzyme, the period of time required for detection of clear bands was recorded and presented in Table XIX. The data in the Table show that the agar-overlay technique was relatively sensitive, with the minimum amount of β-lactamases required for an obvious clear zone to develop being about 1.0 U/ml, or 0.02 units activity in the 20 µl samples tested. Where possible, β-lactamase activity of 5 U/ml was used for iso-electric focusing so as to give clear bands after ten to fifteen mins development; for enzyme preparations with β-lactamase activity lower than 5 U/ml, the over-lay gel was allowed to develop for up to 30 mins, which gave good results. As the iodine in the agar-overlay gel was not stable, after a period of 1½ hours the blue-black colour faded to an extent that accurate positioning of the β-lactamase bands was impossible. Also, as the stain was allowed to develop with time the zone of decolourization spread horizontally, resulting in the enzyme band appearing broader as time progressed. To avoid inaccuracies of band positions incurred by this phenomenon, each β-lactamase activity band was recorded immediately on first appearance.

(c) Iso-electric focusing results

Samples of crude enzyme preparation from all *Photobacterium* strains were iso-electrically focused following the method in Section C7. All samples were focused at least twice with identical band patterns and positions observed each time. In repeated experiments, those β-lactamases exhibiting more than one band always demonstrated the same number of bands in the same relative positions. β-lactamases with similar pI values were checked for identity or non-identity by running them in adjacent tracks. Figure 10 is a sample photograph of iso-electrically focused β-lactamases which have been stained with the agar-overlay gel, showing the resultant clear band positions. As can be

TABLE XIX:

OBSERVATIONS WITH THE TRIAL AGAR OVERLAY STAINING TECHNIQUE AFTER ISO-ELECTRIC FOCUSING

Overlay agar gel, in direct contact with the iso-electric focusing gel, was observed for appearance of clear bands against the blue-black background.

SAMPLE STRAIN	Enzyme Activity u/ml	Enzyme Activity u/ml	Clarity of band	Time of Appearance (min)
P. leiognathi 632	16.15	0.32	+	5
P. leiognathi 202	12.42	0.25	+	5
P. angustum 160	10.77	0.22	+	6
P. logei 851	5.64	0.11	+	9
P. phosphoreum 652	4.28	0.08	+	10
P. leiognathi 644	3.00	0.06	+	12
P. leiognathi 638	2.09	0.04	+	15
P. leiognathi 638 (½ strength)	1.04	0.02	+	20-30
P. leiognathi 638 (¼ strength)	0.50	0.01	-	> 60

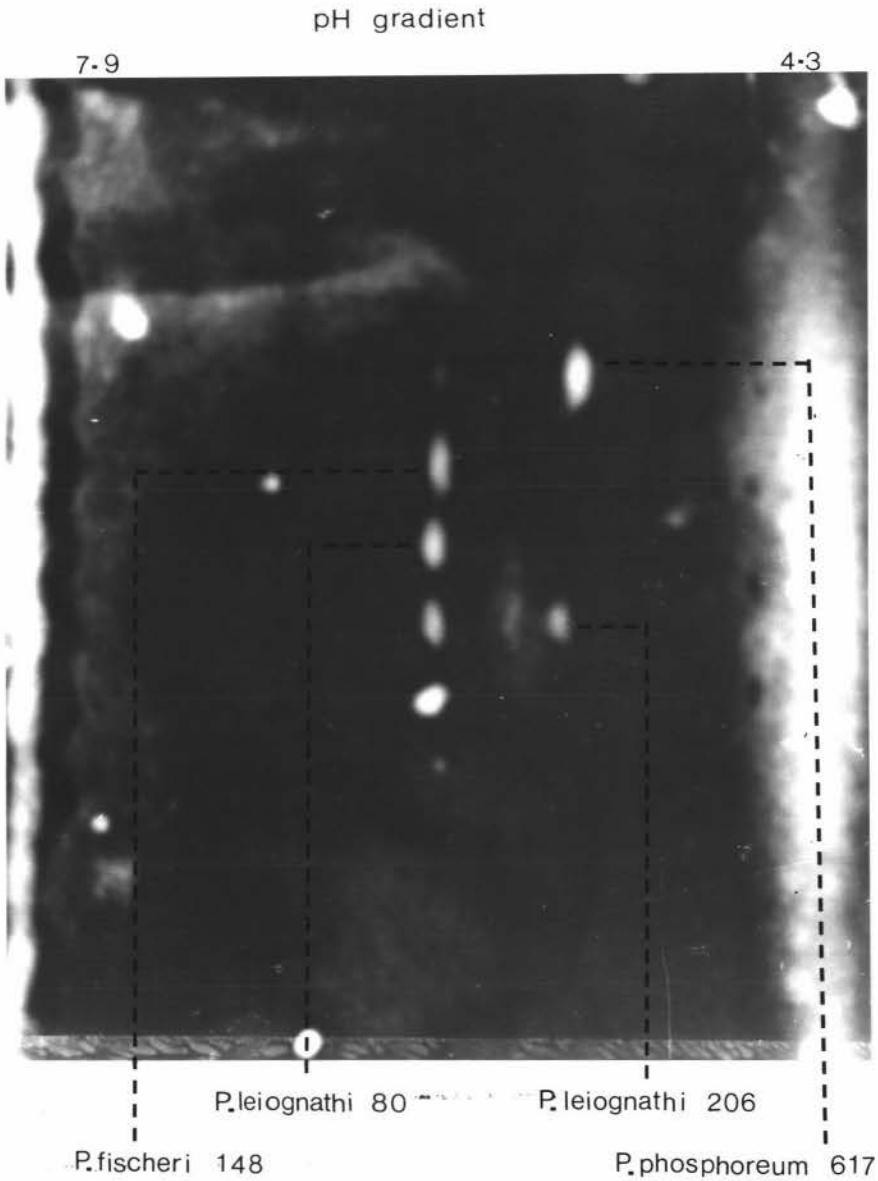


FIGURE 10: Sample photograph of iso-electrically focused β-lactamases stained with the agar overlay gel.

seen from this photograph, the resulting picture is not accurate enough to be used to determine exact band positions, hence the β-lactamase pI's were determined from the acetate sheet records (Section C 7), which method is particularly accurate. The pI's obtained are presented in Figure 11 and it is seen that the β-lactamases produced by strains of *Photobacterium* have pI's ranging from pH 5.1 - 6.9, with 35 strains of the 45 total tested producing a β-lactamase with pI of 6.1 or 6.2 and only 11 strains showing multiple band patterns.

The results show that some pI's are common to β-lactamases from several strains of more than one species. For example, all three strains of *P. logei* and three strains of *P. phosphoreum*, six from *P. fischeri* and two strains of *P. leiognathi* produce a β-lactamse with pI 6.1; ten strains each of *P. leiognathi* and *P. phosphoreum* have a β-lactamase with pI 6.2. All five strains of *P. angustum* produce β-lactamases with identical pI of 6.6, which is also the value of one of the satellite bands in *P. phosphoreum* 174 and 617.

It is also clear from the results obtained that, although β-lactamases which form as one band only may have the same pI value in more than one species, in no case where multiple bands were demonstrated are the band patterns identical in different species.

(d) Slab-gel Electrophoresis

The microheterogeneity observed with some β-lactamases after iso-electric focusing, could be due to enzyme heterogeneity or be an artefact of the technique, such as reaction between ampholytes and the β-lactamase. To determine whether it occurred for the latter reason, the enzymes which demonstrated multiple band patterns were run on the slab-gel electrophoresis system described in Section C8, as such methods are often used as an indicator of enzyme homogeneity (Melling and Scott,

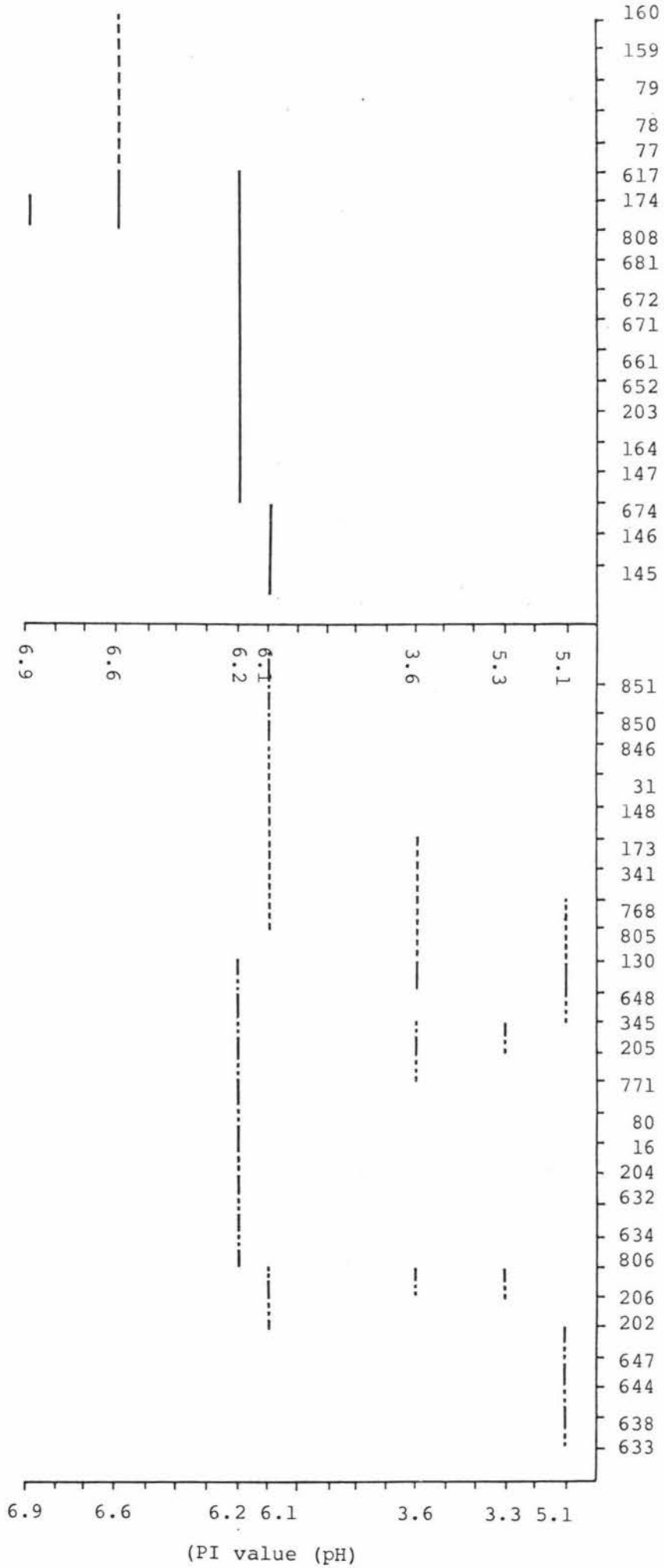


Figure 11: The Iso-Electric Points of β-Lactamases Produced by Strains of *Photobacterium*

The pH gradient across the iso-electric focusing gel was graphed with the position of the β-lactamase bands read directly off the graph.

1972; Davies *et al*, 1974; Sawai and Lampen, 1974; Dale and Smith, 1976; Yamamoto and Lampen, 1976). Unfortunately, this method proved impossible to perform since although the gels could be stained for protein with Coomassie blue stain, staining for β-lactamase activity was not successful as the β-lactamases had been rendered inactive by pH 8.3 of the 'running' buffer (see Section D 1.1b). The gel could not be run at the optimal pH for β-lactamase activity as the enzymes involved had pIs too near this value, hence no migration of the proteins would occur. Staining the gel for protein alone was not sufficient as several bands (about 7) appeared and there was no way of determining which were the β-lactamase ones.

The way to solve this problem is to purify the β-lactamase from the strains so that protein staining after electrophoresis would give information as to whether or not the multiple bands appeared due to an artefact of the iso-electric focusing techique. Purification of the enzymes was not undertaken as for this thesis, it was decided not to pursue the bio-chemical aspects further. Therefore the problem of microheterogeneity was not further resolved.

5. THE GENETIC DETERMINATION OF β-LACTAMASES

The genes determining β-lactamases in Gram-negative bacteria are known to be carried either on the chromosomal DNA or extrachromosomally on plasmids (R-factors). Since a high proportion of *Photobacterium* strains are found to carry plasmid DNA (B. Dymock & K. Smith, this laboratory), it is possible that the β-lactamase (βla^+) genes are located extrachromosomally in these strains. In the remaining strains, no plasmid DNA was found, implying a chromosomal location for the βla^+ genes. In order to attempt to establish the location of the β-lactamase genes two approaches were used: -

(1) Loss of β-lactamase activity under plasmid curing conditions.

(2) Transfer of β-lactamase activity to a new host by conjugation and transormation.

5.1 Plasmid During Experiments

One of the characteristics of extrachromosomal genetic elements is that they may be cured by treatment of the bacterium under a variety of chemical or physical conditions. Plasmid curing has been reported by many workers (Watanabe and Fukasawa, 1961; Kawakami and Landman, 1966; Warren *et al*, 1974) and Novick (1969) pointed out the requirement to establish the coordinated loss of plasmid DNA and a particular marker, in order to claim that the marker was resident on the plasmid. A number of different chemical agents, such as acridine orange, acriflavine, ethidium bromide, mitomycin C and sodium dudecylsulphate, have proved successful in attaining plasmid-free bacteria, with mitomycin C usually considered most effective. However, individual plasmids are known to vary widely in their response to various curing agents and not all plasmids are successfully cured (Novick, 1969; Sebald *et al*, 1975). Plasmid curing experiments alone are not sufficient to determine genetic location but can be used in conjunction with other methods to establish the relationship between β-lactam resistance and plasmids (Del Bene, 1979).

Plasmid curing treatments were performed on *P.leiognathi* 206 to find out whether β-lactamase activity was lost from this strain. If such loss did occur with loss of plasmid DNA, the preliminary conclusion would be that the βla^{+} genes were located extrachromosomally. Serial dilutions of acridine orange (AO), acriflavine (AF), ethidium bromide (EB), mitomycin C (MC) and sodium dodecylsulphate (SDS) were inoculated with 10 - 50 bacteria per tube. The inoculum size was necessarily small to minimize cell contact and thereby limit the possible transfer of the plasmid from

cell to cell. Bacterial growth in the presence of the curing agents was assessed visually after 18 hours incubation at 30°C and the minimum inhibitory concentration (MIC) for each determined (Table XX). Cells from the two tubes with curing agent concentrations immediately below the MIC were plated to give 100 - 200 colonies per plate and the resulting clones subjected to the starch-iodine plate test (see Section C 3.2) to determine levels of β-lactamase activity. Cells from the tubes containing no curing agent were also plated and plate-tested in a similar way, to check for spontaneous loss of βla^+ genes. The results of the plate tests are presented in Table XXI and it is seen that after treatment with the curing agents, all clones of *P. leiognathi* 206 retained wild-type levels of β-lactamase activity. The mitomycin C-treated cells were plated on selective plates containing benzylpenicillin 50 µg/ml, a concentration just below the MIC for *P. leiognathi* cells and above the MIC for βla^- mutants, to determine whether wild-type levels of β-lactamase activity were still maintained by the treated cells. This was carried out as the result of this test are more qualitative than the starch-iodine plate test. The colony counts obtained from the test are recorded in Table XXII, from which it is evident that the cells still retain full β-lactamase resistance levels, even after treatment with mitomycin C.

With successful curing experiments it is usual to achieve loss of the plasmid from 10-50% of the bacteria, however, Tables XXI & XXII show that with the curing agents used, the curing rate of β-lactamase activity in *P. leiognathi* 206 is less than 1%. If it could be demonstrated that the cells had lost the plasmid while retaining their β-lactamase activity, then a chromosomal location for the βla^+ genes could be proposed. As no other plasmid-borne genetic marker is known, the presence or absence of plasmid DNA in *P. leiognathi* 206 cells which had been treated with mitomycin C (2.5µg/ml) was tested by agarose gel electrophoresis and in 15 randomly chosen clones, it was found that the plasmid DNA band was still present (B. Dymock, this laboratory).

TABLE XX:

GROWTH OF *P. leiognathi* 206 IN THE PRESENCE OF CURING AGENTS

All tubes were inoculated with 10-50 cells and incubated 30°C, 18 hours, then growth was assessed visually using turbidity of the solution.

AGENT	CONCNS									
	200	100	50	25	12.5	6.25	3.12	1.56	0.78	0µg/ml
EB	-	-	-	++*	+++*	+++	+++	+++	+++	+++*
AD	-	-	-	++*	+++*	+++	+++	+++	+++	+++*
AF	-	-	-	-	-	-	++*	+++*	+++	+++*
	10	5.0	2.5	1.25	0.625	0.31	0.15	0.08	0.04	0µg/ml
MitG	-	-	++*	+++*	+++	+++	+++	+++	+++	+++*
	1.0	0.5	0.25	0.125	0.06	0.03	0.015	0.008	0.004	0 %sol^{n}
SDS	-	-	-	-	-	++*	+++*	+++	+++	+++*

Growth:
- \- nil
- \+ poor
- ++ good
- +++ normal (= control growth)

* Tubes chosen for plating

TABLE XXI:

PLATE DETECTION OF *P. leiognathi* 206 β-LACTAMASE ACTIVITY FOLLOWING CURING TREATMENTS

Samples of the tubes marked * in Table 20 were plated to give 100-200 colonies/plate and incubated at 30°C for 18 hours. β-lactamase activity levels were assessed visually using the starch-iodine plate test and recorded as mild-type mutant levels.

Curing agent concentration (μg/ml)		Number of colonies tested (total of 5 plates)	Number of colonies without -lactamase activity
O		950	0
E.B.	12.5	899	0
	25.0	815	0
AO	12.5	902	0
	25.0	597	0
AF	1.56	828	0
	3.12	793	0
MC	1.25	743	0
	2.50	651	0
SDS	0.015	653	0
(% soln)	0.030	544	0

TABLE XXII:

MITOMYCIN C - TREATED *P. leiognathi* 206 SELECTIVELY PLATED FOR WILD-TYPE β-LACTAMASE ACTIVITY LEVELS

Mitomycin C-treated cells were diluted to give 100-200 colonies/plate and plated on selective plates with incubation at 30°C for 18 hours. The results are given as the mean of 8 plates.

Mitomycin C Concentration (μg/ml)	Colonies per plate grown on:	
	LA	LA & PenG 50 μg/ml
0	141.3 ± 2.5	140.0 ± 2.0
1.25	139.2 ± 2.5	189.1 ± 3.0
2.5	130.6 ± 4.6	128.0 ± 2.4

The presence of the plasmid after curing treatment, suggests that the plasmid is not curable from *P. leiognathi* 206, and hence the results of these experiments are inconclusive.

5.2 Transfer of β-lactamase genes by conjugation

Transfer of β-lactamase genes from a βla^+ strain to a βla^- mutant strain by conjugation techniques is the most widely used and accepted method for establishing whether the genes specifying the β-lactamase are plasmid-borne or not. When the transconjugants obtained demonstrate levels of β-lactamase activity equivalent to that of the donor parent, it is taken as meaning that the βla^+ genes have been transferred, implying an extrachromosomal location. Generally, the opposite also holds: when no transfer of β-lactamase activity occurs the βla^+ genes are considered to be chromosomal, although this does not necessarily follow (see also later discussion, Section E 8).

Conjugation experiments were performed in an attempt to transfer the βla^+ genes from strains of *Photobacterium* to new hosts, to try and establish whether the β-lactamase activity was plasmid-mediated.

(a) Bacterial strains

(i) Donors:

P. leiognathi 206 and 345
P. angustum 79 and 159
P. phosphoreum 147 and 674
} βla^+

strain 534 = *P. leiognathi* 206.R68.45 βla^+ Nm^r Tc^r

All *Photobacterium* donors demonstrated high levels of β-lactamase activity and carry at least one plasmid (B. Dymock and K. Smith, this laboratory).

Strains from *P. fischeri* and *P. logei* were not used as donors since most of these strains do not carry a plasmid.

(ii) Recipients:

strain P_9Sm^r = *P. leiognathi* 206 βla^- Sm^r
strain A43 = *P. leiognathi* 206 βla^- Sm^r *leu*
E. coli PB1395 F^- *met*$^-$ supE supF hsdR

Strain A43 was used in initial experiments but because of problems with the stability of the Sm^r marker in this strain, P_9Sm^r was used in subsequent experiments.

(b) Selective media

Selection for exconjugants was carried out on either marine minimal agar (MMA) for *Photobacterium* recipients or terrestial minimal agar (TMA) for the *E. coli* recipient. Selective antibiotics were incorporated into the media on the basis of the information presented in Tables XXIIIa & b. In initial experiments, benzyl-penicillin (50 µg/ml) was used to select for βla^+ exconjugants but as background growth of the *E.coli* recipient occurred, this was changed to ampicillin in subsequent experiments. The ampicillin successfully eliminated the background growth problem. Neomycin (50 µg/ml) and streptomycin (250 µg/ml) were also incorporated to select against the donor strains.

(c) Conjugation results

Conjugation experiments were performed following the method described in Section C 9.2. The initial number of cells for mixing was calculated to ensure that at least 10^6 donor cells were transferred to the selective plate in case the frequency of transfer was very low. The mating mixtures were immobilized on solid agar to maximize cell contact since *Photobacterium* strains are motile. The long mating incubation period (24 hours) allowed for a very slow rate of transfer or expression of the plasmid genes, as had previously been found necessary for the transfer of R68.45 plasmid into

TABLE XXIIIa:

SINGLE CELL RESISTANCES TO BENZYLPENICILLIN USED IN SELECTIVE PLATES

Each strain was diluted from an overnight broth culture to give 100-200 colonies/plate, then plated on the selective media. After incubation at 30°C for 18 hours, colonies/plate were counted and recorded according to the following scale: -

Growth: - ++ normal (100-200 colonies/plate)
+ poor (0 - 10 colonies/plate)
- inhibited (0 colonies/plate)

Media: all antibiotics incorporated into MMA*leu*, except colomns marked * where the media was TMA*met*.

Antibiotic Concentration (µg/ml)		Growth of single cells				
		Donors		Recipients		
		Pl 206	534	A_{43}	$PaSm^R$	PB1395*
PenG	100	-	++	-	-	-
	50	++	++	-	-	-
	25	++	++	-	-	-
	12.5	++	++	-	-	+
	6.25	++	++	-	-	++
	3.12	++	++	+	+	++
	1.56	++	++	++	++	++
Nm	50	-	++	-	-	-

TABLE XXIIIb:

SINGLE CELL RESISTANCES TO AMPICILLIN USED IN SELECTIVE PLATES

Media: all antibiotics incorporated into MMA, except colums marked * where TMA*met* was used.

Antibiotic concentration (µg/ml)	GROWTH OF SINGLE CELLS								
	Donors							Recipients	
	*Pl*206	*Pl*345	*Pa* 79	*Pa*159	*Pp*147	*Pp*674	534	PgSm^R	PB1395*
Amp 100	-	-	-	-	-	-	++	-	-
50	++	+	+	+	+	+	++	-	-
25	++	++	++	++	+	+	++	-	-
12.5	++	++	++	++	++	++	++	-	-
6.25	++	++	++	++	++	++	++	±(3)	-
3.12	++	++	++	++	++	++	++	+	+
1.56	++	++	++	++	++	++	++	++	++
Sm 250	-	-	-	-	-	-	-	++	-

P. leiognathi 206 (K. Smith, this laboratory).

Tables XXIVa & b give the results of the attempted transfer of βla^+ between *P. leiognathi* 206 and the recipient strains. The figures given represent the number of clones with recombinent properties obtained on the selective plates. Also presented are the results of the control transfer of the plasmid-borne Nm^r from strain 534, which was carried out under the same experimental conditions. As measured by the appearance of exconjugants manifesting the properties of the recipient strains, along with the plasmid characteristics of ten donor strain 534 (Table XXXa and b), it is evident that successful transfer occurred under the experimental conditions.

Conjugation experiments were also performed using other *Photobacterium* donors to establish whether or not the βla^+ genes could be transferred from these strains. As shown in Table XXVIa & b, the inability to transfer the βla^+ genes appears to be a general property of *Photobacterium* strains.

5.3 Transformation of βla^+ genes

Since all attempts to transfer β-lactamase activity from *Photobacterium* donors to new hosts by conjugation methods were unsuccessful, further attempts were made to transfer the βla^+ genes by transformation techniques. The methods for DNA preparation, preparation of competent cells and the transformation procedure are described in detail in Section C, 9.3, with *P. leiognathi* 206 used as the βla^+ donor and *E.coli* PB1395 as the recipient, since this strain has no known restriction enzyme system. Donor strain 534 was used as a control for the experimental conditions.

TABLE XXIVa:

RESULTS OF CONJUGATION EXPERIMENTS BETWEEN DONOR *P. leiognathi* AND RECIPIENT STRAIN A43

The conjugation mating mixtures were plated on the appropriate slective media in 2 dilutions. The figures represent the mean of 5 experiments.

Selective media & strains	Estimated number of donors/plate	Number of colonies with exconjugant properties				Transfer rate/donor
		4 hours mating		24 hours mating		
		incubation time		incubation time		
		24 hrs	48 hrs	24 hrs	48 hrs	
MMA leu & Sm (250µg/ml) + PnG (50µg/ml)						
206 ⊗ A_{43}	1.0×10^5	0	0	0	0	
	1.0×10^6	0	0	0	0	$< 10^{-6}$
donor 206 alone		0	0	0	0	
recipient A43 alone		0	0	0	0	
MMA leu & Sm (250µg/ml) + Nm (50µg/ml)						
534 ⊗ A_{43}	1.4×10^5	1	1	22	23	
	1.4×10^6	22	34	165	166	$\sim 10^{-4}$
donor 534 alone		0	0	0	0	
recipient A43 alone		0	0	0	0	

TABLE XXIVb:

RESULTS OF CONJUGATION EXPERIMENTS BETWEEN DONOR *P. leiognathi* 206 AND RECIPIENT *E. coli* PB 1395

Selective media & strains	Estimated number of donors/plate	Number of colonies with exconjugant properties				Transfer rate/donor
		4 hours mating		24 hours mating		
		Incubation time. 24 hrs	48 hrs	Incubation time. 24 hrs	48 hrs	
TMA + PenG (60µg/ml)						
206 ⊗ PB1395	1.1×10^5	0	0	0	0	
	1.1×10^6	0	0	0	0	$< 10^{-6}$
donor 206 alone		0	0	0	0	
recipient PB1395 alone		0	0	0	0	
TMA & Nm (50µg/ml)						
534 ⊗ PB 1395	1.2×10^5	0	0	10	10	
	1.2×10^6	3	10	86	104	$\sim 10^{-4}$
donor 534 alone		0	0	0	0	
recipient PB 1395 alone		0	0	0	0	

TABLE XXVa:

CHARACTERISTICS OF 10 INDEPENDENT EXCONJUGANT CLONES FROM THE 534 ⊗ A_{43} CROSS

a) tested by zone inhibition with filter strip soaked in 10 mg/ml Tc solution, controls were strain 534(Tc^R) and A_{43} (TC^S)

R = resistant S = sensitive

b) tested by starch - I_2 plate test

+++ v. strong β-lactamase activity
++ strong ± very weak
\+ poor - none evident

STRAINS	Growth on		Growth on NMA leu		TC^R [a]	β-lactamase [b]
	MMA	MMAleu	& Nm (50μg/ml)	& Sm (250μg/ml)		
Donor 534	+	+	+	-	R	+++
Recipient A_{43}	-	+	-	+	S	±
Exconjugants (10 clones)	-	+	+	+	R	+++

TABLE XXVb:

CHARACTERISTICS OF 10 INDEPENDENT EXCONJUGANT CLONES FROM THE CROSS 534 ⊗ *E. coli* PB 1395

a) tested by zone inhibition by 10mg/ml Tc on R filter strip, controls were strain 534 (Tc^R) and *E. coli* PB 1395 (Tc^S)

R = resistant S = Structure

b) relevant tests performed on LA

STRAINS	Growth on			Growth in presence of		Tc^R [a]
	TMA	TMAmet	LA	Nm (50 µg/ml)	PenG (60 µg/mℓ)	
Donor 534[b]	−	−	+	+	+	R
Recipient PB 1395	−	+	−	−	−	S
Exconjugants (10 clones)	−	+	−	−	−	R

TABLE XXVIa:

RESULTS OF CONJUGATION EXPERIMENTS OF CROSSES BETWEEN *Protobacterium* STRAINS AND $PgSm^R$

Samples of the mating mixtures were plated onto appropriate selective plates in 2 dilutions. The concentration of ampicillin incorporated into the media was that just below the MIC for the relevant *Photobacterium* strain (refer Table 23b)

Selective Media & strains	Esimtated Number of Donors/plate	Number of colonies with exconugant properties				Transfer rate/donor
		4 hours mating		24 hours mating		
		Incubation time 24 hrs	48 hrs	Incubation time 24 hrs	48 hrs	
MMA & Sm (250µg/ml & Amp						
P. leiognathi 345 ⊠ $PgSM^R$	$\times 10^{-5}$	0	0	0	0	
	$\times 10^{-6}$	0	0	0	0	$<10^{-6}$
P. angustum 79 ⊠ $PgSm^R$	$\times 10^{-5}$	0	0	0	0	
	$\times 10^{-6}$	0	0	0	0	$<10^{-6}$
P. angustum 159 ⊠ $PgSm^R$	$\times 10^{-5}$	0	0	0	0	
	$\times 10^{-6}$	0	0	0	0	$<10^{-6}$
P. phosphoreum 147 ⊠ $PgSm^R$	$\times 10^{-5}$	0	0	0	0	
	$\times 10^{-6}$	0	0	0	0	$<10^{-6}$
P. phosphoreum 674 ⊠ $PgSm^R$	$\times 10^{-5}$	0	0	0	0	
	$\times 10^{-6}$	0	0	0	0	$<10^{-6}$
donor 345 alone		0	0	0	0	
" 79		0	0	0	0	
" 159		0	0	0	0	
" 147		0	0	0	0	
" 674		0	0	0	0	
recipient $PgSm^R$ alone		0	0	0	0	
MMA & Sm (250µg/ml) & Nm (50µg/ml)						
534 ⊠ $PgSm^R$	$\times 10^{-5}$	0	0	26	26	
	$\times 10^{-6}$	34	38	192	194	$\sim 10^{-4}$
donor 534 alone		0	0	0	0	
recipient $PgSm^R$		0	0	0	0	

TABLE XXVIb:

RESULTS OF CONJUGATION EXPERIMENTS OF CROSSES BETWEEN *Photobacterium* STRAINS AND *E. coli* PB 1395

Selective Media & strains	Estimated Number of Donors plate	Number of colonies with exconjugant properties				Transfer rate/donor
		4 hours mating		24 hours mating		
		Incubation time 24 hrs	48 hrs	Incubation time 24 hrs	48 hrs	
TMAmet & Amp						
P. leiognathi						
345 ⊠ PB	$\times 10^5$	0	0	0	0	
	$\times 10^6$	0	0	0	0	$<10^6$
P. angustum						
79 ⊠ PB	$\times 10^5$	0	0	0	0	
	$\times 10^6$	0	0	0	0	$<10^6$
P. angustum						
159 ⊠ PB	$\times 10^5$	0	0	0	0	
	$\times 10^6$	0	0	0	0	$<10^6$
P. phosphoreum						
147 ⊠ PB	$\times 10^5$	0	0	0	0	
	$\times 10^6$	0	0	0	0	$<10^6$
P. phosphoreum						
674 x PB	$\times 10^5$	0	0	0	0	
	$\times 10^6$	0	0	0	0	$<10^6$
donor 345 alone		0	0	0	0	
" 79 "		0	0	0	0	
" 159 "		0	0	0	0	
" 147 "		0	0	0	0	
" 674 "		0	0	0	0	
recipient PB"		0	0	0	0	
TMA met & Nm (50µg/ml)						
534 ⊠ PB	10^5	0	0	10	11	
	10^6	4	9	92	106	$\sim 10^{-4}$
donor 534 alone		0	0			
recipient PB :		0	0			

Donor DNA was prepared by three methods (as described in Section C, 9.3):

(a) crude SDS lysis
(b) lyzozyme pretreatment of (a), to ensure cell lysis
(c) partially purified DNA, by phenol extraction of a cleared lysate, followed by ethanol precipitation.

Preparations (a) and (b) were made as large volumes (10 ml) with 0.1 ml samples used in the transformation experiments, but since the samples taken could possibly consist of a high proportion of cellular debris and insufficient DNA, small volumes (0.1 ml) were also prepared directly in the tubes used for transformation. Three DNA donor suspensions were used for these two DNA preparations; *P. leiognathi* 206 alone, strain 534 alone, *P. leiognathi* 206 and strain 534, with the latter two suspensions being controls for the DNA preparation from *P. leiognathi* 206. If transformation is achieved using the preparation of strain 534 alone then it should also be successful with combination donor preparation, when there is no inhibiting effect from the *P. leiognathi* 206 DNA, hence transformation experiments with DNA from *P. leiognathi* 206 alone should therefore also be successful. It is usually unnecessary to purify DNA for transformation, however a partial purification, which enriches for plasmid DNA, was performed on strain 534 in an attempt to transfer the Nm^r, Tc^r and βla^+ genes carried on the R68.45 plasmid. The preparation was checked for presence of DNA by agarose gel electrophoresis and, as expected, was found to contain both the plasmid DNA bands with very little chromosomal DNA (B. Dymock, this laboratory).

The transformation mixture, containing 0.1 ml DNA preparation and 0.2 ml competent *E.coli* PB1395 recipient, was incubated for a minimum of 2 hrs, with samples plated on TMA*met* plates and selection by 12 µg ampicillin/ml or 50 µg neomycin/ml. Tetracycline resistance was determined by zone inhibition with a filter strip soaked in 10 mg/ml solution of tetracycline.

From a total of nine transformation experiments, attempted with all three donor suspensions (*P. leiognathi* 206 alone, strain 534 alone, *P. leiognathi* 206 and strain 534) each prepared by all three preparation methods, no transfer of genetic markers was observed. Further experiments therefore, concentrated on achieving successful transformation with the control donor, strain 534. Four independent series of fresh DNA preparations from strain 534 were made, using all of the three methods, with fresh *E. coli* cells made competent for each experiment and the viability of the cells checked before and after competence treatment. The period of incubation of the transformation mixture was increased from the recommended 2 hours to 4 and 6 hours, with viability of the recipient *E. coli* again verified after these incubation times, by use of a control containing all ingredients of the transformation mixture except the DNA. The longer periods of incubation should have allowed for any delay in expression of the neomycin resistance, which is known to occur in some bacteria. However, it is of note that no transformants were obtained when selectively plated for β-lactamase activity, for which no expression delay has been reported. Also, no loss of *E. coli* viability occurred at any stage during transformation procedure. All experiments, including those involving the control donor, strain 534, were unsuccessful in obtaining transformants.

SECTION E

DISCUSSION

FACTORS AFFECTING β-LACTAMASE ASSAY AND ACTIVITY

Five different methods have been developed for assaying β-lactamase activity: spectrophotometric assays (O'Callaghan *et al*, 1968; Samuni, 1974), the hydroxylamine assay (Boxer and Everett, 1949), the microbiological assay (Ørstavik and Ødegaard, 1971), the iodometric assays (Perret, 1954; Sargent, 1968; Sykes and Nordstrom, 1972) and acidimetric/alkalimetric methods (Henry and Housewright, 1947; Hou and Poole, 1972; Wise and Twigg, 1950). Selection of the method for use depends on the enzyme preparation, whether the β-lactamase is present in intact cells, as crude enzyme or purified form; the concentration of the β-lactamase and the substrate used, whether penicillin or cephalosporin.

The iodometric assay of Perret (1954) is the one most commonly used, particularly for comparative studies such as substrate profile, as it is considered very reliable with results being readily reproducible (Ross and O'Callaghan 1975) and is sufficiently sensitive to detect low levels of β-lactamase activity. The iodometric assay is particularly suitable for penicillin substrates and does not require the β-lactamase to be in a purified form, although, as the assay relies on uptake of free iodine molecules, the presence of starch or similar compounds in the reaction mixture should be avoided. The iodometric assay is not so reliable with extremely low β-lactamase activities or when cephalosporins are used as subtrates.

The β-lactamase from *Photobacterium* demonstrate high activity, with the results of substrate profiles (see Section D, 4.1 and 4.2a) indicating that the enzymes are predominantly active on penicillins and as knowledge of β-lactamase activity was required for comparative purposes, the iodometric assay (Perret, 1954) as described by Ross and O'Callaghan (1975) was used for the β-lactamses from all *Photobacterium*

strains. The results obtained from the assay, using either cell-bound β-lactamase or crude enzyme preparation, were readily reproducible with a measured maximum error of only about 1.5% between any two identical test flasks containg β- lactamase of about 10 units activity/ml. However, when very low levels of β-lactamase activity are measured, the inaccuracy of the assay increases 10-fold. Areas of the technique where errors can occur are in measuring volumes and timing, reading of the spectrophotometer (Bausch - Lomb Spectronic 20) and the volatility of the iodine. When measuring β-lactamase in cells, error can occur from biological variations between cultures on different days and precipitation of ferric ions from the medium.

The most commonly used unit of β-lactamase activity is that of Pollock and Torriani (1953), who defined it for precise conditions of temperature (30°C) and pH (7.0). However, other conditions have been employed by different workers to suit the β-lactamase under investigation. For example, temperatures of 25°C and 37°C have often been used as these were the optimum for the bacteria concerned, but thermolabile β-lactamases have been found which are inactivated at temperatures exceeding 33°C (Smith, 1969). The pH value of the assay mixture has also been altered in some laboratories (Novick, 1962; Richmond, 1963 and 1965) since the pH activity curves obtained with β-lactamases from various Gram-negative bacteria show maximal activity anywhere between pH 5.0 and pH 8.5. The Gram-positive β-lactamases have a narrower range, with their pH optima falling between pH 6.0 and 7.0 and show a rather steep decline of activity in the alkaline range. The use of conditions other than the standard ones of 30°C and pH 7.0, is unfortunate as it limits direct comparison of results from different sources.

The pH curve obtained for the β-lactamase from *P. leiognathi* 206 (see Section D, 1.1b) shows a rather narrow activity range of pH 6.2 to pH 7.6, with the optimum at pH 6.2. However, pH 7.0 was used for assaying as the β-lactamase was only 8% less active at this pH than at the optimum and

using the standard conditions allow direct comparison between our results and those obtained by other workers who had used them. As the standard conditions of both 30°C and pH 7.0 were found to be suitable for assaying the activity of *P. leiognathi* 206 β-lactamase (see Section D, 1.1), the Pollock and Torriani unit of enzyme activity is the one used throughout this work.

The activity of β-lactamase II from *Bacillus cereus* 659H is increased 4 to 5-fold in the presence of 1mM $ZnSO_4$ and has 80% of its maximum activity at 10μM $ZnSO_4$, but very little activity when the zinc ion concentration is decreased to 1μM. The large variations in the activity of β-lactamaseII, with and without zinc ions present, were interpreted by Sabath and Abraham (1966) as meaning the enzyme is zinc-dependent. In the presence of zinc ions, a stimulatory effect of β-lactamase activity is also observed with the enzyme from a staphylococcal mutant, P54 (J. Fleming and R. Ambler, pers. comm.) but it is not thought to be a true case of divalent ion-dependence as Ambler suggests that when using the Perret iodometric conditions and a significant zinc ion concentration, a complex precipitate, of perhaps zinc-protein-phosphate, forms and that it is in this precipitate that the stimulation of activity is noticed. Also of interest, is the report by Kuwabara (1970) of β-lactamase I from *B. cereys* 569H being inhibited 95% by 3mM zinc ion concentration.

The activity of the β-lactamase from *P. leiognathi* 206 remained unchanged at all zinc ion concentrations tested, except at 10μM $ZnSO_4$where a very small increase (less than 13% in enzyme activity was observed (see Section D, 1.3b). The significance of this slight stimulation of activity is not known and it is possible that it resulted from the complex precipitate mentioned by Ambler. At zinc concentrations of 1mM and 1μM, the β-lactamase activity was equivalent to that measured in the absence of zinc ions and hence it is concluded that the *P. leiognathi* 206 β-lactamase is not zinc-dependent. At all zinc concentrations tested, the enzyme

activity was never less than that measured in the absence of zinc, therefore it is also concluded that the β-lactamase is not inhibited by zinc ions.

2. PRODUCTION OF β-LACTAMASE DURING BACTERIAL GROWTH

The production of β-lactamase in wild-type *E. coli* and *ampA* (penicillin-resistant) *E. coli* mutants (Lindstrom *et al*, 1970; Boman *et al*, 1967) and in some strains of *Bacteroides* (Darland and Birnbaum, 1977), is dependent on growth of the bacteria. In other *Bacteroides* strains, the β-lactamase was found to be produced independently of the bacterial growth phase (Salyers *et al*, 1977). While investigating the β-lactamase production in various bacterial cultures, several workers have observed a short sharp increase in β-lactamase activity from cells during late lag/early exponential phase of growth. For example, Sachithanandam *et al*, (1974) noticed the anomalous appearance of β-lactamase produced spontaneously during the lag and exponential phases of growth in a *Staphylococcus aureus* culture, whereas normally the enzyme of this strain needs to be induced (see induction discussion, Section E, 3). In 1978, Sachithanandam *et al* reported the isolation of a potent β-lactamase from a fully penicillin-sensitive strain of *Staph. aureus*, which was only present in significant levels during very early exponential growth phase.

The production of β-lactamase by *P. leiognathi* 206 cells was found to be largely independent of growth of the bacteria (see Fig. 6, Section d, 2.1), although during late lag and very early exponential phases a small peak of β-lactamase activity was observed. This peak of activity was always of reproducible size despite the fact that, at the growth phase in which it occurred, the actual β-lactamase level in the culture was very low, meaning that the inaccurracy incurred with the iodometric assay when measuring the enzyme activity present, is high (see Section D, 1.2). The anomalous peak appears to be similar to those observed by other workers (above) and it is interesting to note that all reports of

such increased β-lactamase activity during lag/early exponential growth phases involve Gram-positive bacteria, whereas *P. leiognathi* 206 is a Gram-negative organism. Perhaps the lack of examples from other Gram-negative bacteria is due to β-lactamase synthesis not having been investigated in other strains.

3. INDUCTION OF β-LACTAMASE

Most Gram-negative β-lactamases are consitutive, meaning that the enzyme production is not influenced by presence of β-lactams in the medium. Those which are reported to be inducible usually belong to Richmond and Sykes Class I group of β-lactamases (see Table III, Section A, 2.3), which are predominantly active on cephalosporins (Hennessey, 1967; Nordstrom and Sykes, 1974; Farrer and O'Dell, 1976). It is notable that in all cases of inducible Gram-negative β-lactamases, very high concentrations of inducer are required (usually greater than 500 μg/ml and often up to 5 mg/ml). Smith (1963) and Hamilton-Miller (1966), both suggest the possibility that induction of β-lactamase which apparently occurs in some Gram-negative species could be 'pseudo-induction'; resulting from the presence of very high levels of β-lactam interacting with the outer membrane of the cells and hence reducing the permeability barrier to certain β-lactams (see Section E, 5).

The β-lactamases from Gram-positive bacteria are induced to high levels (up to 500-fold, Pollock, 1962; Citri and Pollock, 1966) in the presence of β-lactam compounds. Saz and Lowery (1965 and 1979) and Ozer *et al*, (1970) suggest that the presence of the 'inducer' β-lactam serves only to inhibit cell wall synthesis, with such inhibition leading to the formation of non-crosslinked peptidoglycans which are the real inducers of the β-lactamase activity. Evidence to support this theory came from Tynecka and Ward (1975) who showed that such peptidoglycan was released into the medium from *B. licheniformis* cells when incubated in the presence of

β-lactams. However, Nordstrom and Sykes (1974) found that cell wall synthesis inhibitors such as gramicidin and 5-methyltryptophan, did not induce the β-lactamases in *Ps. aeruginosa*. Inhibitors of cell wall synthesis, other than β-lactams, have also ueen found ineffective as β-lactamase inducers(Kollonitsch *et al*, 1973; Hammes, 1973), with the exception of bacitracin (Citri, 1978).

Working with *B. cereus*, Ozer *et al*, (1970) noticed that sometimes high levels of β-lactamase activity arose in the absence of exogenous inducer, indicating that enzyme synthesis is not occasioned solely by addition of penicillins. The presence of the endogenous inducer in the cultures of *B. cereus* paralleled periods of growth phase change such as germination and sporulation times, in both β-lactamase-inducible cells (as defined by penicillins) and constitutive cells. From these observations, Ozer *et al* suggested that β-lactamase, in *B. cereus* at least, plays a role in cell wall/sporecoat metabolism. Sachithanandam *et al* (1974) extracted, isolated and purified the endogenous inducing compound from a *S. aureus* strain and characterised it as a peptidoglycan having a composition consistent with it being a cell wall precursor. The peptidoglycan induced β-lactamase activity when added exogenously to the bacterial culture. From this work, Sachithanandam *et al* hypothesized that β-lactamases are related to transpepidases involved in cell wall synthesis and that the peptidoglycan induces both the transpeptidases and the β-lactamases. This hypothesis is supported by several pieces of evidence. Saz and Lowery (1964 and 1965) showed that low levels of completely synthetic, homogenous cyclic peptides (chemically unrelated to penicillins or cephalosporins) induced β-lactamase in *B. cereus* and *S. aureus* S-10 to levels similar to those reached by induction with benzylpenicillin and in further experiments, found that linear peptides also acted as inducers of β-lactamase activity. Kozarich and Strominger (1978) purified a membrane enzyme to homogeneity and found it had both β-lactamase and transpeptidase activities. Ghuysen (1977) suggested that the active sites of β-lactamases and transpeptidases must be

similar and Yocum *et al*, (1979) showed the D-ala-D-ala-carboxypeptidases and β-lactamases from *B. subtilis* and *B. stearothermophilus* have the same unique amino acid sequence containing a serine residue which is irreversibly acylated by benzylpenicillin, but that only for β-lactamases is hydrolytic deacylation an enzyme-catalyzed process.

All the evidence also supports the hypothesis that penicillins are analogues of terminal D-ala-D-ala cell wall precursors which are substrates for transpeptidases (Tipper and Strominger, 1965) and the theory that β-lactamases evolved by duplication of the locus that specifies transpeptidase synthesis, followed by mutational modifications (Pollock, 1971).

The β-lactamase from *P. leiognathi* 206 was not induced by any of the β-lactams tested (see Section D, 2.2) even when present in high concentrations (up to 1 mg/ml) and hence it was concluded that the enzyme is constitutive. The peak of activity observed in late lag/early exponential phase (although not true induction) could perhaps be related to the early switch on of transpeptidase-specifying genes.

4. CELLULAR LOCATION OF β-LACTAMASE

The β-lactamases of Gram-positive bacteria are usually liberated into the extermal medium of the cells as extracellular enzymes. The Gram-negative β-lactamases remain cell-bound and are located between the outer membrane and the cytoplasmic membrane. Smith and Wyatt (1974) demonstrated that β-lactamases with molecular weights (MWs) not greater than 24,000 are released from the cell by osmotic shock techniques which temporarily damage the outer membrane. Enzymes with MWs greater than 30,000 are not released by osmotically shocking the cells, but require disintegration of the cell wall to liberate them.

As expected, the β-lactamase from *P. leiognathi* 206 was not an extracellular enzyme, but remained cell-bound. The β-lactamase was released by the osmotic shock method and

hence is termed a 'periplasmic' enzyme. The β-lactamases from most other strains of *Photobacterium* were also released by osmotic shock methods (see Section D, 4.2a), suggesting that these enzymes have MWs of 24,000 or less. In only 5 strains, three *P. phosphoreum* and two *P. leiognathi*, did the β-lactamases require sonic disintegration to release them from the cells, as they were not released by osmotic shock. It is therefore, most likely that the enzymes of the latter strains have MWs of greater than 30,000.

5. THE PERMEABILITY BARRIER TO β-LACTAMS

The outer membrane of most gram-negative bacteria acts as a selectively permeable barrier to different β-lactams, limiting the access of the β-lactamase to the substrate, which is termed crypticity of the enzyme. Most Gram-negative bacteria have β-lactamases which are highly cryptic to β-lactams although in some species transmembrane diffusion of cephalosporins, in particular cephaloridine, does occur (Curtis *et al*, 1979; Cornelis and Abraham, 1975). Only three Gram-negative strains have been reported to have no permeability barrier to β-lactam compounds (Sykes and Percival, 1978).

Initial crypticity experiments with the β-lactamase from *P. leiognathi* 206 showed that it was non-cryptic for benzylpenicillin, hence the study of the permeability of the outer membrane was extended to other β-lactam substrates and other strains of *Photobacterium* species. The accessibility to cephaloridine could not be determined in the strains of *P. leiognathi*, *P. angustum* or *P. phosphoreum*, as the β-lactamses produced by these species do not hydrolyse cephalosporins (see Table XVI) profiles were determined using crude enzyme preparation). It was found (see Table XIII) that there was no permeability barrier to benzylpenicillin or ampicillin in any of the strains examined, although a very slight barrier to carbenicillin did exist in most strains. The highest crypticity factor observed for carbenicillin was 3, in *P. phosphoreum* 674, but is of doubtful significance as it is very small compared to the crypticity of β-lactamases from

other Gram-negative species. For example, *E. coli* K12 and *Ps. aeruginosa* have crypticity factors for carbenicillin of 126 and 60 respectively and it is common from Gram-negative β-lactamases to have crypticity factors of 100 to 500 for the substrate benzylpenicillin (Richmond and Sykes, 1973). The lack of a permeability barrier to penicillins in *Photobacterium* strains is an unusual feature for Gram-negative bacteria.

It was observed (see Table XIII, Section D, 2.3a) that in several strains the β-lactamase activity after cell disintegration was less than the activity present in the intact cells, hence resulting in crypticity factors of 1.0. Possible explanations for the loss of β-lactamase activity are that sonication of the cells is releasing protease (s) which inactivate or partially destroy the β-lactamase (hence changing the enzyme's specific activity) or that disruption of the bacterial cell wall allows vesicles to form, within which a proportion of the β-lactamase is enclosed and therefore unavailable to the substrate. However, for any one strain where loss of β-lactamase is evident, such loss is not uniform against all substrates. For example, following cell discruption of *P. angustum* 159 (see Table XIII), the β-lactamase shows 25% less activity on ampicillin, but 70% less activity on benzylpenicillin and slightly more activity on carbenicillin. This substrate-related differential activity of the β-lactamase appears to rule out explanations for the loss of enzyme activity involving destruction of a proportion of the β-lactamase or of the β-lactamase being inaccessible, as it would be expected that in such situations, the decrease in activity would apply to all substrates.

6. CLASSIFICATION OF THE β-LACTAMASE FROM *P. leiognathi* 206

The Richmond and Sykes Class II group of enzymes are predominantly active on penicillins and are characteristically sensitive to inhibition by cloxacillin, but not inhibited by parachloromercuribenzoate (pCMB) (Richmond and Sykes, 1973).

Resistance to inhibition by pCMB, an inhibitor which reacts with free thiol groups, does not exclude the possibility of cysteine residues being present at or near the active site of the β-lactamase, but it does suggest that, if present, the residues are masked or buried deep within the molecule. From the substrate profile for the *P. leiognathi* 206 β-lactamase (see Table XIV, Section D, 3.1) it is seen that the enzyme hydrolyses only penicillin substrates, with no activity demonstrated against cephalosporins, not even nitrocefin which is generally considered to be extremely β-lactamase -labile. (O'Callaghan *et al*, 1972). However, non-utilization of more cephalosporins than the number tested would need to be demonstrated, before it could be said that the β-lactamase is an exclusive penicillinase. The β-lactamase was found to be sensitive to inhibition by cloxacillin but was not inhibited by pCMB. From this information it was concluded that the β-lactamase from *P. leiognathi* 206 belongs to Class II of the Richmond and Sykes classification system.

Since there is strong evidence for β-lactamases being related to transpeptidases (see Section E, 3) it is suggested that similar to such enzymes, all β-lactamases have an essential serine present at or very near the active site. Presence or absence of the serine residue can be determined by sensitivity to phenylmethylsulfonyl fluoride (PMSF), an inhibitor which reacts with available serines. The β-lactamase from *P. leiognathi* 206 was inhibited by PMSF to an apparent maximum of 30% under experimental conditions of 30°C and pH 7.0 (standard iodometric assay conditions). The inhibition maximum is only apparent, as under the conditions used it is possible that the inhibitor, PMSF, was inactivated (see Section D, 3.2).

7. COMPARISON OF THE β-LACTAMSES OF *Photobacterium* STRAINS

To date, no β-lactamase has been described which demonstrates exclusive activity on either penicillins or cephalosporins, although hydrolysis of one of the β-lactam groups may be extremely limited. The chromogenic cephalosporin, nitrocefin, is considered extremely β-lactamase-labile (O'Callaghan *et al*, 1972). Sufficiently sensitive to indicate β-lactamase presence in strains previously thought not to produce the enzyme (Matthew *et al*, 1975) and therefore it is probable that if a β-lactamase was active on cephalosporins at all, it would hydrolyse nitrocefin. Matthew *et al*, (1975) and Matthew and Harris (1976) claim that nitrocefin can be used for screening all bactdrial species for β- lactamases.

Using iso-electric focusing, Matthew and Harris (1976) conducted an extensive survey of chromosomally-mediated β-lactamases from 242 strains of 5 Gram-positive and 16 Gram-negative genera, and they concluded that β-lactamases are of universal occurrence and are not only genus specific, but also species specfic. From these conclusions, Matthew and Harris suggested that iso-electric points of chromosomal β-lactamases could be used as a bacterial characteristic for taxonomic separation of strains.

Iso-electric focusing of β-lactamases often results in a pattern of bands, consisting of one main band and one or more satellite bands (Matthew *et al*, 1975; Matthew and Harris, 1976; Brive *et al*, 1977; Olsson *et al*, 1977; Labia *et al*, 1979). The current explanations of this microheterogeneity are that there is more than one β-lactamase per cell arising from different genetic origins (for example, β-lactamases specified by chromosomal and R-factor genes, Matthew *et al*, 1975) or that the bands are stable conformers of one-gene product, possibly arising from protease action in the cells. The latter suggestion is supported by evidence that the main and satellite bands for some β-lactamases are antigenically the same (Matthew *et al*, 1975).

Microheterogeneity can arise as an artefact of the iso-electric focusing technique (Carlström and Vesterberg, 1967; Frater, 1970; Illingworth, 1972); however, Matthew *et al*, (1975) showed that identical patterns resulted when samples were loaded at different ends of the focusing gel and onto different concentrations of ampholine. Matthew *et al* also claim that when enzymes were purified on QAE-sephadex (Ross and Boulton, 1973) their iso-electric focusing patterns differed from those of crude intracellular enzyme preparations only by the relative intensities of some of the satellite bands. However subsequently, Labia *et al*, (1979) has shown that crude enzyme preparations focused as multiple-banded patterns, but when purified the β-lactamases are free from satellite bands. Brive *et al*, (1977) found that when crude enzyme preparation was held at room temperature or 37°C for 24 to 72 hours prior to iso-electric focusing, the β-lactamase focused as multiple bands, while if the preparations were kept at -20°C or 4°C no satellite bands appeared after focusing. They concluded that at temperatures greater than 4°C degradation of the β-lactamases occurred, with consequent loss of the activity which focused as a main band and subsequent appearance of satellite bands. At present though, no conclusive reason why an apparently single β-lactamase focuses during iso-electric focusing as a main band accompanied by satellite bands, has been reached.

Comparing the β-lactamases from *Photobacterium* by their substrate profiles (see Table XVI, Section D, 4.1) it is seen that all βlactamases from the species *P. leiognathi*, *P. angustum* and *P. phosphoreum* are predominantly active on penicillins and do not hydrolyse cephalexin or cephaloridine. Of the β-lactamases from these three species it is notable that the β-lactamase from *P. phosphoreum* 174 is the only one to show definite activity against the chromogenic cephalosporin nitrocefin; those from the other strains only hydrolysed this compound very slightly or not at all. It would seem therefore, that the proposed use of nitrocefin to screen all bacteria for β-lactamase activity (Matthew *et al*, 1975; Matthew and Harris, 1976) would certainly not be

successful with the β-lactamases from *Photobacterium*. It is interesting to note (Table XVI), that the β-lactamases from the two species, *P. fischeri* and *P. logei*, have very different substrate profiles from the other three *Photobacterium* species, in that the former enzymes are capable of hydrolyzing cephalexin and cephaloridine to some extent and are very active against nitrocefin.

After iso-electric focusing all the enzymes, it was found that the β-lactamases from the genus *Photobacterium* have iso-electric points (pIs) over the range pH 5.1 to pH 6.9. It is seen that the β-lactamases from several strains of different species appear to have the same pI (see Fig. 13) and hence the pI of the β-lactamase could not be used alone, as a taxonomic characteristic suggested by Matthew and Harris (1976) because it would not necessarily separate the species of the *Photobacterium* genus. The β-lactamases from 5 strains of *P. leiognathi*, 2 strains of *P. phosphoreum* and 4 strains of *P. fischeri* each showed microheterogeneity in their iso-electric focusing pattern, in spite of keeping the preparation at 4°C during laboratory handling and at -20°C for storage, as recommended by Brive *et al* (1977). The reason for this is unknown but the amount obtained in this study is very small compared to that observed by other workers. It is worth noting though, that some strains of *Photobacterium* are psychrophiles, therefore a temperature of 4°C may not inactivate protease action in the crude preparation and it is also possible that cell wall synthesis enzymes such as carboxypeptidases are appearing as satellite bands with β-lactamase activity. The way to clarify what is happening in this situation, would be to purify the β-lactamases and then repeat the iso-electric focusing experiments.

When the substrate profiles (Table XVI, Section D, 4.1) of the β-lactamases are considered in conjunction with their pI values (Fig. 13, Section D, 4.2c), some interesting points emerge. The pI of a β-lactamase gives no indication of its biochemical properties in that, β-lactamases may have

very different pIs and similar substrate profiles (Sykes and Matthew, 1976) and this is exemplified in this study. For example, *P. phosphoreum* 174 has a unique iso-electric focusing pattern but a substrate profile very similar to *P. phosporeum* 617; also *P. leiognathi* 206, *P. leiognathi* 632 and *P. phosphoreum* 617 also possess similar substrate profiles but β-lactamases show a variety of pIs. The converse, of β-lactamases having the same pI and different substrate profiles, appears to be frequently true. For example, the iso-electric focusing pattern of the *P. leiognathi* 206 β-lactamase is the same as that for the enzyme from *P. fischeri* 768 yet *P. leiognathi* 206 does not hydrolyze cephalosporins at all, which the *P. fischeri* strain does quite readily. *P. fischeri* 31 has a pI the same as the main band for the *P. leiognathi* 206 β-lactamase while the former hydrolyzes two penicillins, methicillin and cloxacillin, which inhibit the *P. leiognathi* 206 enzyme of the latter. However, there are some instances where the substrate profiles and pIs do seem to correlate quite well. The β-lactamases from the *P. angustum* species all focus at the same pI and all demonstrate very similar hydrolytic activities, which statement also holds for the β-lactamases from *P. logei*. All *P. logei* strains and *P. fischeri* strains except *P. fischeri* 130, have β-lactamases focusing at pH 6.1 and all hydrolyze cephalosporins to some extent.

It is interesting to note that in some cases strains isolated from the same geographical region have the same pIs (for example, strains of *P. angustum* and also *P. logei*) but for other strains this does not necessarily apply. For example, *P. leiognathi* 632 and 633 were isolated from fish in the same area yet have pIs of 6.2 and 5.1 respectively.

8. THE GENETIC DETERMINATION OF β-LACTAMASES

The genes specifying β-lactamases may be plasmid-borne or chromosomally located. Most *Photobacterium* strains contain plasmids and hence all genetic experiments were performed in an attempt to determine the genetic location of the $\beta 1a^+$ genes in *Photobacterium*. Under curing conditions, with all curing agents employed, it was found that all *P. leiognathi* 206 cells retained their β-lactamase activity (see Tables XXI & XXII, Section D, 5.1). It was also found that even all clones of mitomycin C-treated *P. leiognathi* 206 bacteria still exhibited wild-type levels of β-lactamase activity and furthermore, the plasmid was still present in these cells although mitomycin C is considered the most effective agent for attaining plasmid-free bacteria (Novick, 1961). From these results, it was concluded that the plasmid present in *P. leiognathi* 206 was not curable, which is a characteristic reported for some plasmids (Novick, 1969; Sebald, 1975). The fact that plasmid curing attempts were unsuccessful is unfortunate because it means that no firm decision can be made about the location of the $\beta 1a^+$ genes in *P. leiognathi* 206, as the two possibilities of the genes being either chromosomal or of plasmid origin are both still equally probable.

In the conjugation experiments, all *Photobacterium* strains used as donors were known to be carrying at least one plasmid and all the plasmids except that present in *P. angustum* 159, were sufficiently large (B. Dymock and K. SMith, this laboratory) for the plasmid to carry transfer (tra^+) genes (Rubens *et al*, 1976) as well as the β-lactamase information. However, all attempts to transfer β-lactamase activity from *Photobacterium* strains by conjugation methods were unsuccessful, while under the same experimental conditions, the *Pseudomonas* plasmid R68.45, an R-factor commonly used for transfer of genes, chromosome mobilization and genetic engineering (Olsen and Shirley, 1973; Beringer, 1974; Chakrabarty, 1976; Towner and Vivian, 1976; Haas and Holloway, 1976; Jacob *et al*, 1976; Stanisich and Bennet, 1976; Alexander and Jollick, 1977;

Denarie *et al*, 1977) was transferred to both *Photobacterium* hosts, strains P_9 and A_{43}, and to the restrictionless host *E. coli* PB1395. It seems likely therefore, that if the β-lactamases in *Photobacterium* strains were plasmid-mediated, transfer of the enzyme activity to the new hosts would have occurred. The possibility of interference with transfer, expression or replication of the donor βla^+ genes by the plasmid still present in the *Photobacterium* βla^- mutants, P_9Sm^r and A_{43}, did not appear to be the reason for unsuccessful transfer of βla^+ genes as the R68.45 plasmid was successfully transferred to both these recipients. However, there remains the possibility that the βla^+ genes are located on the plasmid and the plasmid is a non-conjugative type similar to the non-transmissable plasmids described by Anderson (1968) and others.

The most conclusive way to show whether genes are chromosomal in location is by demonstrating linkage with other chromosomal genes, but this sort of study can only be undertaken with bacterial species for which genetic recombination systems have been developed in the laboratory. As such a system is not in operation for *Photobacterium* species, transfer of βla^+ genes by transformation was attempted. However, all transformation attempts were unsuccessful, even when involving transfer of the $R_{68,45}$ plasmid. The failure of the experiments was very disappointing; the reason for the failures is unknown, but the most likely explanations probably relate to the *E. coli* cells which were used as the recipient host. Either they were not competent or perhaps they possess a new as yet unidentified restriction system. However, the competence and transformation methods used are known to be successful with *E. coli* PB_{1395} (D. Lane, Department of Cell Biology, University of Auckland, pers. comm.) and the strain will accept plasmid $R_{68.45}$ from *Photobacterium* by conjugation.

SECTION F
CONCLUSION

The β-lactamase from *P. leiognathi* 206 is constitutive, as are many Gram-negative β-lactamses, although the specific activity of the enzyme is increased during change of growth rate phases. The *P. leiognathi* β-lactamase is not liberated into the external environment as extra-cellular enzyme but remains cell-associated. The enzyme is termed a 'periplasmic' β-lactamase as it could be released from the cell by osmotic shock techniques suggesting that it has a molecular weight of less than 24,000.

The β-lactamase from *P. leiognathi* 206 is not dependent on the presence of zinc ion for hydrolytic activity. The enzyme is predominantly active on penicillins and does not appear to hydrolyze cephalosporins at all. It is inhibited by cloxacillin but resistant to inhibition by pCMB. These characteristics therefore suggest that the enzyme belongs to the Richmond and Sykes Class II group of β-lactamases. From the substrate profile information about sample strains from all species of *Photobacterium* it seems likely that the β-lactamases from *P. leiognathi*, *P. angustum* and *P. phosphoreum* all belong to the Class II β-lactamases, as not one demonstrated hydrolytic activity on cephalosporin substrates, except slight hydrolysis of nitrocefin by strain *P. phosphoreum* 174. Somewhat unexpectedly, the two species, *P. fischeri* and *P. logei*, demonstrated very different substrate profiles to the other three species in that all strains tested hydrolyze cephalosporins. From the substrate profiles of these strains it seems the β-lactamases would more likely belong to Class IV or V, but inhibition studies need to be performed before the β-lactamases can be classified positively. The iso-electric focusing studies showed that the pIs of the *Photobacterium* β-lactamases are not species specific and hence, could not be differentiated from each other by this criteria. In addition, there is no correlation between the enzymic properties of substrate profile and pI value, except in *P. angustum* and *P. Logei* strains.

An unusual feature of the *Photobacterium* outer membrane is that it does not appear to act as a permeability barrier for the entry of penicillins. The β-lactamases of *Photobacterium* strains are not cryptic therefore, but have free access to all substrates in intact cells. In most Gram-negative bacteria the β-lactamase is cryptic, with only three species found to have no permeability barrier to β-lactams.

It seems most probable that the β-lactamases of *Photobacterium* strains are chomosonally-mediated since all strains exhibit β-lactamase activity (K. Smith, this laboratory) but not all strains carry plasmid DNA (B. Dymock and K. Smith, this laboratory) and hence, in these strains at least, the β-lactamase must be specified by genes on the chromosome. Further evidence for a chromosomal location of the βla^+ genes comes from the fact that all attempts to transfer the enzyme activity from strains of *P. leiognathi*, *P. angustum* and *P. phosphoreum* known to be carrying plasmids, were unsuccessful. Also of note is that, to date, there has only been one Class II β-lactamase reported to be plasmid-mediated. By correlation of plasmid presence or absence in *Photobacterium* strains to substrate profiles and pIs, it is seen that in some cases the same β-lactamase appears to be produced by both strains which carry plasmid DNA and those which have no plasmid. For example, the β-lactamases produced by all five strains of *P. angustum* have a pI of pH 6.6 and very similar substrate profiles (therefore suggesting that the enzymes are very similar) but only two of the strains carry plasmids. It seems unlikely that such similar β-lactamases would have different modes of genetic determination.

In this study, the β-lactamases from the strains of *P. fischeri* and *P. logei* demonstrated rather different biochemical properties to the other three *Photobacterium* strains. If the genes which specify the β-lactamases in *P. fischeri* and *P. logei* are chromosomally located, the findings in this work would appear to support the proposal of Baumann, Baumann, Bang and Woolkalis (Preprint, 1980, pers. comm.) that these two species do not belong in the genus *Photobacterium*.

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