ANTIBIOTIC RESISTANCE OF

FLAVOBACTERIUM AND RELATED GENERA

by

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This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and to the best of my knowledge contains no copy or paraphrase of any material published or written by any other person, except where due reference is made in the text of the thesis.

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SUMMARY

The genus Flavobacterium is characterized by high levels of resistance to a wide range of antibiotics. Clinical laboratories may experience difficulty in detecting this resistance, the underlying mechanisms of which are unknown. Genetically the genus is so diverse that it may eventually be subdivided into at least four genera. Furthermore within the genus some species are genetically heterogeneous and warrant subdivision into further species. To date these taxonomic reforms have not been possible because of a lack of phenotypic markers which can be correlated with the genetic differences. An additional taxonomic problem which requires clarification is the relationship of Flavobacterium to other genera, particularly Cytophaga and Bacteroides.

In this study eighty nine Flavobacterium study strains and 43 reference strains were tested to investigate the susceptibility of the genus to antibiotics. Cell-free beta-lactamase preparations of selected strains were used to determine the substrate profiles, inhibitor profiles, inducibility and isoelectric points of the beta-lactamases of the genus and some related organisms. Preliminary data were obtained about the roles of beta-lactamases and outer membrane permeability in resistance to beta-lactam antibiotics.

Although two standardised conventional antibiotic susceptibility testing techniques showed poor correlation the antibiotic resistance patterns were nevertheless specific

for certain species and for groups of flavobacteria, indicating that antibiotic resistance is of taxonomic utility for this genus. Flavobacterium aquatile, F. breve, F. meningosepticum and F. odoratum could be readily delineated by their susceptibility patterns. Similarities in the antibiograms and beta-lactamases of F. multivorum, F. spiritivorum and F. thalpophilum was further evidence of the relatedness of these species and supported suggestions that they be recognised as a separate genus.

The beta-lactamases of the genus Flavobacterium are characteristically chromosomally mediated beta-lactamases of Richmond and Sykes Class IV. Many Flavobacterium beta-lactamases were unusual in exhibiting activity against the new beta-lactamase resistant antibiotics cefoxitin and cefotaxime, and occasionally imipenem. This suggested that these enzymes are Class IV oxyiminocephalosporinases. This finding is of taxonomic significance. It is also of clinical and pharmaceutical significance in that it identifies Flavobacterium as a genus capable of inactivating the latest beta-lactam antibiotics and therefore requiring close examination by research chemists if they are to succeed in producing antibiotics which can evade the resistance mechanisms of pathogenic bacteria.

The heterogeneity of beta-lactamases within F. breve, F. gleum, F. meningosepticum, F. odoratum and Group IIb suggested that the beta-lactamases of the genus should be further investigated to delineate new taxa as they represent new phenotypic markers of the type needed for the taxonomic revision of the genus.

1. LITERATURE REVIEW

a. Introduction

In recent years many new beta-lactam antibiotics have been released for therapeutic use (Table 1) and many more are in the developmental stage. A number of these antibiotics are superior to previous agents in potency, resistance to inactivation by beta-lactamases, and in pharmacokinetics (Winston et al., 1984; Dixson and Andrew, 1985). The market has become inundated with beta-lactam antibiotics, especially the cephalosporins, and there is much confusion as to which agents are most appropriate for particular situations (Selwyn, 1980). The release of even more of these antibiotics will exacerbate the confusion to the point where even infectious disease physicians, who are specially trained in the use of antibiotics, will be hard pressed to keep current in this expanding field (Avorn et al., 1987). Notwithstanding this intellectual dilemma the spectrum of activity of the newer beta-lactam antibiotics has been so significantly expanded that there is enthusiastic speculation about their future role. Looking to the future it appears that we may be on the brink of a new golden age of antibiotic therapy in which there will be a non-toxic beta-lactam antibiotic for every known bacterial infection (Richmond, 1981; Thomson and Ward, 1986). Most importantly this would mean that the therapy of severe infections will no longer depend on toxic agents such as the aminoglycosides and vancomycin.

At present such optimism needs to be tempered with caution as there are reports documenting the development of resistance by certain species during therapy with the newer beta-lactam

TABLE 1

Representative New Beta-lactam Antibiotics

Second Generation Cephalosporins
Cefamandole
Cefoxitin (*)
Cefuroxime
Cefotetan (*)
Third Generation Cephalosporins
Moxalactam (**)
Cefotaxime
Cefoperazone
Ceftizoxime
Ceftriaxone
Ceftazidime
Cefsulodin
Expanded Spectrum Penicillins
Mezlocillin
Azlocillin
Piperacillin
Monobactams
Aztreonam
Carbapenems
Imipenem
Clavams
Clavulanic acid

(*) also classified as methoxycephems or cephamycins

(**) also classified as an oxacephem

antibiotics (Sanders and Sanders, 1985; Tipper, 1985). Furthermore there are reports of the failure of routine susceptibility tests to predict when resistance is likely to occur (Sanders, 1984b). There have been unexpected treatment failures with these agents and as a consequence there are considerable reservations about their role as monotherapy for serious infections. It is therefore important that the resistance problems be overcome if the full potential of the new beta-lactam antibiotics is to be realised.

So far resistance to these drugs involves two principal mechanisms - beta-lactamase mediated drug inactivation and reduced permeability of the bacterial outer cell membrane to the drugs (Bush et al., 1985; Werner et al., 1985; Sanders and Sanders, 1986a; Bakken et al., 1987).

The attainment of a new era of safe beta-lactam therapy therefore seems to await two developments - (1) further increases in the spectrum of antibacterial activity of these drugs, and (2) resolution of the resistance problems. Many researchers in industry, acadaemia and medicine have responded to this challenge and currently seek to:

1. elucidate the mechanisms of resistance to the new beta-lactams (Godfrey and Bryan, 1984; Sanders, 1984b; Murakami and Yoshida, 1985; Vu and Nikaido, 1985; Livermore et al., 1986),
2. better define the therapeutic roles of the new drugs to minimize the development of resistance (Sanders and Sanders, 1985; Tipper, 1985),
3. produce new antibiotics which evade the current

resistance problems (Then and Anghern, 1985; Tipper, 1985; Sanders, 1987), and

4. develop convenient antibiotic susceptibility tests which predict when resistance is likely to occur (Sanders and Sanders, 1979; Thomson et al., 1984; Jorgensen, 1985; Thomson and Ward, 1986).

This thesis reports a study of some aspects of the beta-lactamases and antibiotic resistance of the genus Flavobacterium and related organisms. The reasons for the study are as follows.

1. Members of the genus Flavobacterium are resistant to therapeutic levels of many antibiotics including newer beta-lactam antibiotics (Igari et al., 1983; Holmes et al., 1984a; Rubin et al., 1985; Strandberg et al., 1983; Burnakis et al., 1986). Their reported resistance to, and beta-lactamase-mediated hydrolysis of, both imipenem and temocillin is particularly striking. Imipenem, a carbapenem, is one of the most potent broad-spectrum, beta-lactamase-stable antibiotics available (Freimer et al., 1985) and is hydrolysed by the beta-lactamases of very few bacteria,one of which is F. odoratum (Sato et al., 1985). Temocillin is even more resistant to hydrolysis by beta-lactamases. It is the first penicillin with high resistance to Gram-negative beta-lactamases (Slocombe et al., 1981) and so far the only beta-lactamase known to hydrolyse it is that of Flavobacterium strain 80 (Chen and Williams, 1982, 1985). To my knowledge there is only one other report of Flavobacterium beta-

lactamases in the scientific literature. Raimondi et al. (1986) report that seven strains of Flavobacterium meningosepticum have three different types of beta-lactamase. The activity of these beta-lactamases is also unusual in being capable of hydrolysing the relatively stable 7-beta-aminothiazole cephalosporins.

The genus Flavobacterium thus contains organisms of very unusual resistance to beta-lactam antibiotics and, in the case of Flavobacterium 80, appears to contain a unique beta-lactamase. These studies, and the findings of Kono et al. (1980) that F. odoratum N299B contains a plasmid coding for resistance to ampicillin, carbenicillin and erythromycin, and of Nolte and Sussmuth (1987) of chloramphenicol acetyltransferase in Flavobacterium CB60, appear to be the only reports of resistance mechanisms being identified in Flavobacterium.

In view of the broad multi-resistance of flavobacteria and the high level of current research into antibiotic resistance it seems surprising that the genus remains a virtual terra incognita. There is certainly much scope, on scientific grounds alone, for a systematic study of antibiotic resistance in Flavobacterium. In view of the interesting beta-lactamases just mentioned a survey of the beta-lactamases of the genus would provide an opportune starting point.

2. There are also good clinical reasons for studying the beta-lactamases of Flavobacterium. Some flavobacteria are opportunistic pathogens of immunocompromised hosts (Holmes et

al., 1984a; Rubin et al., 1985). Today there are increasing numbers of immunocompromised patients arising from modern medical and surgical practices and from disease states such as acquired immune deficiency syndrome (AIDS) and cancer. The increasing susceptibility of patients to infection has focussed attention on the increasing numbers of opportunistic Gram-negative pathogens, particularly those like flavobacteria which are resistant to a wide range of antibiotics (Finland et al., 1959; Rogers, 1959; Feeley et al., 1975; Buckwold and Ronald, 1979; McGowan, 1983; Fainstein and Bodey, 1985; Klastersky, 1985; Weinstein, 1985; Young, 1985). In this context an investigation of antibiotic resistance mechanisms in Flavobacterium seems timely as it would complement other work relevant to the future of antibiotic therapy in immunocompromised patients.

3. The search for more reliable susceptibility testing procedures is a key issue (Sanders, 1984a). Some current routine testing procedures are unreliable for flavobacteria (Von Graevenitz, 1981; Winslow and Pankey, 1982; Johny et al., 1983). If testing discrepancies for flavobacteria can be correlated with resistance mechanisms (e.g. beta-lactamases) it may be possible to use this knowledge to improve the accuracy of current tests or to develop better tests.

4. In spite of recent advances in the taxonomy of Flavobacterium some aspects remain in an unsatisfactory state because:

(a) heterogeneity within the genus precludes the

allocation of many flavobacteria to discrete species, and

(b) the taxonomic relationships within the genus and with other genera are poorly understood (Holmes et al., 1984).

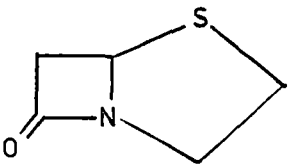
Information about the antibiotic resistance of the genus will generate new data some of which may assist in resolving its taxonomic problems.

To provide a background to this study it is necessary to review the literature of three complex fields of knowledge. The first of these is the mode of action and properties of beta-lactam antibiotics relevant to their interaction with Gram-negative bacteria. The second field is the range of mechanisms by which Gram-negative bacteria resist these drugs, with particular emphasis on beta-lactamases, and the final field is the clinical and taxonomic features of the genus Flavobacterium and its interactions with beta-lactam antibiotics.

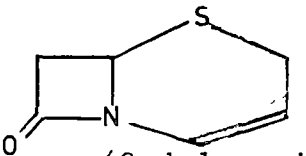
b. Beta-lactam Antibiotics

Antibiotics of the beta-lactam group are the most frequently prescribed antiinfective agents in the world (Selwyn, 1980; Richmond 1981; Bush and Sykes, 1986). They are used predominantly because they couple effectiveness with safety at a level not usually found in therapeutic agents (Jackson, 1979: Selwyn, 1980; Richmond, 1981). The beta-lactam ring, which occupies a central position in all antibiotics of this group is crucial to antibacterial activity. However it also conveys vulnerability to beta-lactamases which can inactivate the antibiotic by hydrolysis of the cyclic amide bond (Sykes and Matthew,1976; Richmond, 1981; Cullman, 1985) or by binding to it without subsequent hydrolysis (Yokota and Azuma, 1980; Then and Anghern, 1982; Sanders and Sanders, 1986b).

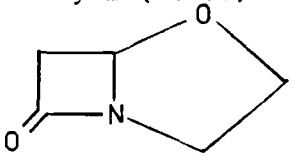
Since Fleming's original work on penicillin in 1928 (Fleming, 1929) a variety of naturally occurring and semisynthetic beta-lactam antibiotics have been produced (Table 1). Generally they have been defined and classified by trivial nomenclature (e.g. as penicillins, cephalosporins etc.) on the basis of the producing organism and a chemical feature of the compound (Brown, 1982). However a more recent classification is based on a defined parent skeleton (Fig. 1). In this classification penicillins and cephalosporins are regarded as derivatives of the penam and cephem nuclei respectively. Clavulanic acid is derived from the clavam nucleus, imipenem and olivanic acid are carbapenems and the other beta-lactams are based on the penem, oxacephem and



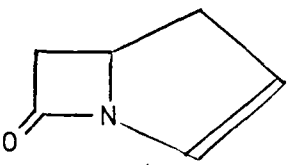
Penam (Penicillins)
4-Thia-1-azabicyclo(3.2.0)heptan-7-one



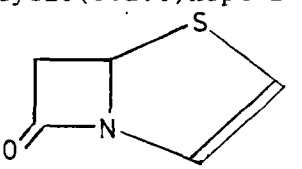
Cephem (Cephalosporins)
5-Thia-1-azabicyclo(4.2.0)oct-2-en-8-one



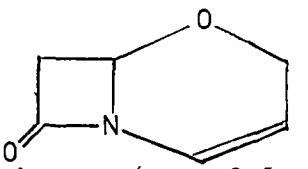
Clavam (e.g. Clavulanic Acid)
4-Oxa-1-azabicyclo(3.2.0)heptan-7-one



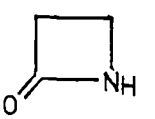
Carbapenem (e.g. Imipenem)
1-Azabicyclo(3.2.0)hept-2-en-7-one



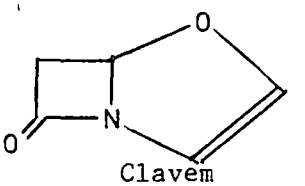
Penem (e.g. SCH 29482 and SCH 34343)
4-Thia-1-azabicyclo(3.2.0)hept-2-en-7-one



Oxacephem (e.g. Cefoxitin)
5-Oxa-1-azabicyclo(4.2.0)oct-2-en-8-one



Monobactam (e.g. Aztreonam)
Azetidin-2-one



Clavem
4-Oxa-azabicyclo(3.2.0)hept-2-en-7-one

Fig. 1. Beta-lactam Skeletons

monobactam ring systems (Brown and Reading, 1983). The nomenclature in this review is mainly trivial because the fully systematic nomenclature is complex and cumbersome (Fig. 1).

1. Penicillin Binding Proteins

The targets for beta-lactam antibiotics are the penicillin binding proteins (PBPs) which are located on the cytoplasmic membrane of the cell. The interaction of the drug with one or more of the PBPs determines the antibacterial effects of beta-lactam antibiotics. Drug potency and the nature of the effects the antibiotic has on the cell ultimately depend on PBP inhibition by acylation. To be effective against Gram-negative pathogens a beta-lactam antibiotic must reach the infective site in the host, penetrate the outer layers of the cell envelope, survive the action of periplasmic beta-lactamases and reach the PBPs in sufficient concentration to form the covalent complexes necessary for inhibition (Tomasz, 1986).

PBPs are quantitatively minor components of the plasma membrane. There are 1,000 to 10,000 PBPs per cell with copy numbers of individual PBPs sometimes as low as 20-30 molecules per cell. They have been most extensively studied in Escherichia coli, in which there are seven major groups of PBPs each having a different physiological function (Table 2). There is also an eighth major PBP (PBP 1C) of uncertain significance. PBPs are numbered in order of decreasing molecular size - a convention by which similarly

numbered PBPs from different organisms are not necessarily related.

In E. coli transpeptidase and transglycoslase activities have been detected in PBP groups 1, 2 and 3 whereas D,D-carboxypeptidase activities have been associated with PBP groups 4, 5 and 6. Most PBPs are thought to be penicillin-sensitive enzymes catalyzing terminal stages in the assembly of the peptidoglycan network of bacterial cell walls. They are probably located strategically to perform as a 'functionally integrated assembly line' (Tipper, 1985).

The selective toxicity of beta-lactam antibiotics for bacterial cells but not for mammalian cells is explained by the substrate analogue theory (Tipper and Strominger, 1965). This is based on the structural similarity between the beta-lactam ring and a transitional state conformation of the carboxy terminal D-alanyl-D-alanine residue in the natural substrate of these penicillin-sensitive enzymes. That is, PBPs which are essential for cell wall synthesis seem to mistakenly 'recognise' beta-lactam antibiotic molecules as their natural substrate(s). It is not certain if cell viability requires complete or only partial activity from any PBP groups (Tipper, 1985).

Selective inhibition of individual PBPs is associated with morphological changes arising from defects in cell wall synthesis. In E. coli cephaloridine and cefsulodin bind selectively to the hundreds of molecules in the multicomponent

TABLE 2

Properties of E. coli PBPs

PBP	Molecular weight (kd)	Molecules per cell *	Selective inhibitors	Functions
1A	92	200	Cephaloridine, cefsulodin	Essential for cylindrical cell wall synthesis
1B	90	250		
2	66	20	Amdinocillin, clavulanic acid, imipenem	Initiation of cylindrical wall growth at sites of septation
3	60	50	Cephalexin, cefuroxime, aztreonam, furazlocillin	Required for septum cross-wall synthesis
4	49	110		Peptidase activity on maturing peptidoglycan
5	42	1800		
6	40	600		

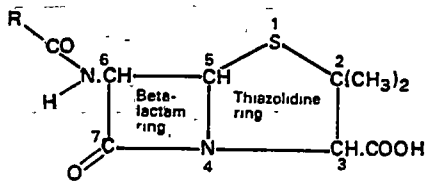
* The 'molecules per cell' are approximations based on measurement of bound radioactivity and assuming stoichiometric penicillin binding.

(From Tipper, 1985)

PBP1 complex, an event which can result in rapid and extensive killing of cells, degradation of cell wall material, and cell lysis (Tomasz,1986). Lysis, when it occurs in response to beta-lactam antibiotics, results from inappropriate activation of autolytic peptidoglycan hydrolases. This is associated with inhibition of PBP 1B (or 1A plus 1B) (Yousef et al., 1985). Amdinocillin, clavulanic acid, and imipenem have a high selectivity for the 20 or so molecules of PBP2 and convert bacilli to osmotically stable spherical cells which may lose viability without extensive cell wall degradation. Piperacillin, mezlocillin and aztreonam bind selectively mainly to the hundreds of PBP3 molecules to inhibit septation, leading to filamentation and limited lysis (Tomasz, 1986) but their activity is bactericidal. The low molecular weight and relatively abundant PBPs 4,5 and 6 are not lethal targets for beta-lactam antibiotics (Tipper, 1985).

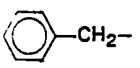
2. Penams

Penicillins are N-acylated derivatives of 6 beta-amino-penicillanic acid (6-APA), which is a derivative of the penam nucleus (Fig. 1). Fig. 2 and Table 3 list penicillins representing the different types of antibacterial activity of the group. Variations in the side chains of the molecules greatly influence properties such as antibacterial activity, resistance to beta-lactamases and amidases, acid stability, protein binding, absorption, distribution and elimination (Selwyn, 1980).

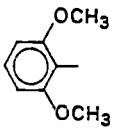


General formula
(with conventional numbering)

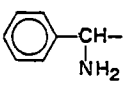
Side chain (R) of derivatives



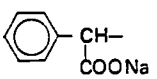
Penicillin G



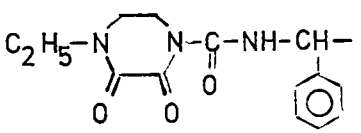
Methicillin (Resistant to staphylococcal beta-lactamases)



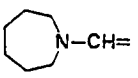
Ampicillin
(Broad spectrum)



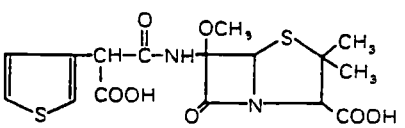
Carbenicillin
(Anti-Pseudomonas)



Piperacillin
(Extended spectrum)



Amdinocillin
(Amdino Penicillin)



Temocillin - complete molecule
(Resistant to Gram-negative beta-lactamases)

Fig. 2. Structures of Representatives of Different Classes of Penams.

TABLE 3

Classification of Penams by Antibacterial Spectrum

Natural Penicillins	Antipseudomonas Penicillins
Penicillin G	Carboxypenicillins
Penicillin V	Carbenicillin
Phenethicillin	Ticarcillin
	Indanyl carbenicillin
Penicillinase-Resistant	Carfecillin
Penicillins	Acylamino Penicillins
Methicillin	Azlocillin
Nafcillin	Mezlocillin
Isoxazolyl Penicillins	Piperacillin
Cloxacillin	Apalcillin
Dicloxacillin	
Flucloxacillin	Amdino Penicillins
Oxacillin	Amdinocillin
	Pivampicillin
Aminopenicillins	
Ampicillin	Gram-negative Beta-lactamase-
Amoxycillin	Resistant Penicillins
Bacampicillin	Temocillin
Cyclacillin	BRL 36650
Epicillin	
Hetacillin	
Pivampicillin	
Talampicillin	

Benzyl Penicillin

The first commercial penicillin was sodium benzyl penicillin, or penicillin G (Fig. 2), a natural fermentation product of *Penicillium chrysogenum*. Although it is primarily active against Gram-positive bacteria, *Neisseria gonorrhoeae*, *N. meningitidis*, spirochaetes, borreliae and some anaerobes it is relatively inactive against most Gram-negative bacteria and lacks beta-lactamase stability. The early emergence of beta-lactamase-producing strains of *Staphylococcus aureus*, which were present before benzyl penicillin was used therapeutically, led to widespread resistance in hospitals within five or six years of its introduction into common use (Richmond, 1981).

Methicillin and the Isoxazolyl Penicillins

At the beginning of 1959 Beecham Laboratories began to produce relatively large quantities of the 6-APA making possible the synthesis of many new antibiotics by simple acylation (Batchelor *et al.*, 1959). The first semi-synthetic penicillin, phenethicillin, was produced in 1959. It was soon followed by methicillin (2,6-dimethoxyphenyl penicillin) which was the first penicillin highly resistant to staphylococcal beta-lactamases (Rollinson *et al.*, 1960), and the isoxazolyl penicillins cloxacillin (Knudsen *et al.*, 1962), oxacillin and dicloxacillin (Gravenkemper *et al.*, 1965), also possessing resistance to staphylococcal beta-lactamases. Although methicillin and the isoxazolyl penicillins have little activity against Gram-negative bacteria they have high resistance to many Gram-negative beta-lactamases. This is not of therapeutic importance but is valuable in the

characterisation of beta-lactamases (Brown and Reading, 1983).

Amino Penicillins

Ampicillin, the first 'broad-spectrum' penicillin, was introduced in 1961. It is more active than benzyl penicillin against Gram-negative bacteria but less active against most Gram-positive bacteria (Rolinson and Stephens, 1961). Amoxycillin, a later analogue, is now more widely used because of better oral absorption and possibly more rapid bactericidal action. Both ampicillin and amoxycillin are vulnerable to a wide range of beta-lactamases and amidases (Selwyn, 1980).

Alpha-carboxy Penicillins

Carbenicillin, the first penicillin with activity against Pseudomonas aeruginosa, was introduced in 1967. It is active against certain Gram-negative bacteria which are resistant to ampicillin but in general is inherently less active than other penicillins against most bacteria (Knudsen et al., 1967). Ticarcillin, a more active analogue of carbenicillin, was introduced in 1973 (Rodriguez et al., 1973). Both carbenicillin and ticarcillin have very poor activity against Gram-positive bacteria.

Acylamino Penicillins

In 1981 three extended spectrum acylamino penicillins (acylureido-penicillins) were released - azlocillin, mezlocillin, and piperacillin. Apalcillin, a further acylamino penicillin, is still in the investigational stage. All are derivatives of ampicillin and are vulnerable to a wide range of beta-lactamases. Their spectrum of activity is

similar to the less active, but more stable, alpha-carboxy penicillins. Their superior activity may arise from their affinity to PBP3 and their ability to rapidly inhibit septation at low concentrations (Brown and Reading, 1983).

Amdino Penicillins

Amdinocillin (mecillinam), which was released in early 1985, has a spectrum limited essentially to a few Enterobacteriaceae. However its unusually selective binding to PBP2 (Spratt, 1975) produces osmotically stable spherical cells which lyse in appropriate environmental conditions. The spherical cells are especially vulnerable to beta-lactam antibiotics which bind to PBP1 and/or PBP3 (Spratt, 1977). This vulnerability to double beta-lactam therapy is thought to stem from synergistic antibacterial activity through a 'two-step-blockade' of cellular function (Eliopoulos, 1986).

Sanders et al. (1987) report an additional mechanism by which amdinocillin appears to potentiate the activity of other beta-lactam antibiotics through causing leakage of beta-lactamase through the outer cell membrane. The leakage of beta-lactamases from the periplasm depletes the cell's defences and permits beta-lactamase-vulnerable antibiotics to exert an antibacterial effect. This is a different mechanism from the 'two-step-blockade' and may arise from changes in outer membrane permeability as a result of PBP2 binding. The effectiveness of this mechanism has been demonstrated in animals. The success in animal trials suggests that it has considerable therapeutic potential for infections caused by those bacteria which develop resistance to the newer beta-

lactams and thus may be a major breakthrough in the quest for a new golden age of antibiotic therapy.

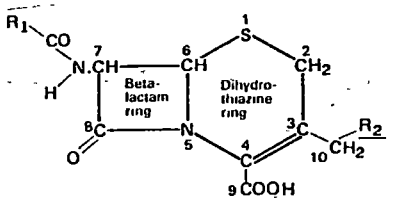
Future Penicillins

Temocillin, a 6-alpha methoxy penicillin, is the first penicillin to combine broad spectrum activity with stability to a broad range of bacterial beta-lactamases. The beta-lactamase stability is attributed to the addition of a 6-alpha methoxy group to the ticarcillin molecule (Fuchs et al., 1984). Temocillin's activity against many Gram-negative beta-lactamase-producing bacteria has been gained at the expense of activity against Gram-positive organisms and P. aeruginosa (Eliopoulos, 1986). As previously mentioned Flavobacterium strain 80 is the only organism known to possess a beta-lactamase which hydrolyses this antibiotic (Chen and Williams, 1982, 1985).

The novel 6-alpha formamido penicillin, BRL 36650, is an even more active, beta-lactamase stable, broad-spectrum penicillin. It is regarded as potentially useful in the treatment of infections by mutant bacteria which are derepressed beta-lactamase producers (Van Landuyt et al., 1986).

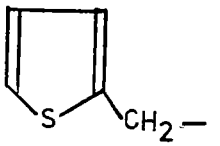
3. Cephems

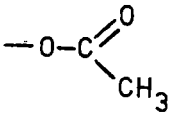
Cephalosporins are N-acylated derivatives of 7 beta-amino-cephalosporanic acid (7-ACA) which is derived from the cephem nucleus (Fig. 1). Fig. 3 lists a number of



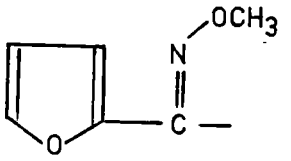
General formula
(with conventional numbering)

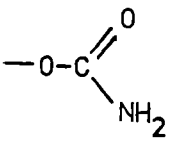
R1 side chain and R2 side chain of derivatives



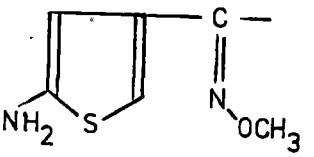


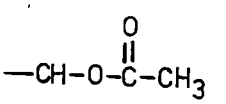
Cephalothin (first generation)



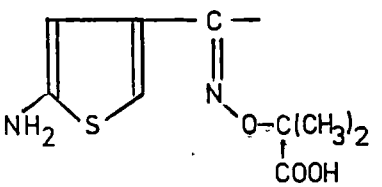


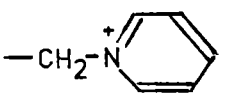
Cefuroxime (second generation)





Cefotaxime (third generation)





Ceftazidime (third generation)

Fig. 3. Structures of Cephems Representative of Different Classes of Antibacterial Activity.

cephalosporins representing the three 'generations'. The division of cephalosporins into generations is an arbitrary but convenient guide which reflects the advances made with the introduction of each group. First-generation cephalosporins (i.e. the early cephalosporins) characteristically possess moderate anti-Gram-positive activity but restricted anti-Gram-negative activity owing to vulnerability to beta-lactamases. The second-generation cephalosporins are more resistant to Gram-negative beta-lactamases and therefore more active against Enterobacteriaceae but tend to be less active against Gram-positive bacteria. Third-generation cephalosporins are the most active against Gram-negative bacteria and some agents have activity against P. aeruginosa. However their anti-Gram-positive activity varies from slight to moderate (Sanders and Sanders, 1986b).

The presence of two side chains (R1 and R2 in Fig. 3) allows an even greater range of semi-synthetic cephalosporins than is possible for the penicillins which have only one side chain. Alterations at the 7-alpha carbon position affect the the antibacterial spectrum and beta-lactamase stability of the antibiotics. Beta-lactamase stability is also conveyed by a methoxy group at C-7. Substitution of an oxygen atom for the usual sulphur atom at position 1 increases antibacterial potency and beta-lactamase stability. Side chain alterations at C-3 affect pharmacokinetics and, to a lesser extent, antibacterial potency (Sanders and Sanders, 1986b).

Emergence of Resistance During Therapy

In 1984 Sanders (1984c) reviewed the newer cephalosporins

and expressed concern about their impact on the hospital environment, where they are among the most frequently used antibiotics. Multiple beta-lactam resistance arising from use of the newer beta-lactam antibiotics appeared to be particularly associated with use of the newer cephalosporins and cephamycins. These drugs were identified as important inducers of chromosomally mediated beta-lactamases, a major factor in multiple beta-lactam resistance. In the same year reports appeared documenting the increasing incidence and impact of multiply beta-lactam resistant bacteria in Australian, American, and French hospitals. The increasing incidence of resistant organisms was associated with use of cefotaxime and moxalactam (Sanders and Sanders, 1986b).

Future Cephalosporins

Cefpirome and BMY 28142 have shown considerable promise during developmental stage testing. These aminothiazolyl-alpha-methoxamino-cephalosporins are highly potent against beta-lactamase-producing Gram-positive and Gram-negative bacteria including P. aeruginosa. Their potency is attributed to a very low affinity for beta-lactamases, a property which is thought to enhance access to PBPs (Phelps et al., 1986). Of particular interest is their apparent reduced potential to induce chromosomal beta-lactamases. These drugs have been proposed as the first members of the 'fourth generation' of cephalosporins. (Sanders and Sanders, 1986b).

4. 7 Alpha-methoxycephems and oxacephems

The cephamycins, or 7 alpha-methoxycephems, resemble

TABLE 4

Classification of Cephems, Methoxycephems(*)

and Oxacephems(**) by Generation

First Generation	Third Generation
Cefadroxyl	Lacking Enhanced Anti-
Cephazolin	Pseudomonas ActivitY
Cephalexin	Cefbuperazone (**)
Cephaloridine	Cefmenoxime
Cephalothin	Cefotaxime
Cephapirin	Ceftriaxone
	Moxalactam (*)
Second Generation	With Enhanced Anti-
Cefaclor	Pseudomonas Activity
Cefamandole	Cefoperazone
Cefmetazole (**)	Cefpimazole
Cefonicid	Cefpiramide
Ceforanide	Cefsulodin
Cefotetan (**)	Ceftazidime
Cefotiam	
Cefoxitin (**)	'Fourth Generation'
Cefuroxime	Cefpirome
	BMY 28142

second generation cephalosporins in spectrum of activity but have enhanced potency against anaerobic bacteria. This similarity makes it convenient to sometimes group both classes of antibiotics together as in Table 1. The trivial nomenclature 'cephamycin' denotes the bacterial source of cephamycins (from Streptomyces spp.) rather than moulds. Cephamycin molecules differ from cephalosporins in having different substituents at the C-3 and 7 alpha positions. Analogous to 7-ACA and the cephalosporins, cephamycin C is the source of synthetic cephamycin derivatives. Cefoxitin, the first commercial derivative (Kosmidis et al., 1973), possesses a 2-thienylmethyl group at the 7 beta position of cephamycin C. Cephamycins have a wider spectrum of activity and are more resistant to 'cephalosporinases' (beta-lactamases which preferentially hydrolyse cephalosporins) than first generation cephalosporins (Selwyn, 1980). This resistance is attributed to the methoxy group at the C-7 position (Brown and Reading, 1983).

If the sulphur atom of the cephem (Figs 1 and 3) is replaced with oxygen a series of analogues called oxacephems is produced. Moxalactam (latamoxef), the most notable, has activity resembling third generation or cephalosporins (Brown and Reading, 1983).

5. Carbapenems and penems

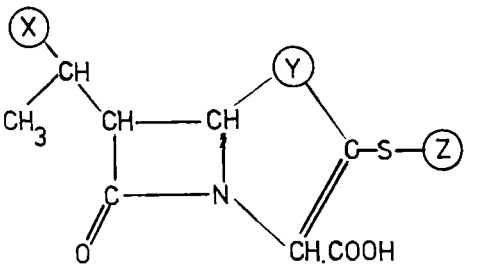
These are newly developed groups of beta-lactam antibiotics with chemical structures differing from penams, cephems, oxacephems, methoxycephems and monobactams (Figs 1,

4). The carbapenems are naturally occurring antibiotics (e.g. thienamycin is isolated from Streptomyces cattleya) whereas the penems are synthetic molecules. Both groups are very similar in possessing a very broad antibacterial spectrum, high resistance to beta-lactamases and susceptibility to hydrolysis by mammalian renal dipeptidases (Norrby, 1986). They exhibit high potency against most aerobic and anaerobic bacteria including multiply-resistant pathogens such as P. aeruginosa, Acinetobacter, B. fragilis and enterococci.

Imipenem (N-formimidoyl-thienamycin), the first commercial carbapenem, has outstanding potency - a feature which is attributed to the relatively small number of antibiotic molecules required to bind to the 20 or so PBP2 molecules per bacterial cell. This is in contrast to the hundreds of molecules of other beta-lactam antibiotics usually required to bind to PBPs 1 and/or 3 to be effective (Winston et al., 1984).

Beta-lactamase inhibition is a feature of all carbapenems. Beta-lactamase inhibition results from the high affinity of these antibiotics for many beta-lactamases leading to the formation of enzyme-inhibitor complexes which are not prone to hydrolysis (Brown and Reading, 1983).

Initially clinicians hoped that imipenem, and subsequent carbapenems and penems, would resolve current resistance problems (Winston et al., 1984). However treatment failures have occurred with imipenem during therapy of P. aeruginosa



General formula

Antibiotic	X	Y	Z
Thienamycin *	OH	C	CH2CH2NH3
Imipenem *	OH	C	CH2CH2CH=N
SCH 29482 **	OH	S	CH2CH3
SCH 34343 **	OH	S	CH2CH2OCONH2

* Carbapenem ** Penem

(From Norrby, 1986)

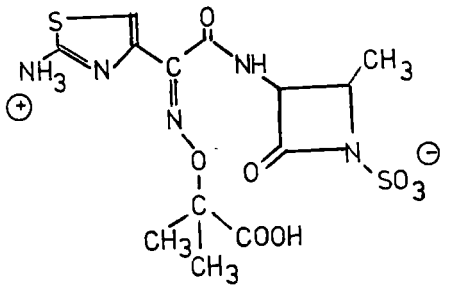
Fig. 4. Chemical structures of some carbapenems and penems.

infections (Winston et al., 1984; Quinn et al., 1986; Pedersen et al., 1987). Furthermore a number of organisms in the hospital environment, some of them opportunistic pathogens, are resistant without previous exposure and may be selected by imipenem therapy. These include F. meningosepticum, F. multivorum, Flavobacterium Group IIb (O'Donnell et al., 1982; Von Graevenitz and Bucher, 1982), F. odoratum (Von Graevenitz and Bucher, 1982; Sato et al., 1985), P. maltophilia, some strains of P. cepacia, P. pseudomallei, Corynebacterium spp. and Streptococcus faecium. An additional resistance problem is the ability of imipenem to induce beta-lactamase production in P. aeruginosa causing increased resistance to other beta-lactam antibiotics (Tausk et al., 1985).

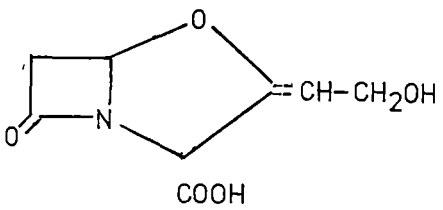
In most respects the penems are similar to the carbapenems. However P. aeruginosa and enterococci are usually resistant to the penems while anaerobic bacteria are usually more susceptible to the penems than to the carbapenems (Norrby, 1986). The vulnerability of all compounds of these groups to hydrolytic inactivation by hydrolysis by the mammalian renal dipeptidase, dihydropeptidase-I (DPH-I), is a major disadvantage which is currently overcome by combining the antibiotics with the DHP-I inhibitor cilastatin (Norrby, 1986).

5. Monobactams

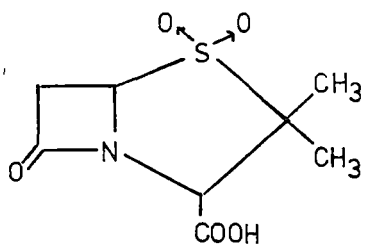
The monobactams are N-acyl derivatives of 3-amino-monobactamic acid (Brown and Reading, 1983). They have a simple monocyclic structure in contrast to the bicyclic



Aztreonam



Clavulanic acid



Sulbactam

Fig. 5. Structures of Aztreonam, Clavulanic Acid and Sulbactam

molecules of other beta-lactam antibiotics (Fig. 1).

Aztreonam, the first monobactam (Fig. 5), is relatively inactive against Gram-positive and anaerobic bacteria but has high potency against many aerobic Gram-negative bacteria, but not Flavobacterium, Acinetobacter, Achromobacter, Moraxella or Alcaligenes denitrificans and faecalis (Brogden and Heel, 1986). It is also resistant to hydrolysis by many beta-lactamases. Its narrower spectrum may be preferred to other newer beta-lactam antibiotics for specific therapy as there is less likelihood of emergence of resistant organisms and diarrhoea arising from disturbance to the natural gut flora. It is not a strong inducer of chromosomally mediated beta-lactamases. Instead it tends to either inhibit them or to act as a poor substrate (Sykes and Bonner, 1985; Williams, 1986). Resistant strains of Pseudomonas have emerged during therapy (Greenberg et al., 1985; Sanders and Sanders, 1985). The mechanism of resistance in P. aeruginosa and other Gram-negative bacteria possessing inducible chromosomal beta-lactamases is the controversial non-hydrolytic beta-lactamase-mediated inactivation (Sanders and Sanders, 1986c).

Carumonam is a new monobactam with greater activity than aztreonam against some strains of Klebsiella oxytoca, Citrobacter freundii, C. diversus, P. maltophilia, P. cepacia and Providencia stuartii. The greater activity against K. oxytoca is attributed to greater beta-lactamase stability (Fass and Helsel, 1985).

6. Beta-lactamase Inhibitors

While the production of beta-lactamase is only one of several mechanisms by which bacteria resist beta-lactam antibiotics it is, for the majority of clinically important bacteria, the major mechanism of resistance (Reading, 1981; Gould and Wise, 1986). The difficulty in developing antibiotics, particularly oral antibiotics, with stability to a wide range of beta-lactamases has stimulated efforts to produce enzyme inhibitors which can be used either as antibiotics or as co-drugs to protect labile antibiotics from inactivation. To date most beta-lactamase inhibitors are beta-lactam compounds (Gould and Wise, 1986).

The inhibitory activity is initiated by covalent binding to beta-lactamases to form long-lived complexes in which neither enzyme nor inhibitor have biological activity. The binding can be reversible or further binding can occur resulting in irreversible inhibition (suicide or progressive inhibitors) (Sanders, 1983; Gould and Wise, 1986).

The first clinically significant beta-lactamase inhibitor, clavulanic acid (Fig. 5), was discovered in 1976 (Howarth *et al.*, 1976). It has little intrinsic antibacterial activity except against penicillin-susceptible *N. gonorrhoeae* and *Legionella pneumophil a*. It is however a potent inhibitor of staphylococcal beta-lactamases and those of Richmond and Sykes Classes II-V (Gould and Wise, 1986). However it does not inhibit Class I beta-lactamases - enzymes which are associated with an increasing number of treatment failures (Sanders,

1983). Clavulanic acid is marketed in combination with amoxycillin and also in combination with ticarcillin.

It now seems that clavulanic acid was incompletely investigated before its release for clinical use. There are increasing reports of curious and unexpected effects arising from use of this compound. Sometimes it behaves as a potent co-drug with synergy arising from a mechanism other than beta-lactamase inhibition. This is apparent when clavulanic acid potentiates the activity of beta-lactam antibiotics against bacteria possessing class I beta-lactamases, the only beta-lactamases not inhibited by clavulanic acid (Clarke and Zemcov, 1984; Tausk and Stratton, 1986). It can also potentiate antibiotics which are resistant to hydrolysis by beta-lactamases. This effect is attributed to the ability of clavulanic acid to selectively alter outer membrane permeability (Bakken et al., 1987). An even more curious and potentially disturbing effect of clavulanic acid is its ability to induce the production of beta-lactamases and enhance bacterial resistance. All reports of this effect concern bacteria possessing inducible class I beta-lactamases (Minami et al., 1980; Crump and Cansdale, 1982; King et al., 1983; Bolivar et al., 1984; Clarke and Zemcov, 1984; File et al., 1984; Tausk and Stratton, 1986). The clinical significance of this effect is unclear (Tausk and Stratton, 1986). In all clavulanic acid is biologically active in a variety of ways and should not be considered solely as a beta-lactamase inhibitor. There is considerable scope for further investigation into the complete range of biological

properties of this agent.

Other beta-lactamase inhibitors include carbapenems, sulphated macrocyclic lactones (e.g. izumenolide), monobactams and penicillanic acid sulfones (e.g. sulbactam and YTR °30) (Gould and Wise, 1986).

Like clavulanic acid, sulbactam is used commercially - in combination with ampicillin and with cefoperazone. Alone sulbactam has little antibacterial activity except against N. gonorrhoeae and Acinetobacter calcoaceticus. Its spectrum of inhibitory activity is similar to that of clavulanic acid but is two to five times less potent. It does however provide greater protection against the beta-lactamases of some strains of Proteus, Providencia, Citrobacter, Enterobacter and Serratia marcescens (Gould and Wise, 1983).

The future clinical importance of beta-lactamase inhibitors is difficult to predict. The currently marketed preparations are not regarded as superior to conventional antibiotic therapy (Conner, 1985). They lack useful activity against some clinically important beta-lactamases and their pharmacokinetic properties are not always matched to their beta-lactam co-drugs. The future of this type of therapy therefore appears to depend on the development of new inhibitors (Gould and Wise, 1986).

c. Resistance of Gram-negative Bacteria to Beta-lactam Antibiotics.

1. The Clinical Problem

The production of antibiotics in Nature by microorganisms is presumably of survival value in reducing the population levels of competitors. Similarly the clinical use of antibiotics by man is aimed at enhancing survival through reducing bacterial numbers to either prevent or treat infection. However in both clinical and environmental settings antibiotics may be ineffective against bacteria which possess mechanisms for resistance (Lacey, 1980; Levy, 1982).

Prior to the antibiotic era the majority of clinically encountered bacteria were susceptible to antibiotics. However since the introduction of sulphonamides in 1935 and penicillin in 1942 subsequent antibacterial therapy has created an intense selection pressure causing antibiotic resistant bacteria to proliferate at the expense of the susceptible majority (Finland, 1971; Lacey, 1980; Levy, 1982). This selection pressure has important implications for the outcome of antibiotic therapy, the microbial ecology of institutions and communities, and for the useful lifetimes of antibiotics (Murray and Moellering, 1978; Jackson, 1979; Gardner et. al., 1980; Levy, 1982; McGowan, 1983; Sanders et. al., 1984; Acar, 1985). Furthermore the emergence of antibiotic resistance has impacted on the types of infectious disease states encountered by contributing to an evolving state of infectious disorders. While minor changes in infectious diseases may have occurred

previously, infectious diseases since the period 1935 to 1942 have increased in frequency and changed in character to present new problems in a way not paralleled in other classes of diseases (Florey, 1956; Rogers, 1959; Buckwold and Ronald, 1979; Weinstein, 1985; Acar, 1985).

Prior to the introduction of antibiotics serious infections encountered in hospitals were caused predominantly by pneumococci, haemolytic streptococci and staphylococci (Buckwold and Ronald, 1979). Gram-negative bacteria were relatively infrequently involved with E. coli being the most important pathogen. Other Gram-negative pathogens encountered were primarily N. meningitidis, N. gonorrhoeae, Klebsiella pneumoniae, Haemophilus influenzae, Salmonella spp. and Proteus mirabilis. Opportunistic pathogens such as Pseudomonas spp., Cardiobacterium hominis, Serratia spp., Providencia spp., Proteus vulgaris, Morganella morganii, Acinetobacter spp., and Flavobacterium spp. were extremely rare causes of infection. Since the introduction of antibiotics these organisms have increased in incidence and importance as causes of nosocomial infection (Finland et al., 1959; Rogers, 1959; Feeley et. al., 1975; Buckwold and Ronald, 1979; McGowan, 1983; Fainstein and Bodey, 1985; Weinstein, 1985; Young, 1985).

As each new antibiotic has become available organisms have, with few exceptions, rapidly developed resistance. This trend has accelerated to the point where organisms such as Pseudomonas and Serratia are frequently resistant to most of the commonly used antibiotics. Since the late 1970s resistance has developed

in certain clinical settings with such rapidity that it threatens the ability of pharmaceutical companies to continue producing effective therapies (Levy, 1982; McGowan, 1983; Acar, 1985; Tipper, 1985; Weinstein, 1985).

Antibiotic use has not been the sole determinant of the changing pattern of bacterial infection. Other modern medical practices have contributed by providing increasing numbers of patients especially vulnerable to infection. Such patients include:

(i) an increasing number of older and sometimes debilitated patients,

(ii) those with tumors and leukaemia who now survive longer, but because of immunosuppression due to disease or treatment are highly susceptible to invasion by almost any organism, and

(iii) the increasing number who undergo immunocompromising procedures and therapies (Klastersky, 1985; Weinstein, 1985).

Such infection-prone patients enhance the acquisition, multiplication and persistence of antibiotic-resistant bacteria and their subsequent transmission (Sherris, 1970).

2. Resistance Mechanisms - Introduction

The difference between the extracellular concentration of a beta-lactam antibiotic and its concentration at the target PBPs is determined by its rate of penetration and its susceptibility to beta-lactamases. The outer membrane of

Gram-negative bacteria (Fig. 6) is selectively permeable. Access to the periplasmic gel (Hobot et al., 1984) is determined by the rate of passive diffusion through the exopolysaccharide layer and the outer membrane (Tipper, 1985; Nayler, 1987). In the case of hydrophilic molecules such as the majority of beta-lactam antibiotics the diffusion is largely through aqueous pores called porins (Nikaido, 1981; 1985). Outer membrane permeability varies widely, being generally high in Neisseria, very low in Pseudomonas, and intermediate in Enterobacteriaceae. Beta-lactamases secreted into the periplasm can reach high concentrations. These along with other proteins and macromolecular solutes may modify or trap antibiotic molecules which traverse the outer membrane and thereby control the concentration of active antibiotic in the vicinity of the PBPs (Tipper, 1985; Nayler, 1987). While outer membrane permeability and beta-lactamases are important determinants of antibiotic resistance, the target PBPs may also affect resistance by undergoing mutational alterations which alter their affinity for antibiotics (Tipper, 1985; Tomasz, 1986).

The rapidity and extent with which bacteria develop resistance varies widely depending on both the types of bacteria and antibiotics involved (Garrod et. al., 1973; Wilson and Miles, 1975; McGowan, 1983).

3. Permeability

The lipid bilayer, which comprises most biological membranes including the outer membrane of Gram-negative

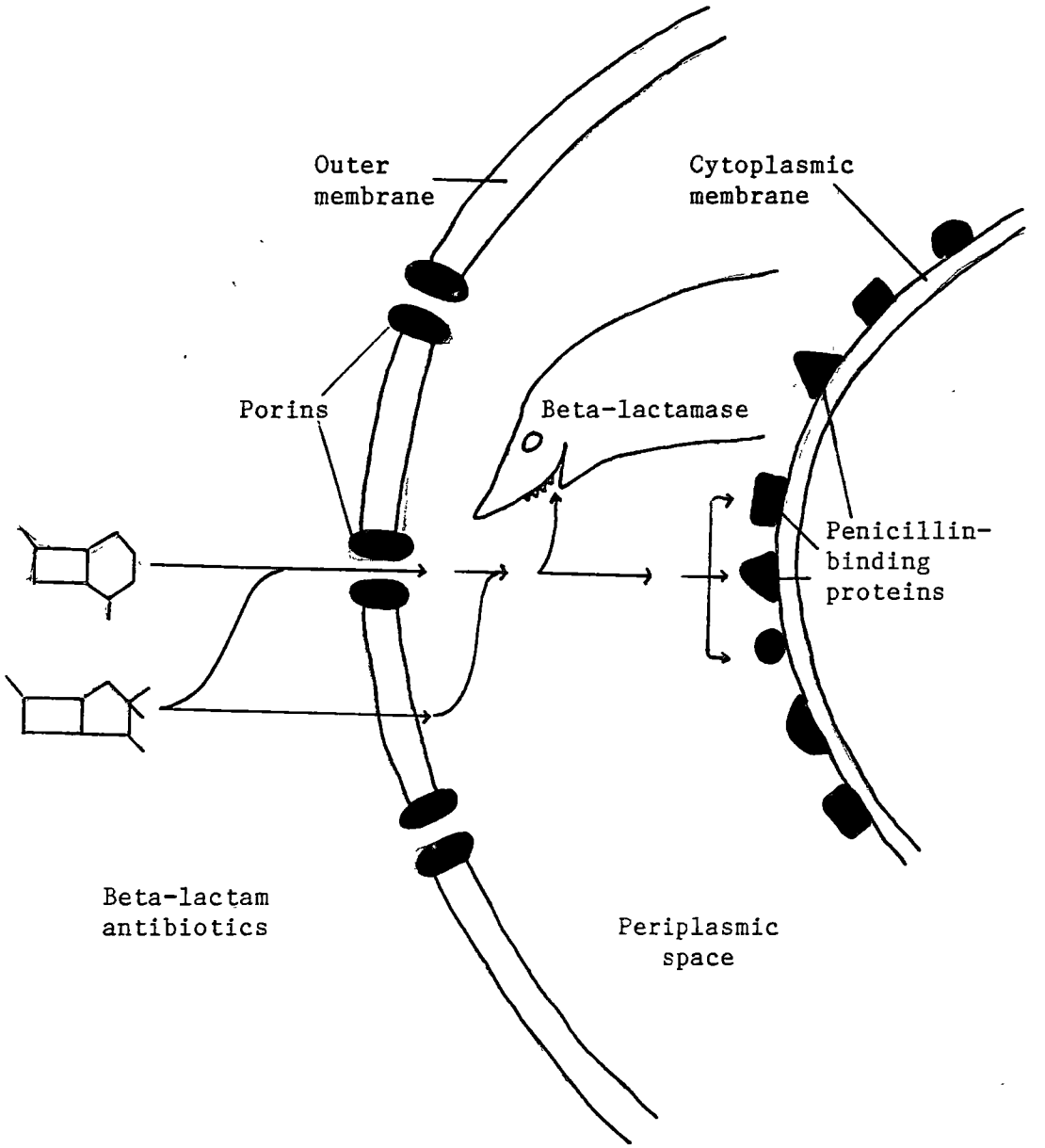


Fig. 6 Interaction of beta-lactam antibiotics with Gram-negative cell.

(Adapted from Normark and Lindberg, 1985)

bacteria (Fig. 6), usually has low permeability to hydrophilic solutes. It is necessary however for the bacterium to take up nutrients from, and excrete waste products into, the external environment. These functions are achieved through porin channels which, in the cases of E. coli and S. typhimurium, allow the diffusion of any solute providing it is small, i.e. less than 600 daltons, and hydrophilic (Nikaido, 1985).

In most Gram-negative bacteria the porins are proteins of MW 35,000-40,000 which are often the most abundant cell proteins e.g. 10^5 molecules per cell. Studies comparing wild types and porin-deficient mutants have demonstrated that it is through the porin channels of E. coli and other Enterobacteriaceae that many beta-lactam antibiotics cross the outer membrane. The Enterobacteriaceae frequently produce multiple types of porin. E. coli K-12 produces OmpF (outer membrane protein F) and OmpC porins. Salmonella typhimurium produces OmpF, OmpC and OmpD porins. Enterobacter cloacae also produces two porin types. All nonenteric species seem to produce a single porin type which is characteristic of the strain. Porins have been identified for P. aeruginosa, N. gonorrhoeae, Aeromonas salmonicida, Brucella spp., Chlamydia trachomatis, P. mirabilis, P. vulgaris, Providencia rettgeri, Morganella morganii, Rhodopseudomonas capsulata and R. sphaeroides (Nikaido, 1985; Mitsuyama et al., 1987). In general comparisons of permeability, and allowing for considerable differences between strains within species, H. influenzae (most permeable) > E. coli > E. cloacae > P. aeruginosa (least permeable) (Nayler, 1987).

While porin channels do not exert configurational specificity the diffusion rates of solutes are greatly affected by their gross physicochemical properties. In E. coli the estimated diameters of the OmpF and OmpC channels are 1.16 and 1.04 nm respectively. These are sizes close to the sizes of beta-lactam molecules. It is thus obvious that the rates of diffusion of the beta-lactam antibiotics is very dependent on interactions with the rims or walls of the porin channels.

Since even very low beta-lactamase activity may inactivate a significant fraction of beta-lactams reaching the periplasm antibiotic efficacy is influenced by the balance between an antibiotic's rate of influx and its rate of removal by beta-lactamases. Effective periplasmic concentrations are therefore not necessarily indicative of high permeability. Many of the newer beta-lactams exhibit relatively poor permeability but are nevertheless quite potent because of relatively high resistance to beta-lactamases (Nikaido, 1985).

Antibiotic Penetration in E. coli

Rates of diffusion through the porin channels of E. coli, and other bacteria, are determined by solute attributes such as hydrophobicity, size and charge, each of which interact with each other. In general zwitterionic compounds penetrate extremely well while negative charges exert a retarding effect. Smaller molecules diffuse faster than larger molecules or similar sized molecules with bulky side chains. The substituted

oxime side chain on the alpha carbon of the substituent group at position 7 of the cephem nucleus causes a 10-fold decrease in penetration rate while the presence of a methoxy group at this position reduces penetration by 20%. Hydrophilicity enhances penetration (Nikaido, 1985).

Porin production in E. coli K-12 is nutrient dependent. On ordinary laboratory media two porins, OmpF and OmpC are produced. The narrower OmpC channel is more selective in terms of hydrophobicity and molecular size. Cephalosporins penetrate two to three times faster than their more hydrophobic penicillin analogues (Nikaido, 1985). This does not mean that cephalosporins are more effective as penicillins are thought to also penetrate through non-porin pathways in E. coli and E. cloacae and possibly other species (Komatsu et al., 1981; Sawai, Hiruma et al., 1982; Yamaguchi et al., 1985). When starved for phosphate E. coli K-12 derepresses an additional porin, PhoE, which favours negatively charged beta-lactam antibiotics. This porin is not however regarded as having clinical significance as serum phosphate concentrations are too high to invoke utilisation of PhoE (Yoshimura and Nikaido, 1985).

The importance of size of solute molecules can be seen from the permeability of arabinose (MW 150) which penetrates approximately one hundred times faster than disaccharides of MW 342. Many beta-lactams have MWs in the range 350-450, a size range which significantly restricts diffusion through the porins, especially the narrower OmpC channel. Compounds of unusually large size, such as piperacillin and cefoperazone,

diffuse slower through the larger OmpF than predicted from their MWs and hydrophobicity values, probably as a result of steric hindrance arising from their very large side chains. The smaller OmpC channel appears to be closed to compounds with molecular weights higher than 500 or those with bulky side chains such as cefoperazone (MW 644), ceftriaxone (MW 552) and ceftazidime (MW 545). Sometimes, however, diffusion rates do not correlate well with MW. For example, cephaloridine (MW 415) penetrates twice as rapidly as lactose (MW 342). This is perhaps explained by the compactness of the cephaloridine ring structure and its increased hydrophilicity due to zwitterionic charges. The most rapidly penetrating beta-lactam is imipenem, which combines small size (MW 299) and zwitterionic charges (Nikaido, 1985).

Antibiotic Penetration in Other Bacteria

Apart from the N. gonorrhoeae channel, it seems that the porin channels of other bacteria behave similarly to those of E. coli. That is, cations penetrate faster than anions, and increasing molecular size and hydrophobicity retard penetration. The only difference is that these effects are less important in organisms with larger channels (Nikaido, 1985).

The resistance of P. aeruginosa to a wide range of antimicrobials is attributed to poor permeability of the outer membrane - 12 to 100-fold lower permeability than the outer membrane of E. coli. Interestingly approximately 0.4% of the 200,000 porin F channels of P. aeruginosa are large and allow passage of polysaccharides of several thousand daltons. However

the remaining 99.6% or so of protein F channels are small and almost impenetrable by beta-lactams (Hancock, 1986; Woodruff et al., 1986).

The high resistance of P. aeruginosa to many beta-lactams seems to be due to the very low permeability of its outer membrane coupled with rapid hydrolysis by its inducible chromosomal beta-lactamase. Those beta-lactams with enhanced anti-pseudomonal activity are probably efficacious because of properties such as high beta-lactamase resistance, inability to induce the chromosomal beta-lactamase, or high affinity for the target PBPs (Nikaido, 1985).

Outer Membrane Protein Changes and Resistance

Recent reports suggest that altered outer membrane proteins are increasing in incidence as mechanisms of resistance. Reports of clinical isolates of E. coli (Bakken et al., 1987), and S. marcescens (Goldstein et al., 1983; Sanders and Watanakunakorn,1986) follow numerous laboratory studies of resistance due to altered outer membrane proteins in E. coli (Komatsu et al., 1981; Sawai, Hiruma et al., 1982), K. pneumoniae (Sanders et al., 1984), E. cloacae (Sawai et al., 1982; Then and Anghorn, 1986) P. mirabilis (Sawai et al., 1982) and P. aeruginosa (Rella and Haas, 1982)

Development of resistance due to reductions in amounts of outer membrane proteins is a potentially important resistance mechanism especially to agents with low intrinsic rates of penetration e.g. cefoperazone, moxalactam, cefuroxime, cefotaxime and aztreonam (Jaffe et al., 1982; 1983). The associated development of resistance to other classes of

antibiotics is a very major cause for concern. Then and Anghern (1986) suggest that there will be increased occurrence of such multiply resistant mutants as a result of use of potent beta-lactamase stable beta-lactam antibiotics and new fluoroquinolone agents.

4. PBP Mutations

Clinically important resistance to beta-lactam antibiotics arising from alterations to PBPs occurs most frequently as methicillin resistance in S. aureus and penicillin resistance in N. gonorrhoeae. This type of resistance has also been documented in Streptococcus. pneumoniae, S. faecium, H. influenzae and P. aeruginosa. To date it appears to be confined to strains of the more invasive human pathogens, particularly cocci, in which beta-lactamase production has not contributed significantly to resistance or has been highly restricted in substrate profile. This type of resistance, and mutations affecting outer membrane proteins, can be expected to become increasingly important in Gram-negative bacilli with increased use of beta-lactamase stable antibiotics such as moxalactam and aztreonam. To reverse this trend it may become necessary for clinical laboratories to use a more molecular approach to routine susceptibility testing and investigate antibiotics appropriate to the outer membrane permeability, beta-lactamase substrate profile and PBP vulnerabilities of pathogens (Tipper, 1985; Tonin and Tomasz, 1986).

d. Beta-lactamases

Although resistance due to beta-lactamase production was rare in clinical isolates at the time penicillin was first used widespread beta-lactamase-mediated resistance was soon encountered in hospital isolates of S. aureus (Munch-Peterson and Boundy, 1962). Similarly the initial effectiveness of broad-spectrum penicillins such as ampicillin and carbenicillin was rapidly reduced by the beta-lactamases of Gram-negative bacteria (Williams, 1977).

Classically beta-lactamases hydrolyse the cyclic amide bond of susceptible beta-lactam antibiotics to produce acid derivatives which have no antibacterial activity. In hydrolysis a covalent bond is formed between the enzyme and the amide part of the beta-lactam ring. The beta-lactamase molecule envelopes the substrate molecule forming an acyl-enzyme complex. Deacylation of the complex yields free enzyme and ring-opened (hydrolysed) substrate. The efficiency of beta-lactamases is high with many of them capable of hydrolysing thousands of labile substrate molecules per second. However when Class 1 enzymes react with "enzyme-stable" beta-lactams the half-lives of the intermediate complexes may be very long (minutes to hours). The antibiotic is then biologically inactive while it is bound to the enzyme prior to release in hydrolysed form. Organisms which can synthesize enzyme rapidly enough to maintain an enzyme excess will be resistant primarily through non-hydrolytic trapping of the substrate by beta-lactamases rather than through hydrolysis. Maintenance of an enzyme excess may be achieved

and maintained by the interaction of enzyme induction and the restrictive effect of the outer cell membrane on antibiotic diffusion into the periplasm (Bakken and Sanders, 1987).

1. Some Methods Used to Characterize Beta-lactamases

The following review discusses relevant aspects of procedures used to classify beta-lactamases. Historically an understanding of the relatedness of beta-lactamases has developed gradually following advances in analytical procedures and the changing spectrum of beta-lactamase-producing pathogens and beta-lactam substrates. The gradual and incomplete development of knowledge has resulted in a confusing array of classification schemes. It now seems probable that future classifications will incorporate both molecular data and evolutionary aspects and be less arbitrary than contemporary classifications (Bauernfeind, 1986). The future utility of some of the following procedures will almost certainly diminish. However to evaluate current investigations of beta-lactamases it is necessary to have some familiarity with the applications and limitations of these procedures.

Screening Strains and Preparations for Beta-lactamase Activity

The most convenient and sensitive screen for the beta-lactamases of Gram-negative bacteria is the chromogenic cephalosporin or nitrocefin technique of O'Callaghan et al. (1972). Nitrocefin working solution (500 mcg./ml.), which is initially straw-coloured, changes to red when its beta-lactam ring is hydrolysed. Occasionally beta-lactamases are not

readily detected by this method. The ROB-1 beta-lactamase discovered in H. influenzae exhibits a negative nitrocefin reaction unless tested with a very dense cell suspension (Medeiros, 1984) and Legionella micdadei beta-lactamase, which is regarded as nitrocefin-negative by some workers, hydrolyses nitrocefin slowly at a pH-dependent rate (Marre et al., 1982).

Cell-free Beta-lactamase Preparations

Beta-lactamase preparations are usually made from cells harvested from broth cultures (Matthew et al., 1975) although some workers may scrape cells off plates (Eley and Greenwood, 1986). The cells are washed at least once with phosphate buffer of neutral or almost neutral pH then lysed by sonication, sand grinding (Matthew et al., 1975), osmotic shock (Timewell et al., 1981), repeated freezing and thawing (Cartwright and Waley, 1984) or French press (Aranoff and Shlaes, 1987). Cellular debris is removed by centrifugation and the resultant supernatant is used as a crude enzyme preparation.

Many workers do not state whether or not enzyme preparations are used immediately or stored. Cuchural et al. (1986) report that an imipenem-hydrolysing metalloenzyme from B. fragilis loses 99% of its activity on freezing or prolonged exposure to room temperature. The majority of workers who describe storage procedures for beta-lactamase preparations freeze preparations at -20°C (e.g. Cullman et al., 1984), or -70°C (e.g. Levesque et al., 1983), or freeze dry preparations (e.g. Tuner et al., 1985).

Substrate Profile

Many assay techniques have been used to measure beta-lactamase activity because there is no technique suitable for all applications. The main source of technical difficulty is the different types of breakdown products formed during hydrolysis of penicillins and cephalosporins. Penicillins are degraded to penicilloic acids which may be assayed directly. Cephalosporins however have a more complex breakdown pattern. The initial products are acetic acid and cephalosporanic acids which rapidly decompose into smaller molecules, the stoichiometry of the reaction varying according to the nature of the cephalosporin (Selwyn, 1980). Thus while it is possible to assay for penicilloic acids there is no single stable reaction product to detect when cephalosporins are hydrolysed. When accurate assays are required to determine beta-lactamase activity against both penicillins and cephalosporins it is not possible to use the same methodology for both types of substrates and results for both types of substrates are therefore not directly comparable.

The iodometric technique, originally described by Perret (1954) and subsequently modified and refined by several workers, is perhaps the most reliable and convenient when attempting to assay for both penicillinase and cephalosporinase activity with a single methodology. It is ideal for assaying most enzymes when using penicillins as substrates as it relies on the reaction of eight equivalents of iodine with the penicilloic acid produced by beta-lactamase action. In the microiodometric technique (Sykes and

Nordstrom, 1972; Sargent, 1968) the amount of iodine which has reacted with the penicilloic acid is estimated photometrically from the production of a blue starch-iodine complex. As previously mentioned this assay is less reliable for cephalosporin substrates and therefore unsuitable if absolute measurement of cephalosporinase activity is required (Sykes and Matthew, 1976).

The spectrophotometric method of O'Callaghan et al. (1968) is a good general assay for cephalosporinase activity. The rate of hydrolysis of the beta-lactam ring is followed by measuring the rate of decrease in optical density at the wavelength of maximum absorption of the ring.

Another method for measuring both penicillinase and cephalosporinase activity is microbiological assay. This method is extremely sensitive, but not very accurate and very time consuming (Sykes and Matthew, 1976). Sometimes it may be necessary to use this technique in research as conventional hydrolysis assays are insensitive to low levels of hydrolysis. For example Levesque et al. (1983) confirmed slow hydrolysis of cefoxitin by MULB-906 from "Achromobacter xylosoxidans" by bioassay.

The 3-dimensional test (Thomson et al., 1984) is an adaptation of the microbiological assay which is used in conjunction with routine disc diffusion antibiotic susceptibility tests to provide qualitative substrate profile information in the routine clinical laboratory. While there

is a need for such information in routine laboratories (Phillips, 1986) the sensitivity and specificity of this technique have not been fully evaluated and it is not known if it is suitable for all beta-lactamases. HPLC studies (Aldous et al., 1985) and microbiological assays (Thomson et al., 1984) have demonstrated that positive 3-dimensional tests result from antibiotic inactivation.

The clover leaf test (Kjellander and Myrbach, 1964) is another qualitative microbiological assay technique and has been used to detect beta-lactamases, chloramphenicol acetyltransferase and erythromycin esterase (Andremont et al., 1982). There is controversy about the significance of occasional positive clover leaf test results produced by organisms which seem to lack particular antibiotic inactivating enzymes. Reig and Baquero (1984) regard these nonspecific results as evidence that the technique is unreliable for the detection of antibiotic inactivation by the genus Bacteroides. Jorgensen (1985) suggests that nonspecific results may arise from resistance mechanisms other than antibiotic inactivation, and also reports three strains of Enterobacteriaceae with positive clover leaf tests which required sonication to detect beta-lactamase activity by the nitrocefin reaction. This latter finding indicates that the clover leaf test may be even more sensitive than the nitrocefin reaction.

Measuring the hydrolytic activity of an enzyme preparation against a number of beta-lactam substrates is useful for comparing and classifying beta-lactamases. Comparisons of

substrate profiles avoid the inherent difficulties associated with comparing different cells which may have wide differences in levels of beta-lactamase expression. Profiles are often expressed as ratios related to a value of 100 for a chosen substrate. Thus a profile of penicillin G 100, ampicillin 50, cephaloridine 200, indicates an enzyme with a rate of hydrolysis of ampicillin which is half that of penicillin G and for cephaloridine the rate is twice that of penicillin G. The quoting of relative enzyme activities can be misleading as the apparently firm values tend to obscure a background of inherent technical difficulties associated with the assays (Richmond and Sykes, 1973).

Substrate profile alone is not a good parameter for beta-lactamase classification as many enzymes, especially chromosomal beta-lactamases, have similar profiles. However substrate profiles provide very valuable information when used in conjunction with analytical isoelectric focussing (IEF). The two methods are complementary for classification purposes and IEF also is necessary to ensure purity of the enzyme preparations to be used for hydrolysis assays (Sykes and Matthew, 1976).

If hydrolysis assays are performed to investigate the stability of various substrates in the presence of bacteria, assay procedures should be taken further than the just-mentioned procedures which are quite satisfactory for classification purposes. In specific stability studies it is necessary to provide kinetic data so that the Michaelis constant and the

maximum reaction velocity can be determined. The ratio of these characteristics is sometimes referred to as "physiological efficiency" or "efficiency of hydrolysis" and is regarded as predictive of the hydrolytic vulnerability of antibiotics *in vivo* (Bush and Sykes, 1986).

Induction Studies

The induction potential of various beta-lactam antibiotics varies with both the drug and the organism being induced. However all studies so far indicate that the cephamycins and imipenem are potent inducers. Results with other drugs vary. Some workers use antibiotic concentrations far in excess of the MIC to induce beta-lactamases and certain antibiotics appear to be potent inducers only when tested in this manner (Sanders and Sanders, 1986). The significance of such results is questionable as *in vivo* induction resulting in therapeutic failure is unlikely to occur when bacteria are exposed to superinhibitory levels of beta-lactam antibiotics. Instead, and as would seem logical, bacteria are more likely to be inhibited or killed (Aranoff and Shlaes, 1987). Clinically, results obtained in this way are irrelevant, and from a scientific viewpoint it can be argued that such results are artifacts arising from technical problems. That is the high levels of enzyme expression reported due to exposure of bacteria to superinhibitory antibiotic levels are not due to induction but rather are derived from computations based on data pertaining to damaged or dead cells in which protein synthesis has ceased or is impaired . Calculations of beta-lactamase activity per unit protein are therefore likely to produce a misleading impression of high levels of beta-

lactamase activity. Sanders and Sanders (1986c). who performed one of the first systematic investigations of this phenomenom, favour inducing with subinhibitory antibiotic concentrations of 1/4 the MIC or 100 mcg/ml, whichever is less.

Ideally an inducer should be be neither toxic nor a substrate (Nordstrom and Sykes, 1974). Several non-beta-lactam compounds possess a limited capacity to induce beta-lactamases (Cullmann et al., 1984) but at present antibiotics, particularly cefoxitin and imipenem, are the most potent inducers.

Beta-lactamase induction is examined after cells have grown during log phase in the presence of an appropriate inducer. Peak induction usually occurs within two hours (Gootz and Sanders, 1983; Minami et al., 1983). After induction cell-free preparations are used to assay beta-lactamase activity.

Inhibition of Enzyme Activity

Chromosomal beta-lactamases are strongly inhibited by beta-lactamase stable penicillins such as the isoxazolyl penicillins. The plasmid-mediated TEM-type enzymes (Table 7) are also inhibited by these compounds but not as absolutely (Sykes and Matthew, 1976). Clavulanic acid is a potent inhibitor of non-chromosomal beta-lactamases (Gould and Wise, 1986). By using a combination of clavulanic acid and a stable penicillin such as cloxacillin it is possible from inhibition studies to obtain preliminary evidence as to the genetic locus

of the enzyme.

Chloride ions and p-chloromercuribenzoate (p-CMB) are non-beta-lactam inhibitors which have been used in classifying beta-lactamases. P-CMB at 0.5 micromolar concentration completely inhibits enzymes with cysteine residues. However even experienced workers have reported misleading results with this agent as sulphydryl groups, even if present, may not be accessible to p-CMB (Sykes and Matthew, 1976; Sykes and Smith, 1979).

Analytical Isoelectric Focussing

The application of flat-bed isoelectric focussing (IEF) in polyacrylamide gel to beta-lactamase characterisation was a major technological advance (Matthew et al., 1975). This technique is highly specific making it possible to identify different beta-lactamases within the one strain. The banding patterns obtained with IEF permit virtual fingerprinting of beta-lactamases through side-by-side comparisons of unknown and reference beta-lactamases on the same gel. This technique is particularly useful to distinguish non-chromosomal beta-lactamases which are otherwise difficult to classify. More recently agarose has been advocated as an alternative matrix to polyacrylamide for IEF. Agarose is regarded as more convenient to use and less toxic. Apart from slight differences in the banding patterns of HMS-1 and PSE-1 beta-lactamases (Table 35) agarose yields identical results to polyacrylamide (Medeiros, 1984).

The IEF banding patterns of plasmid mediated beta-lactamases are unique, comprising a single major band with several accessory or satellite bands which appear late after application of the nitrocefin overlay. The significance of satellite bands is controversial and various explanations have been offered. Suggestions are that the satellite bands arise from isoenzymes (Labia et al., 1976; Brive et al., 1977), differences in the sialic content of the various bands (Jouvenot et al., 1984), proteolysis and deamination of the enzyme after cell disruption (Brive et al., 1977; Kiss et al., 1983) and binding of carbohydrate moieties to the enzyme (Gal et al., 1983).

Another complication of IEF is lack of electrophoretic mobility of some beta-lactamases which results in poor separation into bands and failure to establish a reliable isoelectric point e.g. beta-lactamases of Legionella dumoffi, L. longbeachae (Marre et al., 1982), Bacteroides melaninogenicus (Timewell et al., 1981) and the imipenem-hydrolysing enzyme of B. fragilis (Cuchural et al., 1986). This is suggestive of unusually large enzymes or binding of the enzymes to larger molecules e.g. membrane binding (Marre et al., 1982; Medeiros, 1984).

The taxonomic value of IEF in characterizing chromosomal beta-lactamases is considerable, in some cases (e.g. L. pneumophila) being specific to subspecies level (Marre et al., 1982). Caution is however necessary in interpreting variations in IEF patterns within a species. Okonogi et al. (1986) warn that a range of isoelectric points, such as is

found in P. vulgaris (pI 6.9 to pI 9.0), may arise from variations in amino acid composition due to mutations in the beta-lactamase gene or from different post-translational processing in different strains. For taxonomic relevance it is necessary to relate pI variations within a species to other taxonomic features.

Molecular Weight Determinations

The molecular weights of beta-lactamases, ranging from 14,000 to greater than 250,000, have usually been determined by the gel filtration method of Andrews (1964). Other methods used are sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Weber and Osborn, 1969), equilibrium centrifugation (Yphantis, 1964) or amino acid analysis. Sometimes different values are obtained by different methods e.g. the penicillinase of P. maltophilia has an apparent molecular weight of 118,000 by gel filtration and 26,000 by SDS-PAGE. This type of result suggests that the enzyme consists of four sub-units (Saino et al., 1982). While molecular weight determinations are sometimes useful in identifying beta-lactamases it is more usual for enzymes to be clearly different in substrate profile, response to inhibitors, and isoelectric points and have molecular weights that do not differ by more than the error of the determination method used (Sykes and Matthew, 1976).

Sequencing Techniques

Amino acid and nucleotide sequencing techniques have yielded additional insights into the relationships between

beta-lactamases. These techniques have supplemented previous data to define three evolutionarily distinct classes of beta-lactamases.

Class A beta-lactamases have a serine residue at the active site, have molecular weights of around 29,000, show significant amino acid homology and preferentially hydrolyse penicillins. The active site of these beta-lactamases shows homology with the amino acid sequence around the penicillin binding sites of the D-alanine carboxypeptidases of Bacillus stearothermophilus and B. subtilis. This supports the hypothesis that these beta-lactamases may be derived from PBPs (Yocum et al., 1979; Waxman et al., 1982). Class B beta-lactamase is a metalloenzyme of molecular weight 23,000 with predominantly cephalosporinase activity and is produced by B. cereus (Ambler, 1980). Class C beta-lactamases are the chromosomally-mediated cephalosporinases of E. coli which show significant homology with those of Shigella species and less homology with Klebsiella, Salmonella, Serratia and Pseudomonas species. These enzymes have a comparatively high molecular weight of about 39,000 and also have a serine residue at the active site but have no sequence homology with class A beta-lactamases (Jaurin and Grundstrom, 1981; Bergstrom et al., 1982; Knott-Hunziker et al., 1982).

2. Beta-lactamases of Gram-positive Bacteria

Gram-positive and Gram-negative bacteria differ fundamentally in beta-lactamase production. Gram-positive

beta-lactamases are exoenzymes whereas those of Gram-negative cells are usually retained in the cell (Sykes and Smith, 1979). S. aureus mainly produces inducible plasmid-mediated beta-lactamases which rapidly hydrolyse benzyl penicillin, aminopenicillins, carboxypenicillins, and acylaminopenicillins, but are only feebly active against the penicillinase-resistant penicillins and most cephalosporins. Staphylococcal penicillinases may be distinguished from each other serologically (Bauernfeind, 1986; Greenwood, 1986).

In general streptococci are unable to develop resistance through beta-lactamase production although recent reports of some beta-lactamase-producing strains of Streptococcus faecalis and S. uberis are cause for concern. Nocardia spp., Clostridium spp. and Bacillus spp are other Gram-positive bacteria which produce beta-lactamases. Many Mycobacterium spp. have also been found to produce beta-lactamases (Bauernfeind, 1986; Greenwood, 1986).

3. Richmond and Sykes Classification of Beta-lactamases of Gram-negative Bacteria

In contrast Gram-negative bacteria produce a wide variety of beta-lactamases with a continuous spectrum of hydrolytic activities ranging from predominantly penicillinase to predominantly cephalosporinase activity with a gradation of enzymes hydrolysing both penicillins and cephalosporins between these extremes. As previously mentioned there are a number of different classification schemes for beta-

lactamases and their lack of concordance in nomenclature makes the scientific literature confusing (see table 7).

To overcome the nomenclatural problems Sykes (1982) suggests that the original classification of Richmond and Sykes (1973) be retained as a reference system as it has been cited extensively in the literature and is still suitable as a common reference for future studies.

In this scheme five broad classes of beta-lactamases are defined on the basis of substrate and inhibition profiles and the genetic location of the beta-lactamase genes (Table 5). Within each class there are different types of enzymes. Class I comprises chromosomally-mediated enzymes which are predominantly cephalosporinases. Class II also contains chromosomally-mediated enzymes. These are however predominantly penicillinases. Class III enzymes are plasmid-mediated and are broad spectrum enzymes having similar activities against both penicillins and cephalosporins but being sensitive to inhibition by cloxacillin and resistant to inhibition by p-CMB. Class IV enzymes are chromosomally-mediated and have similar activities to class III enzymes but are resistant to inhibition by cloxacillin and sensitive to inhibition by p-CMB. Some class IV enzymes hydrolyse cloxacillin. Class V enzymes are plasmid-mediated and have predominantly penicillinase activity, including activity against cloxacillin, and are resistant to p-CMB (Richmond and Sykes, 1973; Sykes and Matthew, 1976; Sykes 1982).

TABLE 5

Richmond and Sykes Classification of Beta-lactamases of Gram-negative Bacteria*

Class	Substrate	Gene Locus	Genetic Expression	Representative Bacteria
I	Prefer cephalosporins	Chromosome	Inducible	*Enterobacter* *Citrobacter* *Serratia* Indole-positive *Proteus* *P. aeruginosa*
II**	Prefer penicillins	Chromosome	Inducible	*P. maltophilia*
			Constitutive	*P. aeruginosa* *P. thomasii*
III**	Penicillins and cephalosporins	Plasmid	Constitutive	Enterobacteriaceae *H. influenzae* *N. gonorrhoeae* *P. aeruginosa*
IV**	Penicillins and cephalosporins	Chromosome	Constitutive	*Klebsiella*
V**	Penicillins, isoxazolyl-penicillins, cephalosporins	Plasmid	Constitutive	Enterobacteriaceae *P. aeruginosa*

* Richmond and Sykes classification modified according to Saino *et al.* (1982) and Bakken and Sanders (1987)

** Inhibited by potassium clavulanate

Some aspects of the Richmond and Sykes classification are now inadequate or technically inconvenient (Sykes and Matthew, 1976). The five classes contain very heterogeneous groups of enzymes. Some parameters such as p-CMB inhibition are difficult to determine with crude enzyme preparations. Furthermore with the proliferation of new types of beta-lactam antibiotics there is now a need for a more suitable system for describing substrate profiles to replace the concept of classifying beta-lactamases solely in terms of penicillinase and cephalosporinase activity. More comprehensive terms such as cefuroximase, cefotaximase, and imipenemase have appeared in the literature and these, and other appropriate terms, are needed to describe hydrolytic activity against compounds such as cephamycins, monobactams and carbapenems (Bauernfeind, 1986).

Of relevance to this study Mitsuhashi (1985) uses the term oxyiminocephalosporin beta-lactamase (CXase) for enzymes which hydrolyse the oxyiminocephalosporins (e.g. cefotaxime, ceftizoxime, cefoperazone, cefmenoxime, and moxalactam). Sato et al. (1985) report that F. odoratum produces a type II CXase.

Sykes (1982) however rejects such terms. This rigid position is perhaps ill-considered as the enzymes to which the new terms pertain (Table 9), while not yet widely distributed among clinical isolates, are distinct from those grouped in earlier classifications and can be expected to increase in incidence in the future (Medeiros, 1984; Bauernfeind, 1986). It would therefore seem that workers would be better equipped

to work with such enzymes if there was a more appropriate nomenclature.

Another problem with the Richmond and Sykes classification is that knowledge of the genetic location of the enzyme is not always particularly easy to determine. In addition the validity of this parameter is questionable. Prior to the discovery of transposons the importance of the parameter was obvious. Beta-lactamase genes were either incorporated in chromosomal or plasmid DNA. There were known to be hundreds of chromosomal beta-lactamases and more than twenty plasmid-mediated beta-lactamases. However it is now known that if the genes on either structure are located on transposons, or 'jumping genes', the genetic locus can be on either the chromosome or the plasmid. This mobility of genes has not yet greatly impinged on classification schemes. However transposons must eventually detract from the emphasis given to the genetic locus in current classifications (Pechere and Levesque, 1983; Bauernfeind, 1986).

4. Chromosomally-mediated Beta-lactamases

Using the increased sensitivity of analytical IEF to detect beta-lactamases Matthew and Harris (1976) surveyed a variety of Gram-positive and Gram-negative bacteria and concluded that virtually all bacteria produce at least one chromosomally-mediated beta-lactamase. In many organisms, particularly penicillin and ampicillin susceptible ones, the amount of beta-lactamase produced may be very low. Increased

enzyme production (hyperproduction) may be associated with resistance.

Hyperproduction can result from induction (derepression), increases in the number of beta-lactamase genes on the chromosome, or alterations in gene regulation. Richmond and Sykes Class I enzymes are usually inducible with enzyme production under repressor control. They are usually produced at a low basal level until exposure of the bacterium to an inducer upon which enzyme synthesis can be increased over 1,000-fold. After withdrawal of the inducer enzyme synthesis returns to the low basal level. Mutants occur in which the repressor is altered allowing transcription to occur freely and constitutive beta-lactamase production occurs at high levels. The frequency of mutations to the stably derepressed state is often quoted as 10^{-7} to 10^{-9}. In E. cloacae however Korfmann et al. (1986) report a higher range of mutational frequencies of 10^{-4} to 10^{-7}. With some exceptions chromosomally-mediated beta-lactamases tend to be specific for genus, species and subspecies exhibiting only slight variations in isoelectric point. They therefore constitute a potential framework for bacterial classification (Matthew and Harris, 1976; Sykes and Matthew, 1976; Sykes and Smith, 1979).

The universal occurrence of beta-lactamase and the failure of laboratories to produce viable cells which lack beta-lactamase support the suggestion that the enzyme has an essential physiological role (Sykes and Matthew, 1976).

Most chromosomally-mediated beta-lactamases are

cephalosporinases which hydrolyse cephalosporins at least five to ten times as rapidly as benzyl penicillin (Sykes and Smith, 1979). Sawai, Kanno and Tsukamoto (1982) differentiate chromosomal cephalosporinases into two major groups on the basis of substrate specificity:

(a) typical cephalosporinases with negligible penicillinase activity, and

(b) cephalosporinases with broad substrate specificity which hydrolyse cephalosporins and labile penicillins.

Typical cephalosporinases are produced by many species and include the constitutive enzymes produced by E. coli, E. cloacae and B. fragilis and the inducible beta-lactamases of E. cloacae, S. marcescens and P. aeruginosa. Broad spectrum cephalosporinases are typified by the inducible beta-lactamase of P. vulgaris.

Very few strains of Gram-negative bacteria are reported to produce chromosomal penicillinases. Sykes and Matthew (1979) cite reports of penicillinase-producing strains of P. mirabilis, M. morganii (previously known as Proteus. morganii), Pseudomonas thomasii and P. aeruginosa. In the former two organisms however the evidence for the genetic loci being chromosomal is not convincing.

The L-1 beta-lactamase of P. maltophilia is an inducible penicillinase which appears to be chromosom ally mediated. It is not inhibited by clavulanic acid, but is almost completely inhibited by EDTA whereupon activity is recovered following

the addition of zinc ions, and to a lesser extent other divalent cations such as Mn2+ and Ca2+. L-1 is thus a zinc metalloenzyme like beta-lactamase II from B. cereus 569. L-1 is unusual in its substrate profile, hydrolysing imipenem at a significant rate. It is also unusual in appearing to consist of four subunits (Saino et al., 1982).

P. maltophilia contains a second beta-lactamase, L-2, an inducible cephalosporinase. This enzyme significantly hydrolyses monobactams and ceftizoxime which resist most beta-lactamases. It is inhibited by clavulanic acid but not by EDTA. The combination of the L-1 and L-2 enzymes in P. maltophilia may explain the high resistance of this organism to beta-lactam antibiotics (Saino et al., 1984).

Enzymes such as these with unusual hydrolytic activity against imipenem, monobactams, or third generation cephalosporins are rare in clinical specimens at the present time but can be expected to increase in occurrence. These are the previously-mentioned chromosomal CXases of Mitsuhashi (1985) which are divided into two subgroups on the basis of substrate and inhibition profiles (Table 6). Type I CXases are predominantly cephalosporinases whereas Type II CXases are predominantly penicillinases which also hydrolyse imipenem and are not inhibited by clavulanic acid. Apart from the Legionella gormanii enzyme the Type II CXases are metalloenzymes.

Yersinia enterocolitica also exhibits unusual beta-lactamase production with most strains producing two distinct

TABLE 6

Classification of Mitsuhashi (1985) for Chromosomal Beta-lactamases Which Hydrolyse Newer Beta-lactams

Type I CXases	:	Type II CXases
B. fragilis group	:	*F. odoratum*
P. vulgaris GN 7919	:	*P. maltophilia* L-1 enzyme
P. cepacia GN 11164	:	*L. gormanii* ATCC 33297
P. maltophilia L-2 enzyme	:	*B. fragilis* TAL 2480

beta-lactamases which differ in inducibility, substrate profile, sensitivity to p-CMB, molecular weight and isoelectric point (Cornelis and Abraham, 1975).

Occasionally there are differences of opinion about the substrate profiles of chromosomal beta-lactamases. An example is the broad spectrum beta-lactamase of K. pneumoniae which is considered by some workers to be a penicillinase. There is also dispute about the chromosomal beta-lactamases of legionellae which Thornsberry and Kirven (1978) and Fu and Neu (1979) report to be primarily cephalosporinases. However Marre et al. (1982) report high penicillinase activity in all legionellae except L. micdadei which appears to lack a beta-lactamase.

5. Beta-lactamases Mediated by Plasmids and Transposons

Beta-lactamases determined by plasmids and transposons are detected more frequently than chromosomal beta-lactamases. This is because they are constitutively produced in greater amounts than most chromosomal beta-lactamases (Medeiros, 1984; Bakken and Sanders, 1987). Quantitative expression of plasmid-mediated beta-lactamase genes varies widely from strain to strain and species to species e.g. the same plasmid coding for Richmond and Sykes Type Vb enzyme codes for different levels of enzyme expression in E. coli and P. mirabilis (about 1% of the E. coli level) (Richmond and Sykes, 1973).

The various schemes for classifying non-chromosomal beta-

lactamases are particularly confusing (Table 7). Matthew (1979) and Matthew et al. (1979) divide the plasmid-mediated beta-lactamases into three groups on the basis of substrate specificity:

(a) broad spectrum penicillinases,

(b) oxacillinases, and

(c) carbenicillinases.

With certain reservations, it is possible to identify plasmid-mediated beta-lactamases from their iso-electric points, or pI values (Sykes and Smith, 1979). Table 8 lists some of the properties used to characterize these beta-lactamases - isoelectric points, inhibition profiles using cloxacillin, p-CMB and sodium chloride, and molecular weight. Immunological cross-reactivity has been widely used to characterize beta-lactamases but some of the results are confusing, possibly because the beta-lactamase preparations used to raise antisera contained more than one beta-lactamase (Sykes and Matthew, 1976; Medeiros, 1984; Bauernfeind, 1986).

Recent studies of clinical isolates have detected rare novel plasmid-mediated beta-lactamases. Some of these exhibit unusual features. The CEP-2 enzyme discovered in Achromobacter species preferentially hydrolyses cephalosporins (Medeiros, 1984), and the CTX-1 enzyme discovered in K. pneumoniae is more active against third generation cephalosporins than against cephalothin (Sirot et al., 1987).

TABLE 7

Nomenclature of Plasmid-mediated Beta-lactamases*

Matthew	Mitsuhashi	Pitton	Labia & Philippon	Richmond & Sykes
Penicillinases - broad spectrum				
TEM-1	Type 1a	TEM-1 type 1		IIIa
TEM-2	Type-1b			IIIa
SHV-1		TEM-1 type 2		IV
HMS-1				
Oxacillinases				
OXA-1	Type II			Va
OXA-2	Type III			Vb
OXA-3				V
Carbenicillinases				
PSE-1	Type IV		CARB-2	V
PSE-2				V
PSE-3			CARB-4	V
PSE-4			CARB-1	V

* Adapted from Medeiros (1984)

TABLE 8

Some Plasmid- and Transposon-mediated Beta-lactamases[1]

	Inhibited by Clox	pCMB	NaCl	Mol. Wt.	pI	Transposon mediated
Penicillinases-broad spectrum						
TEM-1	+	-	-	22,000	5.4	+
TEM-2	+	-	-	23,500	5.6	+
SHV-1	+	+/-	-	17,000	7.6	+
HMS-1	+	+	-	21,000	5.2	
ROB-1*				19,800	8.1 (5.55)[2]	
LCR-1*				44,000	6.5 (5.85)[3]	
Oxacillinases						
OXA-1	-	+/-	+	23,300	7.4	+
OXA-2	-	-	+	44,600	7.45, 7.7	
OXA-3	-	-	+	41,200	7.1	
OXA-4*	+		-	23,000	7.5	+
OXA-5*	-		+	27,000	7.62	+
OXA-6*	+		-	40,000	7.8	+
OXA-7*	-		+	25,000	7.65	
Carbenicillinases						
PSE-1	-	+		26,500	5.7	+
PSE-2		+		12,400	6.1	
PSE-3		-		12,000	6.9	
PSE-4	-	-		32,000	5.3	+
AER-1*				22,000	5.9	Tn 798

TABLE 8 (continued)

Some Plasmid- and Transposon-mediated Beta-lactamases[1]

	Inhibited by Clox	pCMB	NaCl	Mol. Wt.	pI	Transposon mediated
Cephalosporinase						
CTX-1*					6.3	
CEP-2*				36,200	8.1	

* Novel beta-lactamases

1. From Medeiros (1984), Medeiros et al. (1985), Sirot et al. (1987).
2. 5.55 = additional nonmobile band of E. coli sonicate, possibly membrane-bound or unusually large molecule.
3. 5.85 = pI obtained under different experimental conditions.

Transposons determine a number of the well established plasmid-mediated and novel beta-lactamases of Gram-negative bacteria (Table 8). The mobility of genes on plasmids and transposons enables beta-lactamases to spread to different taxonomic groups of bacteria. This means that the prevalence of different beta-lactamases will vary in time and place. The most frequently isolated strains with novel or more than one plasmid-mediated beta-lactamase are from South American and Asiatic countries - a finding which suggests that indiscriminate antibiotic prescribing in these regions has stimulated the bacterial flora to actively develop new resistance mechanisms (Medeiros, 1984).

6. Beta-lactamases and Antibiotic Resistance of Gram-negative Bacteria

In determining the contribution of beta-lactamases to the resistance of an organism it is necessary to consider the interaction of beta-lactamases, outer membrane permeability, and PBP alterations. In many Gram-negative organisms the beta-lactamase is only a contributor to a highly complex defence system protecting the cell from the effects of beta-lactam antibiotics (Sykes and Matthew, 1979). While high levels of beta-lactamase expression usually have a marked effect on resistance one cannot discount the role of very small quantities of beta-lactamase as very low enzyme levels have been shown to significantly contribute to resistance (Richmond and Sykes, 1973). Even low levels that may only be detected by IEF may be important (Nikaido, 1985).

TABLE 9

Some Beta-lactamases Which Hydrolyse the New Beta-lactam Antibiotics

Antibiotic	Beta-lactamase Type	
	Chromosomal	Plasmid
Cefuroxime or Cefotaxime	*P. cepacia* GN 11164	OXA-1
	P. vulgaris GN 7919	OXA-4
	B. fragilis MULB-1008	OXA-5
	K. oxytoca R 30	OXA-6
	K. aerogenes K1 (indole +)	OXA-7
	MULB 906	PSE-1
	L. gormanii	PSE-2
		CTX-1
Ceftazidime	*K. oxytoca*	OXA-1
		OXA-2
Moxalactam	*K. oxytoca* 3859	OXA-4
	MULB 906	OXA-5
	L. gormanii	PSE-2
		PSE-3
Monobactams	*K. oxytoca* 3859	
	K. aerogenes K-1 (indole +)	
	P. maltophilia L-2	
	K. pneumoniae SC 10,436	
Imipenem	*P. maltophilia* GN 12873	
	L. gormanii	

Adapted from Levesque *et al.* (1983), Medeiros (1984), Saino *et al.* (1983), Sirot *et al.* (1987).

e. The Genus Flavobacterium

1. Introduction

The genus Flavobacterium (Skerman et al., 1980; Holmes, Owen and McMeekin, 1984) is somewhat of a 'phylogenetic pot pourri' (Paster et al., 1985). Bacteria recognised by Holmes, Owen and McMeekin (1984) in Volume 1 of Bergey's Manual of Systematic Bacteriology and by Holmes et al. (1983) and Holmes, Owen et al. (1984a) as authentic Flavobacterium species are aerobic, yellow-pigmented, non-fermentative, nonmotile, oxidase-positive Gram-negative rods with a low DNA mol% G+C (range 31-46). However the Approved Lists of Bacterial Names (Skerman et al., 1980) includes a number of additional "flavobacteria" which clearly do not comply with the current genus description e.g. Gram-positive, motile, gliding and high G+C species. An additional problem is the decision of the Judicial Commission not to endorse the proposal (Holmes and Owen, 1979) to make Flavobacterium breve the type species of the genus (Wayne, 1982), but instead to retain as type species Flavobacterium aquatile, an organism of unclear relationship to other organisms in the genus. In all the taxonomy of Flavobacterium is confused and refractory and there is considerable scope for clarification, reform and reconciliation.

There are also unresolved phylogenetic considerations. Recent genetic evidence confirms close relationships between Flavobacterium and Cytophaga, a genus containing bacteria which glide on solid surfaces (Callies and Mannheim, 1980;

Bauwens and de Ley, 1981) and there is evidence of a previously unsuspected but specific genetic relationship between these bacteria and the anaerobic genus Bacteroides (Páster et al, 1985; Weisburg et al., 1985). The relationships of Flavobacterium species to gliding bacteria and Bacteroides species therefore require clarification. It appears likely that the resolution of these issues will lead to major taxonomic reform, possibly with the removal of previously "firm" taxonomic boundaries based on gliding motility.

Our understanding of the roles of Flavobacterium species is limited. They can be however of considerable environmental and clinical importance. They are known to cause food spoilage, degrade a wide range of environmental and man-made substances (e.g. sewerage, pesticides and petroleum products) and cause uncommon but extremely antibiotic resistant, infections - some of which are associated with high mortality rates (Du Moulin, 1979; Shewan and McMeekin, 1983; Holmes, 1987).

2. Taxonomic History

The genus Flavobacterium has been heterogeneous since its inception in the first edition of Bergey's Manual (Bergey et al., 1923) when flavobacteria often had little more than colour in common. This heterogeneity was reduced in subsequent editions of Bergey's Manual with the deletion of polarly flagellated species in 1939 (Bergey et al.), Gram-

positive bacteria in 1957 (Breed et al.), and organisms exhibiting gliding motility and spreading growth in 1974 (Weeks). The latter revision left the genus in two distinct sections with Section I containing nonmotile low mol% DNA strains (range 32-43% G+C) and Section II containing high mol% DNA strains (range 63-70% G+C) which were either motile or nonmotile (Weeks, 1974). In the 1st edition of Bergey's Manual of Systematic Bacteriology Holmes, Owen and McMeekin (1984) restrict the genus to low %G+C strains. Currently there are ten species, the heterogeneous taxon (Group IIb), and additional strains which do not belong to currently described taxa (Table 10).

Environmental isolates, which grow at temperatures from 5-30°C, are frequently difficult to identify whereas clinical isolates, which are usually capable of growth at 37°C, are easier to accomodate in described species (Owen and Holmes, 1981; Thomson and McMeekin, 1981; Holmes, Owen and McMeekin, 1984).

Special genetic procedures have recently provided valuable insights into the phylogenetic relationships of the genus. There are three main procedures, each having a different role - DNA:DNA and DNA:rRNA hybridizations and comparative analysis of oligonucleotides of 16S rRNA. The DNA:DNA reassociation technique is useful for investigating closest relationships. DNA:rRNA hybridization is used mainly to determine relationships at the intra-family level. Oligonucleotide sequence analysis allows detection of distant phylogenetic relationships (Ludwig et al., 1983).

3. Testing for Special Characters

In routine laboratories Flavobacterium species may be misidentified as other taxa and vice versa. The separation of Flavobacterium from other taxa, particularly the yellow-pigmented high %G+C bacteria and gliding bacteria, may be difficult. Certain tests are especially useful for differentiation but however may be difficult to assess. The problems associated with these tests are reviewed briefly in the following.

Pigmentation

The yellow pigment which is characteristic of the genus Flavobacterium is now thought to be of the flexirubin type described by Reichenbach et al. (1981). The hue and intensity of pigmentation of different strains varies considerably and may be influenced by the growth medium, temperature and duration of incubation, and by exposure to daylight (Holmes, Owen and McMeekin, 1984). Pigmentation may be enhanced by growth on casein, milk and starch agar. Flexirubins are widely distributed among strains of Flexibacter, Cytophaga and Flavobacterium (Fautz and Reichenbach, 1980; Reichenbach et al., 1980; Reichenbach et al., 1981; Weeks, 1981).

Gram Reaction and Morphology

Some Gram-positive bacteria are easily decolourised during Gram staining and may appear to be Gram-negative (Hayes et al., 1979). Weeks (1974) recommends the Kopeloff-Beerman

TABLE 10

Species of the Genus Flavobacterium and Group IIb

F. aquatile	F. odoratum
F. breve	F. multivorum
F. balustinum	F. spiritivorum
F. gleum	F. thalpophilum**
F. meningosepticum	Group IIb

Species Incertae Sedis

F. acidificum	F. indoltheticum
F. acidurans	"F. lutescens"
F. capsulatum	F. oceanosedimentum
F. devorans	F. okeanokoites
F. esteraromaticum	F. resinovorum
F. ferrugineum	"F. rigense"
F. halmophilum	"F. tirrenicum"
F. heparinum	F. uliginosum

Data of Holmes et al. (1983), Holmes, Owen and McMeekin (1984) and Holmes, Owen et al. (1984)

** Data of Holmes et al. (1983)

modification of Gram's procedure (Conn, 1957). Confirmation is possible by testing for growth on crystal violet agar (2.5 x 10^{-5} and 1 x 10^{-6} w/v) or by treating a thick cell suspension with a few drops of either sodium dodecyl sulphate (10% w/v) or potassium hydroxide (3% w/v) which will rapidly lyse Gram-negative cells to markedly increase viscosity (Holmes, Owen and McMeekin, 1984a).

Gliding Motility and Spreading Growth

The demonstration of gliding motility and spreading growth on solid media, which distinguish Flavobacterium from Cytophaga and Flexibacter, presents difficulties of technique and interpretation. It is essential to culture the organism on a medium of low nutrient concentration such as that of Anacker and Ordal (1959). Gliding motility is enhanced by surface moisture with best results obtained on freshly poured plates incubated in a humid atmosphere (Henrichsen, 1972). Gliding may be observed by microscopic examination of the margin of growth using a high power dry lens (Henrichsen, 1972) or by adding a coverslip and using oil (Hayes, 1977). Perry (1973) recommends placing small glass beads on the agar to provide pools of liquid of varying depths before overlaying with a coverslip.

The interpretation of gliding varies. Henrichsen (1972) defines gliding as the orientation of bacteria into organised bundles with continuous movement in the direction of the long axis. Perry's (1973) definition includes movement which is not continuous in one direction. He therefore records Flavobacterium aquatile as a glider, a controversial

interpretation at variance with other workers and the subject of considerable taxonomic speculation (Holmes, Owen and McMeekin, 1984a, b).

In theory spreading colonies should be the manifestation of gliding motility. However this is not always the case. NCIB 9059 "Flavobacterium pectinovorum" (now regarded as Cytophaga johnsonae) shows true gliding motility but fails to spread (Lund, 1969; McMeekin et al., 1971; Perry, 1973; Hayes, 1977). This means that the observation of spreading growth alone is not sufficient to detect gliding motility. It is therefore necessary to use microscopic observation for reliable detection of this character.

DNA Base Composition

The accurate determination of DNA base composition requires special equipment. For routine differentiation of high G+C and low G+C strains it is convenient to use the ultraviolet (UV) light sensitivity test of McMeekin (1977). By calibrating a UV light source with known high and low G+C strains such as Pseudomonas paucimobilis (high G+C) and Flavobacterium breve (low G+C), it is possible to determine presumptively in which G+C category unknown strains belong. For example, McMeekin (1977) reports that by using an exposure time of 90 seconds, low G+C strains suffer at least a 10^5-fold kill whereas high G+C strains suffer less than a 10^3-fold kill when placed 55 cm from a 15 watt germicidal lamp in a laminar flow cabinet prior to incubation.

Antibiotic Resistance

Major discrepancies have been noted when disc diffusion and MIC techniques have been used in parallel for F. meningosepticum (Maderazo et al., 1974; Aber et al., 1978; Winslow and Pankey, 1982; Johny et al., 1983; Bruun, 1987) and Flavobacterium Group IIb (Von Graevenitz and Grehn, 1977). Von Graevenitz and Grehn (1977), Winslow and Pankey (1982) and Johny et al, (1983) conclude that disc diffusion testing is likely to give a false impression of susceptibility and recommend that susceptibility detected by disc testing should be confirmed with MIC tests.

Bruun (1987) however suggests that MIC testing is unnecessary for F. meningosepticum as only two agents, vancomycin and fusidic acid, are responsible for major discrepancies and that accuracy can be achieved with disc tests if the interpretive criteria are modified.

In this review results achieved with different testing methodologies, some of which are now considered suboptimal, have been combined. This was necessary to compile representative antibiograms for individual species from published reports many of which comprised only single or small numbers of strains. For the purpose of consistency and to avoid contradictions MICs have been interpreted according to NCCLS criteria (Thornsberry et al., 1985) which in some cases vary from the criteria originally reported. In some cases this has meant that some strains originally classified as susceptible are now classified otherwise. The requirement to analyse the literature in this manner points to the need for a

comprehensive study of the antimicrobial susceptibility of the genus using standardized methods and current antimicrobials.

4. Flavobacterium aquatile

Controversy surrounds F. aquatile, which is the type species of the genus despite never being formally proposed as such (Holmes and Owen, 1979). Only one strain (not an original strain) is maintained in culture collections - strain Taylor F36 (Weeks, 1955) - and this strain does not conform to the description of F. aquatile which is described as motile and peritrichous (Bergey et al., 1923). A further problem is evidence that strain Taylor F36 may be an atypical cytophaga and not a strain of Flavobacterium (Shewan and McMeekin, 1983; Holmes, Owen and McMeekin, 1984). For these reasons Holmes and Owen (1979) regard F. aquatile to be so inappropriate as type species that they propose rejection of its name as a nomen dubium and its replacement as type species by F. breve with strain NCTC 11099 as the neo-type strain. This proposal was considered and rejected by the Judicial Commission (Wayne, 1982).

The evidence for a relationship with cytophagas is strong. Numerical taxonomic studies of phenotypic characteristics (Hayes, 1977), cellular fatty acids (Oyaizu and Komagata, 1981), chemotaxonomic studies of rRNA cistrons (Bauwens and de Ley, 1981), oligonucleotide cataloguing (Paster et al., 1985) and electron microscopic observations of nonflagellar appendages (Thomson et al., 1981) all indicate that F.

aquatile is more closely related to strains of Cytophaga than to Flavobacterium species.

5. Flavobacterium breve

F. breve and F. aquatile have similar taxonomic histories. They are the only species of Flavobacterium surviving from the first edition of Bergey's Manual (Bergey et al., 1923). Both present problems in that the original descriptions were meagre and none of the original strains are extant. The current revised description of F. breve is based on the study of Holmes et al. (1978) of strain ATCC 14234, a fish isolate described in the 8th edition of Bergey's Manual of Determinative Bacteriology (Weeks, 1974), and six subsequent isolates.

DNA reassociation studies of ten strains of F. breve confirm that it "constitutes a relatively homogeneous species" and is distinguishable from allied bacteria (Owen and Holmes, 1980). However only six of the ten strains are closely related while four, including ATCC 14234, show a high degree of base sequence divergence (48% divergence or greater). This genetic heterogeneity is attributed to the differences between strains of human clinical origin and those of animal origin (Owen and Holmes, 1980), a conclusion which is debatable since the homogeneous group of six strains is not entirely of human origin (five clinical strains and a strain from a snake) and the other group of strains is not entirely of animal origin (one clinical and three nonclinical strains). Inferences about the environmental determination of genetic heterogeneity

are difficult to justify on the basis of such small numbers of strains.

The choice of clinical strain NCTC 11099 as the neotype strain of F. breve on the grounds that it is more representative than the previously described nonclinical strain ATCC 14234 represents a recent trend in Flavobacterium taxonomy. Since Holmes and various co-workers began the task of revising existing species descriptions and creating new species, descriptions of the species of Flavobacterium have been based largely on studies of small numbers of mainly clinical isolates (Holmes et al., 1977; 1978; 1981; 1982; 1983; Holmes, Owen et al., 1984). While this might seem oblivious of the fact that flavobacteria are only rarely isolated from clinical specimens (Von Graevenitz, 1981) and the large majority of strains occur in nonclinical environments, the reason for this trend is that nonclinical taxonomic workers have been relatively inactive and there has been a paucity of information about nonclinical strains. The taxonomy of the genus has thus moved by default in a clinical direction (Thomson and McMeekin, 1981). The inherent danger of this, i.e. developing a classification based on a minor, unrepresentative portion of the genus, has been realised in that a large number of environmental strains have proved impossible to identify using current species descriptions (Owen and Holmes, 1981; Thomson and McMeekin, 1981).

Viewed in this context and with the benefit of hindsight it could be argued that the decision not to select the environmental ATCC 14234 as type strain of the species because

TABLE 11

Susceptibility of F. breve to

Antimicrobial Agents

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1. Agents to Which Most Strains are Susceptible

cephaloridine

chloramphenicol

erythromycin

2. Agents to which most Strains are Resistant

ampicillin	gentamicin
amikacin	kanamycin
carbenicillin	penicillin
cefamandole	polymyxin B
cephalothin	rifampicin
	tobramycin

3. Agents to Which Susceptibility is Variable

cefoxitin	sulphonamides
moxalactam	tetracycline
piperacillin	

Data of Holmes et al. (1978), Jooste et al. (1985) and Gilardi (1987).

it is 'unrepresentative' was ill-considered.

The original "short canal bacillus" (F. breve) described by Mori (1888) was pathogenic for guinea pigs, mice and rabbits, but not for pigeons. Isolates conforming to the current species description (F. breve) are not known to be pathogenic to man but have been isolated from a wide range of clinical specimens. Their resistance to most antibacterial agents indicates that treatment would be difficult if F. breve is found to cause infections (Table 11). The nonclinical strain ATCC 14234 however is far more susceptible (Holmes et al., 1978; Jooste et al., 1985; Gilardi, 1987).

In a numerical taxonomic study which included antibiotic susceptibility tests, Jooste et al. (1985) found that NCTC 11099 and nine dairy strains of F. breve formed a well defined and distinct cluster only when data from the antibiotic susceptibility tests were included. This implies that antibiotic resistance is an important taxonomic attribute of F. breve and may explain why Holmes et al. (1986) failed to identify correctly five out of fourteen known F. breve strains using a computer-based probabilistic method which lacked antibiotic susceptibility data.

6. Flavobacterium balustinum (and Group IIb)

The the original description of F. balustinum (Harrison, 1929) needs revision (Holmes and Owen, 1981). At present strain NCTC 11212 is the only authentic strain. Biochemically F. balustinum is regarded by some workers as indistinguishable

from Group IIb but different in having a lower %G+C (Holmes and Owen, 1981). Other workers however report numerical taxonomic studies in which F. balustinum fails to cluster with Group IIb strains (Yabuuchi et al., 1983; Jooste et al., 1985). The latter workers clearly differentiated the F. balustinum-containing cluster from clusters containing the type strain of F. gleum and Group IIb strains only when antibiotic susceptibility data were included in the comparisons. The F. balustinum-containing cluster was much more susceptible to streptomycin, carbenicillin, bacitracin, tetracycline and actinomycin D. This is further evidence of the taxonomic significance of antibiotic resistance in Flavobacterium.

The name Group IIb (also known as King's or Weaver's or CDC Group IIb or Pickett and Pedersen's group 2) was initially given to nine clinical strains similar to F. meningosepticum (King, 1959). Group IIb has subsequently become a taxonomic repository for difficult-to-classify, heterogeneous, saccharolytic Flavobacterium strains and probably contains several species (Owen and Holmes, 1978). However the matching of phenotypic and genotypic differences to delineate the species within this group has so far proven difficult (Shewan and McMeekin, 1983). Perhaps this stems from a reluctance to include antimicrobial susceptibility data in species descriptions. To date F. balustinum and F. gleum have been delineated from within Group IIb and other taxa are in the process of being defined (Holmes, Owen et al., 1984).

Not all workers agree with Owen and Holmes (1978) and Holmes, Owen et al. (1984) that it is possible to identify species within Group IIb. Price and Pickett (1981) report that despite Group IIb's considerable heterogeneity it is impossible to find even two tests by which to delineate phenotypic subgroups. For this reason they consider it a single species for which they propose the name "F. aureum". Yabuuchi et al. (1983) also consider Group IIb a single species and propose the name "Flavobacterium indologenes". Holmes et al. (1986) advocate disregard for these proposals and retention of the original designation, Flavobacterium Group IIb.

In many respects Group IIb resembles F. meningosepticum (Table 18). Although Group IIb is more heterogeneous both taxa have similar biochemical profiles, fatty acid patterns and antimicrobial susceptibility patterns. Their main difference is in hue of pigmentation with Group IIb exhibiting a bright yellow pigment while F. meningosepticum is only weakly yellow (von Graevenitz and Grehn, 1977; Moss and Dees, 1978; Owen and Holmes, 1981).

Group IIb is the probably the most frequently isolated Flavobacterium from clinical environments. It causes rare systemic and soft tissue infections in compromised patients. Widespread skin and respiratory tract colonisation may occur (Owen and Lapage, 1974; Stamm et al., 1975; Monteil et al., 1979; Zajc-Satler et al., 1979; Owen and Holmes, 1981; Von Graevenitz, 1981). In one study Du Moulin (1979) reported the airway colonisation of 195 seriously ill patients in a

TABLE 12

Susceptibility of Group IIb to Antimicrobial Agents

1. Agents to Which Most Strains are Susceptible

cefoperazone	ceftriaxone
cefotetan	moxalactam
cefoxitin	nalidixic acid
ceftazidime	vancomycin
ceftizoxime	

2. Agents to Which Most Strains are Resistant

ampicillin	kanamycin
aztreonam	nitrofurantoin
carbenicillin	penicillin G
cefamandole	polymyxin B
colistin	streptomycin

3. Agents to Which Susceptibility is Variable

amikacin	erythromycin
cefotaxime	gentamicin
cephalothin	imipenem
chloramphenicol	neomycin
clindamycin	sulphonamides
cotrimoxazole	tetracycline

Data of Du Moulin, 1979; Price and Pickett, 1981; O'Donnell et al., 1982; Yabuuchi et al., 1983; Husson et al., 1985; Schell et al., 1985.

respiratory-surgical unit over a 70-month period with an organism which was assumed to be Group IIb. However from its description it could have been either Group IIb or F. gleum. Epidemiological studies suggested that the municipal water supply was the source of the organism and that prior antibiotic therapy made the patients susceptible to colonization.

Group IIb is usually resistant to a wide range of antibacterial agents. Cefoxitin and some of the third generation cephalosporins are reported to have good in vitro activity (Table 12).

7. Flavobacterium gleum

F. gleum was described by Holmes et al. (1984b) on the basis of 12 Group IIb strains which were phenotypically and genetically homogeneous. Most strains were clinical isolates of unknown significance with four of the 12 from vaginal specimens. Phenotypically F. gleum is similar to F. balustinum, F. meningosepticum, F. indoltheticum and Group IIb. From Du Moulin's description (1979) it is possible that F. gleum was involved in airway colonisation of 195 patients in a respiratory-surgical intensive care unit. Features which distinguish F. gleum from the other Flavobacterium species are listed in Table 18. Differentiation from Group IIb is not easy however because there are no tests for which both taxa are clearly different.

8. Flavobacterium meningosepticum

King (1959) proposed the name F. meningosepticum for an organism previously designated as Group IIa, which was known to cause meningitis in the new-born (Shulman and Johnson, 1944; Brody et al, 1958; Vandepitte et al., 1958). Unlike most other Flavobacterium species, F. meningosepticum may not produce conspicuous pigmentation - a feature which helps to distinguish it from the biochemically similar taxa F. balustinum, F. gleum and Group IIb (Holmes, Owen and McMeekin, 1984; Holmes, Owen et al., 1984). Currently fifteen serovars (A to O) of F. meningosepticum are described (Holmes, 1987).

The species is genetically heterogeneous. Using DNA:DNA reassociation studies Sottile et al. (1973) and others (Owen and Snell, 1976; Callies and Mannheim, 1980; Ursing and Bruun, 1987) report that the type strain, NCTC 10016, is genetically atypical of the species. From a study of 52 strains Ursing and Bruun (1987) report two main hybridization groups within the species showing 40-55% relatedness. NCTC 10016 belongs to the minor group of four strains. Of particular clinical interest is the report that the larger group of 48 strains contained four subgroups with two of the subgroups containing 95% of the strains isolated from cerebrospinal fluid (CSF) (Ursing and Bruun, 1987; Bruun and Ursing, 1987). This finding suggests that these two subgroups have an increased pathogenic potential. While there is currently insufficient phenotypic evidence to divide F. meningosepticum into further species the CSF strains were different from other strains in two characteristics. They produced a weak yellow pigment and grew

at 40°C whereas non-CSF strains were non-pigmented and failed to grow at 40°C (Bruun and Ursing, 1987). A less clear-cut difference was noticed in susceptibility to antimicrobial agents. The CSF strains tended to more susceptible to beta-lactam antibiotics and more resistant to most other agents (Bruun, 1987).

Because of the potentially serious outcome of infections with F. meningosepticum, especially neonatal meningitis, it seems important to investigate this further. If future taxonomic studies delineate the pathogenic strains as a distinct species it will be possible to target it in surveillance studies and devise strategies to prevent or control its spread.

The type strain NCTC 10016 is also atypical in its fatty acid pattern (Fautz et al., 1981) and it failed to cluster with other Flavobacterium strains in phenotypic analyses (McMeekin et al., 1972; Thomson 1982). Thus from a number of lines of evidence it is unrepresentative of the species. Strain NCTC 10585, on the other hand, is highly related to the majority of F. meningosepticum strains and would perhaps be a more appropriate choice for type strain (Owen and Snell, 1976). An additional doubt about the taxonomic position of NCTC 10016 is the light microscopic observations of Webster and Hugh (1979) that it possesses nonfunctional flagella. This however is not confirmed by Thomson et al. (1981), using electron microscopy, and until proven otherwise NCTC 10016 should continue to be regarded as a non-flagellate bacterium.

Clinically F. meningosepticum causes meningitis in neonates and, less frequently, in adults (many authors, reviewed by Holmes, 1987). It also causes bacteraemia (George et al., 1961; Olsen et al., 1965), nosocomial pneumonia (George et al., 1961; Teres, 1974), subacute bacterial endocarditis (Werthamer and Weiner, 1972; Yamakado et al., 1975), endophthalmitis following keratoplasty (Le Francois and Baum, 1976), bovine endocarditis (Giorgi et al., 1975) and feline meningitis (Sims, 1974).

In neonatal meningitis the fatality rate is about 55%. Hydrocephalus is a complication in most survivors and it is rare to have no sequelae (Von Graevenitz, 1981; Holmes, 1987). Premature infants, defined as birth weight of less than 2500 grams, appear to be particularly vulnerable and comprise more than half the reported cases with mean onset age of 8 days (Eeckels et al., 1965). It is not confined to premature infants however and is reported in infants up to the age of 19 months (Zappula et al., 1979).

The high fatality rate in neonatal meningitis is probably a consequence of the resistance of F. meningosepticum to a wide range of antibiotics. Prior to the 1980s most strains of F. meningosepticum were found to be susceptible only to agents such as erythromycin, novobiocin and vancomycin which are commonly used against Gram-positive organisms (Holmes, 1987). Empiric therapy based on the microscopic observation of Gram-negative rods was therefore unlikely to indicate the need for these drugs and correct therapy would have been delayed until

completion of accurate antibiotic susceptibility tests.

Asymptomatic upper respiratory tract colonisation of neonates is common during epidemics of F. meningosepticum meningitis (Brody et al., 1958; Cabrera and Davis, 1961; Seligmann et al., 1963; Hazuka et al., 1977). Colonisation is more prolonged in those receiving specific antibiotic therapy (Hazuka et al., 1977). However not all respiratory isolates are harmless. F. meningosepticum is reported as the causative agent of pneumonia in five infants, four of whom died. These cases occurred during a nursery outbreak of meningitis due to serovar C. All five cases yielded positive blood cultures and one had meningitis (George et al., 1961).

F. meningosepticum infections in older persons are rare and usually milder. Infected adults tend to be debilitated or immunocompromised (Lapage and Owen, 1973; Mani et al., 1978; von Graevenitz, 1981; Burnakis et al., 1986; Hirsch et al., 1986). Despite the organism's opportunistic predisposition it has not yet been reported as a pathogen in AIDS patients. Not all adult cases are mild. Four of six adults with meningitis due to F. meningosepticum died (Holmes, 1987). Chloramphenicol treatment is reported as successful in nosocomial pneumonia in a fourteen year old girl who received ampicillin prior to isolation of F. meningosepticum (Teres et al., 1974). Nosocomial pneumonia is also reported in a 45-year old patient with poliomyelitis on a respirator (Nadarajah and Tan, 1979). Forty three strains are reported in sputa and tracheal exudates of 30 patients in an intensive care unit

(Richard et al., 1979).

In reviewing the clinical literature Holmes (1987) comments that "F. meningosepticum strains isolated from blood cultures are generally of no clinical significance". This observation is qualified by noting however that "significant isolates have been reported in association with neonatal meningitis". This generalization is at variance with the author's review in which he cites fatal cases of nosocomial pneumonia in infants (George et al., 1961) and a fatal case of subacute bacterial endocarditis in a previously healthy 18-year old female (Yamakado et al., 1975), all of these fatalities being accompanied by positive blood cultures. While not resulting in fatalities there are other reports of significant bacteraemias (Burnakis et al., 1986; Hirsch et al., 1986). In view of these cases it would be prudent not to ignore F. meningosepticum in blood cultures.

Wound isolates of F. meningosepticum are reported in association with contaminated chlorhexidine solutions (Coyle-Gilchrist et al., 1976). Urinary isolates are rare and generally not significant. Out of a total of 27,600 genital tract specimens, 88 strains of F. meningosepticum were isolated, mainly from the female urethra (Olsen and Ravn, 1971). No clinical significance was attributed to F. meningosepticum in these cases but the possibility of the female genitals being a source of nosocomial infection with F. meningosepticum was conjectured.

The previously mentioned suggestion that pathogenic

strains of F. meningosepticum may differ from non-pathogenic strains in ability to grow at 40°C (Bruun and Ursing, 1987) is supported by other data. Olsen (1966) reports that seven environmental strains and three strains from the blood of adult hyperthermic patients with self-limiting disease fail to grow at 38°C whereas King's original serotype A-F strains from infant meningitis and bacteraemia grow at temperatures up to 41°C. In this study the non-meningitis/bacteraemia strains were from a temperate climate whereas the disease-causing strains were from warmer climates. In Holmes' review (1987) the majority of cases of neonatal meningitis were from warmer climates. Another interesting point is that Holmes quotes strong evidence that the pathogenic strains were of environmental (waterborne) origin rather than maternal origin. The evidence therefore points to geography being important in the epidemiology of F. meningosepticum neonatal meningitis with pathogenic strains being derived from waters of countries with warm climates (Olsen, 1966).

In meningitis cases intravenous erythromycin therapy can be ineffective and resistance is known to develop (George et al., 1961; Hazuka et al., 1977; Rios et al., 1978; Ferlauto and Wells, 1981). However successful therapy with intrathecal erythromycin and intrathecal vancomycin is reported (Plotkin and McKitrick, 1966), as is successful intrathecal and intravenous therapy with erythromycin (Maderazo et al., 1974) and cotrimoxazole (Lapage and Owen, 1973). Intravenous and intraventricular rifampicin is also reported to be successful (Chandrika and Adler, 1982). Intravenous cotrimoxazole (Johny

et al., 1983) and combined intravenous erythromycin and clindamycin (Burnakis et al., 1986) have been successful. Although some strains are susceptible to chloramphenicol its failure in combination with spiramycin is reported (Agarwal and Ray, 1971).

It appears that intrathecal or intraventricular administration is necessary for reliable attainment of therapeutic CSF drug levels with these agents. Some of the newer beta-lactam agents may however prove more successful in coupling good activity against F. meningosepticum (Table 13) with better CSF penetration. Experience to date however emphasizes the need to be aware of:

- the capability of F. meningosepticum to develop resistance during therapy,
- the possibility of false susceptibilities being indicated by disc diffusion testing,
- the possible need for intrathecal administration, and
- the need for frequent monitoring of CSF drug levels

(Holmes, 1987). Even though some of the new agents show good in vitro activity it would be wise to give consideration to these matters until the efficacy of the new agents is proven. The high mortality rate in meningitis cases precludes complacency about the treatment of this condition when it is caused by F. meningosepticum.

Details of resistance mechanisms in F. meningosepticum are largely unknown at present, especially mechanisms by which resistance develops during therapy. Hazuka et al. (1977) report the step-wise development of resistance to

sulphisoxazole, erythromycin and vancomycin during, or shortly after, a patient's therapy with these agents. Cross-resistance to clindamycin also emerged concurrently. Although the isolates were referred to the Antimicrobics Investigations Section at the Centre for Disease Control (CDC) in Atlanta the only investigations performed by CDC were confirmatory MICs. No assessment of resistance mechanisms was made. Hazuka *et al.* (1977) postulated the acquisition of a resistance factor from some other organism in the environment. However this is most unlikely as no other organisms were cultured from the patient's spinal fluid and the stepwise increments in resistance suggest the occurrence of at least three separate events. It therefore remains to be established how *F. meningosepticum* can develop resistance to sulphisoxazole, erythromycin, vancomycin and clindamycin during therapy.

In other Gram-negative rods erythromycin resistance can develop in Enterobacteriaceae as a result of decreased cell wall permeability, alteration of the target site, or drug inactivation. In addition resistance to either one group of agents or cross resistance to all macrolides, lincosamines and streptogramines can occur in *E. coli* and *B. fragilis* by alteration of 23S ribosomal RNA which leads to reduced affinity between the antibiotics and the ribosome. The resistance can be either inducible or constitutive and may be temperature dependent (Callihan *et al.*, 1984; Weisblum, 1984; Welch and Southern, 1984; Courvalin *et al.*, 1985). No mechanisms of vancomycin resistance are known (Crossley, 1986). Perhaps mechanisms of these types occur in *F.*

TABLE 13

Susceptibility of F. meningosepticum

to Antimicrobial Agents

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1. Agents to Which Most Strains are Susceptible

cefoperazone	doxycycline	novobiocin
cefoxitin	lincomycin	piperacillin
ceftizoxime	mezlocillin	rifampicin
ciprofloxacin	minocycline	triacetyloleandomycin
cotrimoxazole		

2. Agents to Which Most Strains are Resistant

ampicillin	cephalexin	nitrofurantoin
aztreonam	cephaloridine	oxacillin
bacitracin	cephalothin	penicillin G
carbenicillin	cloxacillin	polymyxin B
cefamandole	colistin	spectinomycin
cefazolin	imipenem	temocillin
cefsulodin	kanamycin	tobramycin
ceftazidime	methicillin	viomycin
cefuroxime	netilmicin	

TABLE 13 (cont)

3. Agents to Which Susceptibility is Variable

amikacin	fusidic acid	streptomycin
cefotaxime	gentamicin	sulphonamides
chloramphenicol	moxalactam	tetracycline
clindamycin	nalidixic acid	vancomycin
erythromycin	neomycin	

Data of King (1959); Olsen et al. (1965); Watson et al. (1966); Olsen (1969); Altmann and Bogokovsky (1971); Werthamer and Weiner (1972); Lapage and Owen (1973); Coyle-Gilchrist et al. (1976); Hazuka et al. (1977); Aber et al. (1978); Mani et al. (1978); Price and Pickett (1981); Igari et al. (1983); Johny et al. (1983); Strandberg et al. (1983); Yabuuchi et al. (1983); Burnakis et al. (1986); Raimondi et al. (1986); Bruun (1987); Gilardi (1987).

meningosepticum. However the information provided by Hazuka et al. (1977) is too scanty for evaluation and the development of resistance to vancomycin is suggestive of a previously unrecognised mechanism.

Kelsey et al. (1982) report increasing rifampicin MICs (from 1 to 128 ug/ml) in a patient with F. meningosepticum ventriculitis. The organism could not be eradicated from the CSF with combination rifampicin and erythromycin therapy but was eradicated with combination mezlocillin and cefoxitin therapy.

On the basis of the rarity of detectable plasmids and the broadness of antibiotic resistance, Holmes, Owen and McMeekin (1984) suggest that chromosomal genes may be involved in the resistance of Flavobacterium strains.

There is only one report of beta-lactamases in F. meningosepticum. Raimondi et al. (1986) report three different beta-lactamases in seven strains of F. meningosepticum, including reference strain NCTC 10585. The enzymes have a broad spectrum of hydrolytic activity including significant activity against the 7-beta-aminothiazole cephalosporins but are primarily penicillinases. They have similar molecular weights by gel filtration (approximately 20,000 daltons) but differ in isoelectric patterns. Four strains have beta-lactamases with a major band at pI 7.9 and two minor bands for which pI values are not reported. A further two strains have a major band at pI 7.8 with a minor band at 8.2 and the remaining strain has a major band at 7.65

(pers. comm.) and minor bands at 7.6 and 8.0. The beta-lactamase of strain NCTC 10585 is not inducible. All beta-lactamases are regarded as chromosomal because plasmids have not been detected in these strains.

These enzymes are inhibited by clavulanic acid, cloxacillin and pCMB. The addition of clavulanic acid (2 mcg./ml.) to various beta-lactam antibiotics reduces MIC values to varying degrees. Cephalosporin MICs, particularly ceftazidime, are reduced much more than penicillin MICs by clavulanic acid. This is unusual in an enzyme which is predominantly a penicillinase and is thought by Raimondi et al. (1986) to be possibly due to the antibacterial activity of a subinhibitory amount of clavulanic acid. (All strains have an MIC of 8 mcg./ml. for clavulanic acid).

Raimondi et al. (1986) conclude that F. meningosepticum possesses a class IV enzyme of the Sykes and Matthew (1976) classification (presumably the same as class IV of the Richmond and Sykes classification) because it is chromosomally mediated, constitutive, has a broad substrate profile and is inhibited by pCMB and clavulanic acid.

9. Flavobacterium odoratum

Bacterium faecale aromaticum (Stutzer, 1923), later renamed F. odoratum (Stutzer and Kwaschnina, 1929) was originally isolated from the faeces of patients with intestinal infections. The name subsequently lapsed until Holmes et al. (1977) revived it and proposed extant reference

strain ATCC 4651 (NCTC 11036) as neotype strain.

Owen and Holmes (1978) report DNA relatedness of 7-100% in a study of ten strains of F. odoratum. Seven of the strains form a group with G+C of 31-32% and greater than 80% base pairing. Within this group two colony types occur after 24 hours incubation on nutrient agar at 37°C. These are:

Colony type 1: effuse, spreading, 3-4 mm diameter, raised, shiny centre and dull matt surface.

Colony type 2: like type 1 but smaller 1.0-1.5 mm and growth less luxuriant.

The second group of three strains, which includes the neo-type strain, has G+C of 34-36% with less than 28% of nucleotide sequences in common with the first group. These strains produce a third colony type:

colony type 3: smooth, shiny, convex, 0.5-1.0 mm, no spreading edge but after 24 hr like type 1.

On the basis of this heterogeneity Holmes, Owen and McMeekin (1984) suggest that it may be warranted to divide F. odoratum into two or three species. Considering that the G+C range of this species (4.7 mol%) substantially exceeds that of any of the other Flavobacterium species, and even the heterogeneous Group IIb (range 3.5 mol%), subdivision of F. odoratum appears to be warranted. However the genetic differences cannot yet be correlated with phenotypic differences other than colonial morphology (Shewan and McMeekin, 1983).

F. odoratum is a rare opportunistic pathogen which is most frequently isolated from urine but has also been isolated from peritoneal fluid and soft tissue infections (Holmes et al., 1977; Davis et al., 1979; Kohut, 1979; Von Graevenitz, 1981). It caused ventriculitis in a six week-old infant with hydrocephalus (Macfarlane et al., 1985). In this case intravenous cefotaxime was unsuccessful and was discontinued, but intraventricular cefotaxime rapidly sterilized the ventricular fluid to effect a cure. F. odoratum may also be an indicator of pathological processes in the intestine as it has been isolated from the faeces of patients with abdominal typhus, acute gastroenteritis, and relapsing fever, but not from normal faeces (Stutzer and Kwaschnina, 1929).

Even allowing for considerable strain variation F. odoratum has extremely broad antibiotic resistance (Table 14). To date there are no reports of discrepancies between MIC and disc diffusion methods for this species in tests of antimicrobial susceptibility. However from the literature some drugs appear to be more active in disc tests than in MIC tests.

Kono et al. (1980) report the presence of a plasmid of molecular weight 41.6 +/- 1.7 megadaltons in F. odoratum strain N299B isolated from infected urine. The plasmid coded for resistance to ampicillin, carbenicillin and erythromycin. An attempt to transform the plasmid into a strain of E. coli was unsuccessful.

TABLE 14

Susceptibility of F. odoratum to Antimicrobial Agents

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1. Agents to Which Most Strains are Susceptible

josamycin	nitrofurantoin
rifampicin	piperacillin

2. Agents to Which Most Strains are Resistant

amikacin	cefotetan	kanamycin
aztreonam	ceftazidime	penicillin G
carbenicillin	cephalothin	polymyxin B
cefamandole	clindamycin	streptomycin
cefmenoxime	colistin	tetracycline
cefoperazone	gentamicin	tobramycin
cefotaxime	imipenem	

3. Agents to Which Susceptibility is Variable

ampicillin	cephaloridine	erythromycin
cefoxitin *	chloramphenicol	moxalactam *
ceftriaxone	cotrimoxazole	nalidixic acid

Data of Kohut (1979), Holmes et al. (1979), Sato et al. (1985), Schell et al. (1985), Andreoni (1986) and Gilardi (1987).

* Most strains are reported as resistant to this agent in MIC studies and susceptible in disc diffusion studies.

There is only one report of a beta-lactamase in F. odoratum. Strain GN 14053 constitutively produces a broad spectrum beta-lactamase with a molecular weight of about 26,000 and a pI of 5.8 (Sato et al., 1985). This enzyme is more active against cephalosporins than penicillins and is unusual in its ability to rapidly hydrolyse some of the oxyiminocephalosporins and imipenem which makes it a Mitsuhashi type II CXase (Table 6). It does not hydrolyse cefsulodin, ceftazidime or moxalactam. The enzyme is inhibited by EDTA, iodine, pCMB, mercuric chloride and copper sulphate, but not by clavulanic acid, sulbactam, imipenem or cephamycin derivatives. Sato et al. conclude that the enzyme is a metalloenzyme because activity is restored after complete inhibition by 5 mM EDTA by the addition of divalent cations (e.g. Zn2+, Fe2+).

This beta-lactamase appears to be an important determinant of beta-lactam resistance as in eight strains of F. odoratum there is a direct correlation between MIC values and the amount of enzyme produced.

10. Flavobacterium multivorum (King's or CDC Group IIk, type 2 "Sphingobacterium multivorum")

The name F. multivorum was proposed for Group IIk-2 by Holmes et al. (1981) on the basis of its yellow pigmentation, low %G+C, menaquinones, extracellular DNase, cellular fatty acid profile, aerobic metabolism, oxidase and catalase production (Dees et al., 1979; Holmes et al., 1981; Pickett et al., 1981). A subsequent proposal that it be named

"Sphingobacterium multivorum" (Yabuuchi et al., 1983) was made on the basis of its production of high concentrations of sphingophospholipids as cellular lipids. These authors propose that all Flavobacterium species, like F. multivorum, which produce high concentrations of sphingophospholipids as cellular lipids should be assigned to a separate genus, Sphingobacterium.

F. multivorum is possibly unique among flavobacteria in possessing a single nonfunctional flagellum (Levine et al., 1980; Pickett et al., 1981). However this is a controversial issue as other workers have failed to observe the flagellum (Holmes et al., 1981; Yabuuchi et al., 1983; Hayward and Sly, 1984).

F. multivorum shows DNA (Levine et al., 1980) and phenotypic heterogeneity (Weaver et al., 1972; Oberhofer, 1979; Pickett et al., 1981; Yabuuchi et al., 1981; Hayward and Sly, 1984; Gilardi, 1987). The environmental strains examined by Hayward and Sly (1984) are biochemically more active than the clinical strains examined by other workers. Hayward and Sly (1984) point out that clinical isolates are not necessarily representative of the genus Flavobacterium which occurs in terrestrial and freshwater habitats and suggest that the descriptions of new species of Flavobacterium should not place too much stress on a few biochemical features as these may merely reflect the source as well as the mode of isolation of the strains. As an example their biochemically more active strains were isolated from soil by culturing for bacteria capable of degrading dextran. It may therefore have been that

this technique detected only the more biochemically active strains of F. multivorum and not the less active ones. This type of consideration raises the question of Flavobacterium species descriptions having been based on biased sampling techniques, such as the recent clinical emphasis, and suggests that the current species descriptions may be too restrictive.

The 16 isolates examined by Hayward and Sly (1984) show asymmetric division. A small proportion of cells appear to bud to release spherical cells. This phenomenom was previously shown in electron micrographs of F. breve (Weeks, 1981) and F. aquatile (Thomson, 1982).

F. multivorum occurs in soil (Hayward and Sly, 1984; Pichinoty et al., 1985) where its ability to degrade dextran is another feature shared with Cytophaga (Hayward 1975; Janson, 1975). It is a rare pathogen which causes neonatal meningitis (Mizuta et al., 1974), peritonitis (Dhawan et al., 1980) and septicaemia (Potvliege et al., 1984; Freney et al., 1987). It is also recorded as occurring in cavity fluid, synovial fluid and the spleen (Yabuuchi et al., 1983). Like other Flavobacterium species it is resistant to a wide range of antimicrobial agents (Table 15). Freney et al. (1987) report successful treatment of this organism in a 57 year old septicaemic lymphoma patient with a combination of the new quinolone perfloxacin and cotrimoxazole. Seven strains of F. multivorum showed endotoxin-like activity in limulus amoebocyte lysate testing (Smalley, 1982).

TABLE 15

Susceptibility of F. multivorum to Antimicrobial Agents

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1. Agents to Which Most Strains are Susceptible

azlocillin	perfloxacin
erythromycin	piperacillin
nalidixic acid	rifampicin

2. Agents to Which Most Strains are Resistant

ampicillin	cephalothin
amikacin	gentamicin
aztreonam	kanamycin
cefamandole	penicillin G
cefoxitin	polymyxin B
	tobramycin

3. Agents to Which Susceptibility is Variable

carbenicillin	cotrimoxazole
ceftazidime	moxalactam
ceftriaxone	sulphonamides
chloramphenicol	tetracycline
clindamycin	vancomycin

Data of Yabuuchi et al. (1983), Freney et al. (1987), Gilardi (1987).

11. Flavobacterium spiritivorum (Group IIk, type 3)

The name F. spiritivorum was proposed by Holmes et al. (1982) for thirteen strains which differed from F. multivorum in producing acid from ethanol and mannitol. F. spiritivorum is also closely related to F. thalpophilum, and S. mizutae (Yabuuchi et al., 1983) through being less active proteolytically and producing high cellular concentrations of sphingophospholipids. This is considered adequate justification for these organisms to have separate generic status (Holmes and Owen, 1981; Holmes et al., 1983; Yabuuchi et al., 1983). F. spiritivorum is another organism which may provide additional evidence of a close relationship between Flavobacterium species and gliding bacteria. Yabuuchi et al. (1983) report that it spreads on 0.3% agar but does not seem to on 0.5% agar. However true gliding is unconfirmed.

All strains of this species so far reported are of clinical origin, predominantly from blood, urine and the female genital tract (Holmes et al., 1982; Yabuuchi et al., 1983). The clinical significance of the species is unknown but infections would be difficult to treat because it is resistant to a wide range of antimicrobial agents (Table 16).

12. Flavobacterium thalpophilum

The name F. thalpophilum was proposed on the basis of seven strains which differed from F. multivorum in producing acid from adonitol, growth at 42°C and in having a significantly higher G+C range (44-46 mol%) (Holmes et al.,

TABLE 16

Susceptibility of F. spiritivorum to Antimicrobial agents

---

1. Agents to Which Most strains are Susceptible

cotrimoxazole

piperacillin *

rifampicin

sulphonamides

2. Agents to Which Most Strains are Resistant

ampicillin	clindamycin	penicillin G
amikacin	colistin	polymyxin B
carbenicillin	erythromycin	streptomycin
cefamandole *	gentamicin	tetracycline
cefoxitin *	kanamycin	tobramycin
cephalothin	moxalactam *	

3. Agents to Which Susceptibility is Variable

chloramphenicol

nalidixic acid

Data of Holmes et al. (1982), Yabuuchi et al. (1983), Gilardi (1987)

* One strain only reported

TABLE 17

Susceptibility of F. thalpophilum to Antimicrobial Agents

---

1. Agents to Which All Strains are Susceptible

cotrimoxazole

rifampicin

2. Agents to Which All Strains are Resistant

ampicillin	gentamicin
amikacin	kanamycin
cefamandole	penicillin G
chloramphenicol	polymyxin B
erythromycin	tobramycin

3. Agents to Which Susceptibility is Variable

carbenicillin	moxalactam
cefoxitin	piperacillin
cephalothin	tetracycline

Data of Holmes et al. (1983), Gilardi (1987).

TABLE 18

Identification of Flavobacterium Taxa *

Test	1	2	3	4	5	6	7	8	9	10
Acid produced from :a										
Glucose	+b	v	+	+	+	v	-	+	+	+
Adonitol	-	-	-	-	n	-	-	-	-	+
Ethanol	-	-	+	v	v	v	-	-	+	-
Lactose	+b	-	-	-	-	v	-	+	+	+
Mannitol	-	-	-	-	-	v	-	-	+	-
Raffinose	-	-	-	-	-	-	-	+	+	+
Sucrose	+b	-	-	v	-	-	-	+	+	+
Casein digestion	+	+	+	+	+	+	+	-	-	-
Esculin hydrolysis	-	-	+	+	+	+	-	+	+	+
Indole production	-	+	+	+	+	v	-	-	-	-
Nitrate reduction	n	-	+	v	n	v	-	-	-	+
Nitrite reduction	n	-	-	v	v	v	+	-	-	-
Starch hydrolysis	n	-	-	v	n	-	-	-	-	-
Urease production	-	-	-	v	v	v	+	+	+	+
ONPG	-	-	-	v	v	+	-	+	+	+
Growth at 42oC	-	-	-	v	n	v	-	-	-	+

Data of Holmes et al. (1983), Holmes, Owen and McMeekin (1984), Holmes et al. (1984).

1. F. aquatile
2. F. breve
3. F. balustinum
4. Group IIb
5. F. gleum
6. F. meningosepticum
7. F. odoratum
8. F. multivorum
9. F. spiritivorum
10. F. thalpophilum

TABLE 18 (Continued)

a	ammonium salt-sugar medium
+b	delayed reaction
v	variable
n	no result recorded
e	Ehrlich reagent

1983). Like F. multivorum and F. spiritivorum, F. thalpophilum is not markedly pigmented and is less active proteolytically than other Flavobacterium species. The inclusion of this species widens the breadth of the G+C range of the genus and increases the justification for a new genus comprising these species (Holmes and Owen, 1981; Holmes et al., 1983; Yabuuchi et al., 1983). However at present there are insufficient phenotypic characters to support the creation of a new genus (Holmes et al., 1983).

The seven strains on which the species is based were from clinical specimens with blood and wounds the most common sources. Their clinical significance was not determined. However if F. thalpophilum proves to be pathogenic it may be difficult to treat because of its resistance to a wide range of antimicrobial agents (Table 17).

13. Flavobacterium Species Incertae Sedis

The species listed in Table 10 as incertae sedis do not, in most cases, conform to the revised genus description (Holmes et al., 1982) and their inclusion in the Approved Lists of Bacterial Names (Skerman et al., 1980) is confusing. The majority of these species should be deleted for the following reasons.

F. acidificum

This organism is motile (Holmes and Owen, 1979) and is considered to be a strain of Erwinia herbicola (A.T.C.C., 1980, cited Holmes and Owen, 1981). The G+C composition is

52.7 mol% (Callies and Mannheim, 1978).

F. acidurans

The G+C composition is 66.3 mol% (Holmes and Owen, 1979). Holmes and Owen (1981) suggest that this organism could possibly be grouped with F. oceanosedimentum, F. okeanokoites and F. resinovorum in the same genus - possibly Empedobacter if that name is resurrected.

F. capsulatum

The G+C composition is 63 mol% (Weeks, 1974). Ribosomal RNA cistron analysis places it in a distinct rRNA superfamily that includes P. paucimobilis as its nearest taxon (Bauwens and De Ley, 1981).

F. devorans (P. paucimobilis, CDC or Weaver's Group IIk, biotype 1)

This organism is motile and has a G+C composition of 69 mol%.

F. esteraromaticum

This organism is Gram-positive and has a G+C composition of 69 mol% (Weeks, 1974). It is probably a coryneform (Bauwens, 1980).

F. ferrugineum

This organism contains menaquinones (Callies and Mannheim, 1978) and its G+C composition is 48.6 mol %. Its taxonomic position based on rRNA cistron similarity is unclear but it is

not a member of the Flavobacterium/Cytophaga complex (Bauwens and De Ley, 1981).

F. halmophilum

The G+C composition is 49.7 mol% and its respiratory quinones are ubiquinones (Callies and Mannheim, 1978). Genetically it appears to be related to "Alcaligenes aquamarinus" (Bauwens and De Ley, 1981).

F. heparinum (C. heparina)

This is a gliding organism with a G+C composition of 49.7 mol% (Perry, 1973). Although it was transferred to Cytophaga as C. heparina by Christensen (1980) DNA:rRNA hybridization data of Bauwens and De Ley (1981) indicates it may not belong to the Flavobacterium/Cytophaga complex.

F. indoltheticum

This organism is nonmotile and the G+C composition is 33.8 mol% (Holmes and Owen, 1981). Numerical taxonomic analysis and the DNA base composition suggest that this species is very similar to and would be indistinguishable from F. breve. Holmes, Owen and McMeekin (1984) suggest that it may be considered a later synonym of F. breve.

"F. lutescens"

The G+C composition is 65 mol% (Weeks, 1974). DNA:rRNA hybridization evidence indicates it is a member of the genus Pseudomonas (Bauwens and De Ley, 1981).

F. oceanosedimentum

The G+C composition is 67.5 mol% (Carty and Litchfield, 1978).

F. okeanokoites

This organism is peritrichous (Holmes and Owen, 1979) and on the basis of DNA:rRNA hybridization data does not belong to the Flavobacterium/Cytophaga complex (Bauwens and De Ley, 1981).

F. resinovorum

This organism is peritrichous and has a G+C composition of 66.4 mol% (Holmes, Owen and McMeekin, 1984).

"F. rigense"

This organism is peritrichous and has a G+C composition of 69 mol% (Weeks, 1974).

"F. tirrenicum"

This organism is nonmotile, has a G+C composition of 34.4 mol% (Callies and Mannheim, 1978) and has rRNA cistron similarities to the F. aquatile sub-group of Bauwens and De Ley (1981). This species is not included in the Approved Lists (Skerman et al., 1980) but if the name was revived "F. tirrenicum" could be included in the genus. The type strain is ATCC 15997.

F. uliginosum

This organism has a G+C composition of 32 mol% (Weeks, 1974). DNA:rRNA hybridization data indicates it is related to

the gliding organisms C. lytica, C. marinoflava, C. salmonicolor, F. aurantiacus subsp. excathedrus and possibly McMeekin's yellow organism 42 (Bauwens and De Ley, 1981). A relationship to cytophagas is also indicated by the ability of F. uliginosum to degrade agar (Holmes and Owen, 1981).

14. Genus Weeksella

Holmes, Steigerwalt et al. (1986a, 1986b) propose a new genus Weeksella, comprising two species Weeksella virosa (previously Flavobacterium Group IIf, or Pickett and Manclark Group 3, or Olsen and Ravn Group 1) and W. zoohelcum (previously Flavobacterium Group IIj). This proposal follows the suggestion of Holmes and Owen (1981) that these species be excluded from Flavobacterium on the basis of their greater susceptibility to antibiotics, their lack of saccharolytic activity, lack of obvious yellow pigmentation and because they tend to be parasites rather than free-living organisms. Weeksella is included in the family Cytophagaceae.

f. Taxonomy and Antibiotic Resistance of the Genus Flavobacterium: Current and Future Status

Since reviving F. odoratum as a species on the basis of nine clinical strains and one environmental strain (Holmes et al., 1977), Holmes and various co-workers have continued to publish species descriptions based on small collections of predominantly clinical strains. As a result the genus is now fashioned in a growth at 37°C positive, clinical direction with most species being genetically heterogeneous yet unable to accomodate many so-called atypical environmental strains. The major benefit to accrue from this has been the provision of a taxonomic framework for the genus.

To elaborate briefly the problems with the present scheme, Bruun (1982) could not fit all of 184 clinical and environmental strains into existing taxa and noted "that most of the different biochemical patterns represented gradually merged into each other, with certain patterns, however, occurring more frequently than others and corresponding to the already described taxa". Additional strains could have been accomodated in F. breve and F. odoratum by expansion of the species descriptions. However with other strains it was impossible to tell if they belonged to Group IIb or to the F. multivorum-F. spiritivorum complex. Bruun compared the situation to that of "the green fluorescent pseudomonads where very many different biochemical patterns occur gradually merging into each other, but with some occurring more frequently than others; a situation which makes subdivision into stable, recognizable species extremely difficult".

Further study of the strains led Bruun (1983) to again express concern about the taxonomy of the genus commenting that "unfortunately the tentative groups can hardly be said to represent satisfactory taxonomic units as they stand, and further studies are undoubtedly needed before a better scheme for the arrangement of the genus Flavobacterium can be made". Hayward and Sly (1984) also experienced problems with the species description for F. multivorum being too restrictive for some dextranolytic strains from soil and Thomson (1982) reported strains intermediate between species and strains which could not be assigned to described taxa.

In discussing the heterogeneity within defined taxa, McMeekin and Shewan (1983) urged resistance to the temptation to erect new species until it is possible to relate the genetic differences to easily determined phenotypic traits. However this suggestion was ignored and two further species were described, F. thalpophilum (Holmes et al., 1983) and F. gleum (Holmes et al., 1984b), again on the basis of small numbers of clinical strains.

As a consequence of Holmes and co-workers plunging industriously into the taxonomic reconstruction of the genus there are now, and will continue to be, problems in accomodating strains, particularly non-clinical strains, into what are essentially clinical species.

At this point in the history of the genus however it should not be the shortcomings of the present scheme which are

of prime importance but rather the future taxonomic direction of the genus. The gist of the matter is this. There is now a taxonomic framework which, although imperfect in some respects, supersedes the previously unwieldy classification of Weeks (1974) which contained two very different %G+C ranges (Weeks, 1974). However there still remains the confusing and enormously heterogeneous collection of Gram-positive and Gram-negative Flavobacterium species of the Approved Lists (Skerman et al., 1980). The lack of taxonomic cohesion of this group is a disgrace. This type of taxonomic arrangement confuses undergraduates, makes their teachers blush, and either annoys or makes clinicians laugh. The important question therefore is not the taxonomic significance of relatively small studies of clinical strains but rather, now that we do have the first ever coherent taxonomic framework for the genus, how should it be most usefully employed?

This question can be answered by reviewing those areas in which the current classification is complete and correct, those areas in which it is deficient, and considering the importance of points on which not all parties agree. This is done in the following:

1. Heterogeneity Within Species.

Most clinical species of Flavobacterium comprise a core of highly related strains with a number of 'outliers' (atypical strains). Food and environmental strains are often atypical (Owen and Holmes, 1981). The following taxa are genetically heterogeneous: F. breve (Owen and Holmes, 1980), F.

meningosepticum (Sottile et al., 1973; Owen and Snell, 1976; Callies and Mannheim, 1980; Ursing and Bruun, 1987), F. multivorum (Levine et al., 1980), F. odoratum (Owen and Holmes, 1978), and Group IIb (Owen and Snell, 1976). From %G+C values F. odoratum (range 4.7 mol%) is the most diverse taxon of the genus and can be subdivided into two groups with less than 28% of nucleotide sequences in common.

Most workers agree that further subdivision of the taxa is warranted but given the absence of good correlation between genetic differences and phenotypic markers it is not recommended at present. New phenotypic characters are required. Two species should be special priorities for subdivision, F. odoratum, because it is more genetically diverse than the other taxa, and F. meningosepticum, because of the practical importance of the potential correlation between pathogenicity and genotype, and also because the type strain NCTC 10016 is atypical of the species.

2. Heterogeneity Within the Genus

On the basis of genetic and phenotypic data the genus can be subdivided into distinct groups (Bauwens and De Ley, 1981; Holmes and Owen, 1981; Holmes et al., 1983; Yabuuchi et al., 1983; Holmes, Owen et al., 1984) which contain some bacteria not belonging to Flavobacterium:

Group 1: F. aquatile (and "F. pectinovorum", "F. tirrenicum" and C. johnsonae)

Group 2: F. odoratum

Group 3: F. balustinum, F. breve, F. gleum, F. meningosepticum and Group IIb

Group 4: F. multivorum, F. spiritivorum, F. thalpophilum (and S. mizutae).

It is agreed by all except Oyaizu and Komagata (1981), who recommend that all Flavobacterium strains should be transferred to the genus Cytophaga, that generic status is appropriate for the groups (Bauwens and De Ley, 1981; Holmes and Owen, 1981; Yabuuchi et al., 1983; Holmes, Owen and McMeekin, 1984). So far only one proposal has been made, that Group 4 become the genus Sphingobacterium (Yabuuchi et al., 1983). Holmes et al. (1983) however argue against this maintaining that there are insufficient phenotypic characters to justify the new genus.

If subdivision of the genus as a whole is to be delayed perhaps priority could be given to making Group 1 a separate genus at the earliest opportunity. F. aquatile is a type species which is atypical of Flavobacterium. Furthermore this group also contains C. johnsonae and F. pectinovorum which are both gliding bacteria and therefore also atypical of Flavobacterium. The rules of nomenclature would necessitate this group retaining the generic title Flavobacterium and the other species currently recognised as Flavobacterium would have to be renamed.

3. The Flavobacterium-Cytophaga Boundary

In addition to evidence that F. aquatile is an atypical cytophaga there is further evidence of a relationship between Flavobacterium and Cytophaga. Both genera have similar %G+C ranges, respiratory quinones and cellular fatty acid compositions and there is strong genetic evidence of relationships between certain strains of both groups (Bauwens and De Ley, 1981; Paster et al., 1985). Overall the genetic diversity of the Flavobacterium/Cytophaga complex is as broad as the entire family of Enterobacteriaceae. However by DNA:rRNA hybridization studies, strains of Cytophaga and Flavobacterium cannot be separated from each other (Bauwens and De Ley, 1981). Phenotypically the most reliable character for distinguishing the genera is gliding motility but it is impossible to distinguish cytophagas which are aberrant in this trait from Flavobacterium (Holmes, Owen and McMeekin, 1984).

Although there is unanimity that the boundary between Flavobacterium and Cytophaga is indistinct there is contention about the taxonomic implications of this. Oyaizu and Komagata (1981) recommend amalgamation of all taxa into an enormous, all-encompassing genus (Cytophaga) while other workers advocate subdivision of the complex into several genera.

In essence the issue centres on one character - gliding motility. If new phenotypic characters can be found to better delineate the groups within the complex the emphasis and confusion about gliding motility should dissipate and any

nexus between Flavobacterium and Cytophaga should be seen in clearer perspective.

4. Bacteroides

Oligonucleotide cataloguing indicates that Flavobacterium, Cytophaga and Bacteroides "form a phylogenetically coherent and major cluster of eubacteria, a eubacterial 'phylum'" (Paster et al., 1985). That this relationship was not previously suspected is understandable since the Flavobacterium/Cytophaga complex and Bacteroides species are phenotypically very different and are characterized in very different ways.

The more striking phenotypic differences between these two groups have masked the less obvious features they share. The majority of Bacteroides species have %G+C values in the same range as Flavobacterium and are also nonmotile. Both groups are unique in containing organisms which have sphingophospholipids as a major membrane component. Like Flavobacterium, many Bacteroides species are phosphatase-positive and two Bacteroides species, B. ureolyticus and B. gracilis, resemble Cytophaga in their ability to spread and to pit into agar. It is also interesting that B. ureolyticus is oxidase-positive while B. gracilis demonstrates "twitching" movements. Further similarities exist in the antibiotic susceptibility patterns with many organisms of both groups susceptible to chloramphenicol, clindamycin, cefoxitin, erythromycin, minocycline and acylamino penicillins but resistant to

penicillin, amoxycillin, colistin and aminoglycosides (Holdeman et al., 1984; Tables 11 to 17).

Many Bacteroides species produce beta-lactamases. It is striking that F. odoratum and B. fragilis TAL 2480 both possess rare imipenem-hydrolysing Mitsuhashi type II CXases (Table 6). More detailed investigation of these enzymes and investigations of the beta-lactamases of other species might provide further insights into the relationships of these organisms, particularly if the beta-lactamases are chromosomally-mediated.

There is considerable scope for further investigations of this relationship. Because it is regarded as a distant relationship (Paster et al., 1985; Woese et al., 1985) on the basis of oligonucleotide sequence studies of small numbers of strains, the Bacteroides connection should not impinge on taxonomic reform within the Flavobacterium/Cytophaga complex. However because studies to date are of such a limited nature it is not possible to preclude the possibility of a more intimate relationship. The sequences of only two environmental strains of Flavobacterium (F. aquatile and F. breve ATCC 14234) have been studied. Clinical strains of F. breve and strains of other Flavobacterium taxa need to be assessed. If more extensive studies indicate the relationship is more intimate than is currently suspected the taxonomic implications for these groups could be both fascinating and far-reaching.

6. The Significance of Antibiotic Resistance in Flavobacterium

Holmes, Owen and McMeekin (1984) suggest that antibiotic resistance in Flavobacterium is largely controlled by chromosomal genes. Marre et al. (1982) note that IEF of chromosomal beta-lactamases can be of great taxonomic value, sometimes being specific to subspecies level. If the beta-lactamases of Flavobacterium are chromosomal, a survey of the beta-lactamases of the genus could shed light on some of its relationships. In theory it should be possible to classify the entire genus according to its chromosomal beta-lactamases. This, then, might be a new approach for investigating the taxonomic affinities of the heterogeneous genotypes and the unclassified environmental strains.

The Flavobacterium beta-lactamases which have been described so far are unlike those of most other bacteria. One beta-lactamase, that of Flavobacterium strain 80 (Chen and Williams, 1982, 1985) is unique in hydrolysing temocillin. Such unusual enzymes offer considerable potential as distinctive phenotypic markers.

The taxonomic potential of other resistance mechanisms in Flavobacterium also needs to be assessed. Multiple antibiotic resistance is usually suggestive of a permeability barrier or multiple resistance mechanisms. Perhaps resistant Flavobacterium strains have narrower porins than other bacteria. How does F. meningosepticum develop resistance during therapy? These are issues which have been ignored by

researchers.

The elucidation of the non-beta-lactamase mechanisms may be technically more difficult and less informative than a survey of beta-lactamases at the present time. The study of porins and antibiotic penetration rates is expensive, technically complex, and prone to error. Investigations of beta-lactamases can be less demanding technically and more likely to yield useful results because the taxonomic significance of beta-lactamases is established (Matthew and Harris, 1976; Marre et al., 1982) and there is considerable literature to which new data can be related.

With its high levels of resistance and unusual beta-lactamases the genus Flavobacterium is a hole in the spectrum of the new beta-lactam antibiotics. It can therefore be regarded as a yardstick by which to measure how much future progress is necessary in the development of beta-lactam antibiotics. Knowledge of the resistance mechanisms of the genus may lead to the development of improved antibiotics by indicating the level of potency required, or new types of molecular configurations which will allow antibiotics to evade bacterial defence mechanisms. In this respect Flavobacterium is a terra incognita which if explored may yield new understandings about the interaction of antibiotics with bacteria.

From the preceding it is obvious that a lengthy research agenda could be established to investigate the taxonomic

problems of the genus and the many implications of its antibiotic resistance. New approaches to these issues are required. It is important that the recent progress in both taxonomy and antibiotic therapy be consolidated. What is needed is a broadly based study which will point to the various areas of research which are likely to be most profitable. In this respect this thesis aims to further answer the question about how the current taxonomic framework of Flavobacterium can be most usefully employed by examining the genus in the light of antibiotic resistance data and better establishing the nature of relationships within Flavobacterium and also with some of the other bacteria of taxonomic interest.

II. MATERIALS AND METHODS

a. Bacterial Strains

The names, origins and code numbers of the 43 reference and 89 study strains are shown in Table 19. The study strains had been previously described by Thomson (1982) except for strains R41, R42, R43, R47, R48, R49, R50, R51, R54, R55, R56, R58 and R59, and the MP strains which were donated by Prof. M.J. Pickett, University of California, Los Angeles. The reference strains were donated by Dr B. Holmes, National Collection of Type Cultures, Colindale, London, Dr H. Reichenbach, Gesellschaft für Biotechnologische Forschung, Braunschweig, and Prof. C.C. Sanders, Creighton University, Omaha.

b. Characterisation Tests

Strains R41, R42, R43, R47, R48, R49, R50, R51, R54, R55, R56, R58 and R59 were subjected to the characterisation tests listed in Table 20. The taxonomic reference strains, the antibiotic reference strains, the MP strains, and the previously described study strains were accepted as authentic on the basis of colonial morphology and a small number of confirmatory tests from Table 20 e.g. oxidase, phosphatase, UV sensitivity, motility, urease, indole production and acid production from glucose and raffinose.

c. Antibiotics

All antibiotic discs except temocillin and augmentin were manufactured by Oxoid Limited, Basingstoke, England. The

temocillin and augmentin discs were manufactured by BBL, Cockeysville, USA. The aztreonam discs were a gift from the Squibb Institute, New Jersey and the temocillin discs were a gift from Beecham Research Laboratories, Victoria. Disc strengths were ampicillin 25 mcg, augmentin (amoxycillin 20 mcg/ clavulanic acid 10 mcg), aztreonam 30 mcg, carbenicillin 100 mcg, cefamandole 30 mcg, cefoperazone 30 mcg, cefotaxime 5 mcg, cefoxitin 30 mcg, cefsulodin 30 mcg, ceftazidime 30 mcg, cefuroxime 30 mcg, cephaloridine 30 mcg, cephalothin 30 mcg, chloramphenicol 30 mcg, cloxacillin 10 mcg, erythromycin 15mcg, gentamicin 10 mcg, imipenem 10 mcg, moxalactam 5 mcg, norfloxacin 10 mcg, sulphamethoxazole 300 mcg, temocillin 30 mcg, ticarcillin 75 mcg, trimethoprim 2.5 mcg.

All antibiotic diagnostic powders and tablets except penicillin and trimethoprim were gifts from their respective manufacturers. These included ampicillin, lithium clavulanate, ticarcillin (Beecham Research Laboratories, Victoria, Australia), cefamandole, cefoperazone (Pfizer Ltd, N.S.W., Australia), ceftazidime, cephalothin (Glaxo Australia Pty Ltd, Victoria), cefsulodin (Ciba-Geigy Ltd, Basle, Switzerland), cefotaxime, gentamicin (Roussell Pharmaceuticals Pty Ltd, N.S.W., Australia), cefoxitin, imipenem (Merck Sharp & Dohme (Australia) Pty Ltd, N.S.W.), aztreonam (The Squibb Institute, New Jersey), penicillin G potassium salt (Sigma Chemical Co., Missouri), trimethoprim (Burroughs Wellcome Co., Research Triangle Park, North Carolina), erythromycin (Faulding, South Australia), nitrocefin (Oxoid, England) and chloramphenicol (Parke-Davis Pty Ltd, N.S.W.). Standard

TABLE 19

Origin of Strains Included in the Study

1. Taxonomic Reference Strains

Strain Code	Name as Received	Source or reference
ATCC 15997	"F. tirrenicum"	1
ATCC 17061	C. johnsonae	1
ATCC 29551	C. aquatilis	1
DSM 527	Flexibacter elegans	1
NCIB 8694	F. aquatile	2
NCIB 10150	C. johnsonae	1
NCTC 10016	F. meningosepticum	2
NCTC 10585	F. meningosepticum	2
NCTC 10795	F. gleum	2
NCTC 11033	F. multivorum	2
NCTC 11036	F. odoratum	2
NCTC 11099	F. breve	2
NCTC 11162	F. breve	2
NCTC 11179	F. odoratum	2
NCTC 11180	F. odoratum	2
NCTC 11343	F. multivorum	2
NCTC 11386	F. spiritivorum	2
NCTC 11387	F. spiritivorum	2
NCTC 11432	F. gleum	2

Sources: 1. H. Reichenbach 2. B.Holmes

TABLE 19 (Continued)

2. Antibiotic Reference Strains

Strain Code	Name as Received	Enzyme	Source or reference
MISC 99	*K. pneumoniae*	IVa	3
MISC 100	*E. coli*	IIIa (TEM)	3
MISC 101	*E. coli*	Ib	3
MISC 102	*E. cloacae*	Ia	3
MISC 103	*K. pneumoniae*	IVb	3
MISC 104	*P. mirabilis*	IIa	3
MISC 105	*K. pneumoniae*	IIIa (TEM)	3
MISC 111	*E. cloacae* P99+	Ia	3
MISC 112	*E. cloacae* P99-	lacks enzyme	3
MISC 11	*E. coli*	III (TEM 1)	3
MISC 120	*E. coli*	III (TEM 2)	3
MISC 121	*E. coli*	V (OXA-1)	3
MISC 122	*E. coli*	V (OXA-2)	3
MISC 123	*E. coli*	V (OXA-3)	3
MISC 124	*P. aeruginosa*	III (PSE-1)	3
MISC 125	*P. aeruginosa*	V (PSE-2)	3
MISC 126	*P. aeruginosa*	III (PSE-3)	3
MISC 127	*E. coli*	III (HMS-1)	3
MISC 128	*E. coli*	III (SHV-1)	3
MISC 130	*P. aeruginosa*	III (PSE-4)	3
164 CD	*P. aeruginosa*	Id	3
ATCC 25922	*E. coli*		4
ATCC 25923	*S. aureus*		4
ATCC 27853	*P. aeruginosa*		4

3. C.C. Sanders 4. American Type Culture Collection

TABLE 19 (Continued)

3. Study Strains

Name as Received	Strain Code
F. breve	U31
F. gleum	U62, R46, R48
F. meningosepticum	R43, R44, R58, MP816, MP817, MP818, MP819, MP820, MP894, MP963, MP966, MP903, MP969, MP970, MP972, MP1702, MP1742, MP2215, MP2347, MP2348
F. multivorum	R42, MP1170, MP1174, MP1177, MP1180, MP1201, MP1204, MP1205, MP1210, MP1213, MP1231, MP1236, MP2324
F. odoratum	U59, R25, R28, R37, R55, R59
F. spiritivorum	R41, R51, MP2242
F. thalpophilum	MP997, MP1207, MP1232
Group IIb	U1, U37, U39, U42, U43, U58, U70, R34, R40, R45, R47, R49, R50, R54, R56, R862, MP7, MP8, MP305, MP525, MP547, MP548, MP582, MP592, MP616, MP628, MP639, MP1098, MP1409, MP1846
Unclassified Strains	U10, U33, U44, U93, U105, R5, R10, R12, R19, R39

Sources:

1. R strains:
   R5 and R10 (received as H051 and H026 ex J.M. Shewan),
   R12 and R45 (received as 25 and un-named strain ex T.A. McMeekin)
   R19, R43 received as NCTC 11099 and NCTC 10016 from B. Holmes

TABLE 19 (Continued)

Sources of Study Strains (Continued)

R41, R42 received as A2840 and A2841 ex M. Peel)

R47 (received as un-named food strain ex R. Tucker)

R58, R59 - American clinical strains (ex R. Clark)

Other R strains - Australian clinical strains

2. U strains - poultry processing water

3. MP strains - M.J. Pickett (isolation sites not provided)

TABLE 20

Morphological, Physiological and Biochemical Characterisation Tests

Test	Method	Test	Method
Colonial morphology	(a)	Esculin hydrolysis	(k)
Motility	(b)	Acid from adonitol	(1)
Anaerobic growth	(c)	Acid from glucose	(1)
Pigmentation	(a)	Acid from maltose	(1)
UV sensitivity	(d)	Acid from arabinose	(1)
Oxidase	(e)	Acid from inositol	(1)
O-F test (glucose)	(f)	Acid from salicin	(1)
Phosphatase	(g)	Acid from sucrose	(1)
Indole production	(h)	Acid from trehalose	(1)
Nitrite reduction	(i)	Acid from dulcitol	(1)
Urease	(j)	Acid from mannitol	(1)
ONPG	(k)	Gelatin hydrolysis	(m)
DNase	(k)	Arginine dihydrolase	(n)
H2S production	(k)	Ornithine decarboxylase	(n)
Acid from lactose	(k)	Lysine decarboxylase	(n)
Acid from xylose	(k)	Catalase	(i)
Starch hydrolysis	(i)	Citrate utilisation	(n)
Pigment on tyrosine agar	(i)	Filament formation	(o)

TABLE 20 (Continued)

(a) nutrient agar, 48 hrs; (b) wet mount and motility test medium (BBL Microbiology Systems, Cockeyville, Maryland); (c) Oxoid Anaerobic System; (d) McMeekin (1977); (e) Kovacs (1956); (f) Hugh and Leifson (1953); (g) Barber and Kuper (1951); (h) 1% w/v tryptone water (Oxoid CM87); (i) Cowan and Steel (1970); (j) Christensen (1946), cited Cowan and Steel (1970); (k) Uni-N/F-Tek (Flow Laboratories, Inc., Virginia); (l) Ammonium-salt sugar medium; (m) Charcoal gelatin discs (Oxoid BR10), 7 days incubation; (n) API 20E, Montalieu-Vercieu, France); (o) microscopic examination after overnight incubation at 37°C in tryptone soya broth (Oxoid CM129) - a filament is defined as a cell for which the length is at least 10x cell width.

solutions of each antibiotic were prepared on the day of use according to the manufacturer's instructions.

d. Disc Diffusion Antibiotic Susceptibility Tests

Isolates were tested according to the Calibrated Dichotomous Scheme (CDS) of Bell (1975; 1984). Briefly, the inoculum was prepared from a subculture on nutrient agar (Oxoid CM3) by passing a sterile Nichrome SWG.24 wire of diameter 0.56 mm vertically through a selected colony until it touched the surface of the agar. The inoculum was then suspended in 2.5 ml of sterile normal saline (this suspension contains 10^6 to 10^7 cfu/ml) and evenly distributed over a pre-dried plate of depth 2 mm of Sensitest Agar (Oxoid CM409). The plate was then tilted to drain and excess inoculum drawn off with a Pasteur pipette. After the surface of the plate had been air-dried for 15 to 45 minutes the antibiotic discs were applied and the plate was incubated at 35°C for 18 hours. Annular radii of inhibition zones were measured with Vernier calipers and recorded.

By this method rapidly growing bacteria are determined to be susceptible to an antibiotic if the annular radius of the inhibition zone is 6mm or greater and resistant if the zone is smaller. However this, and all disc tests, are known to yield inaccurate results with slow-growing strains (Bell, 1984). Therefore for slow-growing strains of Flavobacterium the validity of the 6 mm cut-off point to determine susceptibility was uncertain.

There were two further concerns with interpretation of this test. Some of the antibiotics (aztreonam, cefamandole, cefoperazone, cefoxitin, cefsulodin, ceftazidime, cefuroxime, cephalothin and erythromycin) had not been calibrated for Gram-negative bacteria. Furthermore some strains (NCIB 8694, NCIB 10150, ATCC 29551, U1, U10, U33, U37, U39, U42, U43, U44, U70, R10, R12, R39, R40 and R45) could not be tested strictly according to the method as they failed to grow sufficiently at 35°C. Four strains did not grow sufficiently on Sensitest agar. NCIB 8694 was tested on Cytophaga agar, NCIB 10150 and ATCC 29551 were tested on Sensitest Agar with 5% defibrinated horse blood added and U70 and was tested on CLED medium (Oxoid CM301). While these technical problems may have led to antibiotic-organism interactions beyond the calibration of the CDS method this did not necessarily invalidate disc tests for these organisms as disc testing was used in this study for additional purposes beyond strict interpretation of susceptibility.

One important function of the disc tests was to screen strains and antibiotics for phenomena of potential interest for further testing. The second important function of the disc tests was their integral role in 3-dimensional susceptibility testing and clover leaf testing (see below). These considerations made it important to include disc testing in this study.

e. 3-Dimensional Testing

Beta-lactam antibiotics were concurrently tested with both the CDS method and the 3-dimensional technique (Thomson et al., 1984) to investigate enzymatic drug inactivation. The 3-dimensional inoculation was performed as follows just prior to placing the antibiotic discs on the plate.

The point of a sterile number 11 scalpel blade was passed vertically into a selected typical colony of the test organism. The blade was then agitated to either side to give just visible inoculum on both sides of the point. The inoculum-laden scalpel was then used to inoculate the plate by means of the Pearce 3-Dimensional Inoculator (Biomedical Engineering Department, Repatriation General Hospital, Hobart, Australia). This inoculation involved stabbing the scalpel point eight times into the agar in predetermined fashion (Fig. 7). The plate was then rotated three times with the scalpel blade remaining inserted in the agar after the final stab-inoculation. This provided an evenly distributed circular slit of inoculum (the 3-dimensional inoculation) extending from the surface of the agar to the bottom of the plate. After this the plate was removed from the Inoculator and the antibiotic discs were placed precisely on the agar 3 mm inside the circular 3-dimensional inoculation. An Oxoid Disc Dispenser Mark II was used to accurately place the discs.

After incubation presumptive evidence of enzymatic inactivation of the antibiotics was detected at the margin of the inhibition zone adjacent to the 3-dimensional inoculation

(Figs 8, 9). Inactivation of drug as it diffused through the slit resulted in a distortion or discontinuity in the usually circular inhibition zone. No distortion of the zone margin at the intercept with the slit indicated that there had been no significant inactivation of the antibiotic.

This procedure was varied when testing drugs to which the antibiotic reference strains were resistant. For these tests the bacterial growth immediately adjacent to the disc masked any 3-dimensional effects. To overcome this a susceptible assay organism was used for the surface inoculation, i.e. E. coli ATCC 25922 or S. aureus ATCC 25923, and the one- and two-disc procedure of Thomson et al. (1984) was used. This procedure entailed using the edge of a ruler as a guide for the scalpel blade to produce a straight-line 3-dimensional inoculation, after which one or two antibiotic discs were placed on the agar 3 mm to one side of the slit.

This more sensitive procedure was initially employed only with the strains containing known beta-lactamases. It was important to assess the potential of the technique against a range of known beta-lactamase types. The more sensitive procedure was used only for study strains of particular interest.

f. 3-Dimensional and Clover Leaf (3D+CL) Testing

In clover leaf testing (Kjellander and Myrbach, 1964) an agar plate is spread with a susceptible assay strain and the

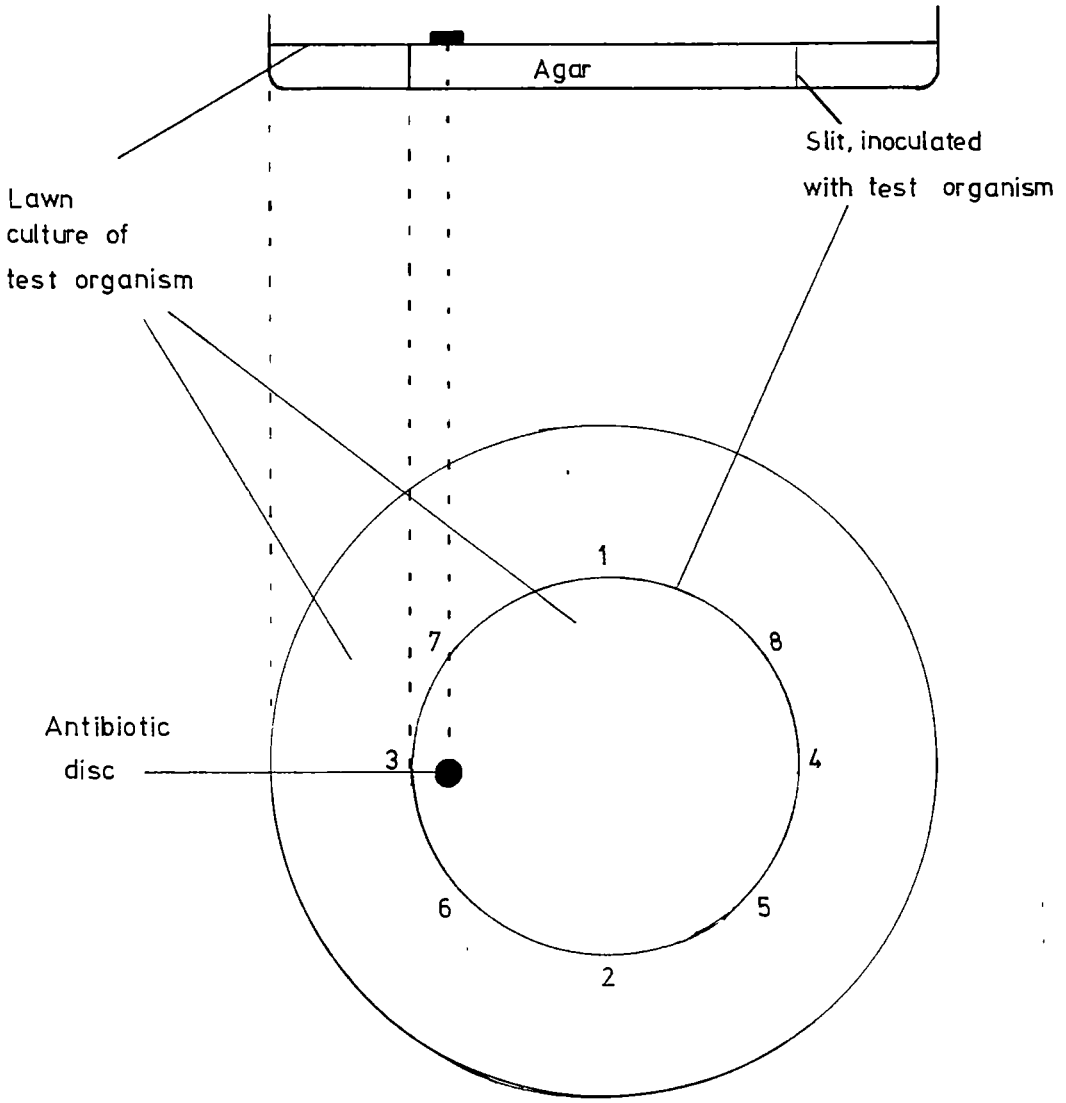


Fig. 7. Diagramatic representation of the 3-dimensional inoculation. Above: sideview showing the slit of inoculum extending from the surface to the bottom of the agar. Beneath: viewed from above, the circular slit of inoculum is 3 mm outside the ring of antibiotic discs (one disc only shown). The numbers 1 to 8 indicate the sequence of stab inoculations of the test organism prior to the three rotations of the plate. During the rotations the bacterium-laden blade distributes the inoculum in a circular slit.

antibiotic disc placed in the centre. A heavy inoculum of the test strain was streaked radially from the disc and the plate was incubated under conditions suitable for growth of both test and assay strain. An indentation in the inhibition zone of the assay strain along the streak of the test strain indicated enzymatic inactivation of the antibiotic by the test strain.

As the study progressed it was found that there were occasional differences between the results of the 3-dimensional and the clover leaf tests. A combination of the two techniques was adopted to provide a more comprehensive presumptive test for beta-lactamase mediated drug inactivation. This was achieved by performing the more sensitive one- or two-disc 3-dimensional tests with a radial streak of test inoculum away from the disc on the opposite side to the 3-dimensional inoculation (Fig. 12). A positive result by either or both of the 3-dimensional or clover leaf tests was regarded as a positive 3D+CL test. Strains NCTC 11099 and R19 were each tested five times to investigate the reproducibility of this technique.

g. Minimum Inhibitory Concentration (MIC) Testing

Strains for MIC testing were selected from those capable of growth at 35°C after CDS and 3-dimensional testing. The organisms were all reference strains of Flavobacterium and strains of species which complemented the reference strains by presenting, for each species, as wide a range of susceptibility to beta-lactam antibiotics as possible. By testing the most resistant, the most susceptible, and a median

strain for each species (plus other unusual strains) it was hoped to investigate any heterogeneity of the species in their interactions with beta-lactam antibiotics. Named species were preferred to Group IIb and unclassified strains to establish a framework by which difficult to classify strains might be compared with the taxonomic framework based on Holmes, Owen and McMeekin (1984). Control strains E. coli ATCC 25922 and P. aeruginosa ATCC 27853 were also included.

The antimicrobials tested were chosen on the basis of published beta-lactamase stabilities and their potential taxonomic significance as determined by the disc tests in this study.

MICs were determined for each antibiotic using an agar dilution procedure. Antibiotic plates were prepared with Mueller-Hinton agar (MHA) (BBL) and inoculated with 10^5 cfu in Mueller-Hinton broth (MHB) (BBL) using a Steers replicating device (Steers et al., 1959). The antibiotic concentrations ranged from 0.06 to 128 mcg/ml. All drugs were tested simultaneously and the MIC was defined as the lowest concentration preventing visible growth after incubation for 18 hours at 35°C in air. The presence of five or fewer colonies was ignored. Differences in MICs greater than twofold were considered significant. Results were defined as susceptible, moderately susceptible or resistant according to National Committee for Clinical Laboratory Standards (NCCLS) interpretive standards (Barry et al., 1981; Thornsberry et al., 1985).

Additional MIC tests were performed to investigate the roles of beta-lactamases and permeability in resistance to cephems using cefamandole and cefotaxime alone, with 2 mcg/ml lithium clavulanate added, with 1 mM disodium ethylenediaminetetraacetate (EDTA) added, and with both lithium clavulanate and EDTA added. For these MIC tests S. aureus ATCC 25923 was used as a control instead of P. aeruginosa ATCC 27853. Apart from this single difference the same organisms were tested in both MIC studies.

h. Enzyme Preparations

Induction cultures were prepared by making a 1:20 dilution of an overnight MHB culture in fresh medium (final volume 100 ml) and incubating it at 35°C with shaking (150 rpm). Due to unavailability of 100 ml vessels each 100 ml volume was dispensed into 10 ml aliquots in centrifuge tubes. After two to three hours cefoxitin was added to the tubes to give a concentration of 1/4 the MIC or 100 mcg per ml, whichever was lesser. Incubation was continued for two to three hours after which protein synthesis was arrested by the addition of 0.1 ml of 1 mM solution of 8-hydroxyquinoline (Sigma Chemical Co., Missouri) to each centrifuge tube. The cells were then centrifuged at 3,000 x g for 20 min at room temperature in an IEC Centra-4X centrifuge (International Equipment Company, Bedfordshire, England). The resultant cell pellets were combined and washed twice with 0.1 M phosphate buffer, pH 7.0, then resuspended in 3 ml of the same buffer and disrupted by sonication at 6 microns peak to peak for 40 to 120 sec at 0°C in an MSE 150 watt ultrasonic disintegrator (Mk 2). Cell

debris was removed by centrifugation at 3,000 x g for 30 min. The cleared sonic extracts were distributed into three aliquots and frozen at -23°C. The protein content of an aliquot from each organism was determined using Bio-Rad reagent (Bio-Rad Laboratories, Richmond, Calif.) in a Multistat III Plus microcentrifugal analyser (Instrumentation Laboratory) with Validate N (Organon Technika 917613) as a standard. The same procedure was used for uninduced enzyme preparations except that cefoxitin was not added after two to three hours preincubation. For the purposes of this study these preparations were designated crude sonicates.

Beta-lactamase activity was assessed to determine sample application volume for IEF by the semiquantitative method of Vecoli et al. (1983) as follows. 150 ul of 50 ug/ml nitrocefin solution was added to 50 ul of crude sonicate. The sample application volume was determined to be 1 ul of sample for each second taken for the development of a pink colour.

Some crude sonicates with insufficient beta-lactamase activity for IEF were concentrated as follows. The preparations were dialyzed overnight and centrifuged at 25,000 x g using a high speed rotor in the Centra-4X centrifuge. (This rotor would only take volumes of less than 1 ml and was therefore unsuitable for preparation of crude sonicates from 100 ml volumes of broth cultures). The supernatants were concentrated in Centricon 10 microconcentrators (Amicon Corporation, Danvers, Massachusetts). Some preparations required further

concentration by freeze drying.

i. Beta-lactamase Assays

Beta-lactamase assays were performed using a Beckman DB-GT grating spectrophotometer. Cephalosporinase activity was determined in UV spectrophotometric assays by the method of Gootz et al. (1982) using crude sonicates and 100 uM concentrations of each substrate. Hydrolysis at 37°C was followed over one to ten minutes by monitoring the change in the optical density (OD) of the drug at the wavelength associated with maximal absorbance for its beta-lactam ring. This was 264 nm for cephalothin and 263 nm for cefoxitin. Hydrolysis resulting in a change of < 0.001 OD units could not be detected accurately in this assay. Organisms with an induction ratio of greater than 3.0 in the presence of cefoxitin were considered to be producers of an inducible beta-lactamase.

Penicillinase activity was measured by a modification of the microiodometric method of Perret (1954) (Ross and O'Callaghan, 1975). Reaction mixtures containing 0.9 ml 0.1 M phosphate buffer (pH 7.0), 1.0 ml penicillin G (200 uM), 1.0 ml iodine-starch solution and 0.1 ml crude sonicate were maintained at 30°C for the duration of the assays. Absorbance at 620 nm was measured at 0, 15, and 20 minutes. A commercial penicillinase preparation (BBL 11897) was used as a control.

j. Isoelectric Focussing and Inhibition Studies

Beta-lactamases were focussed in agarose gels (105 x 230 x 0.5 mm) containing pH 3 to 10 ampholytes (Pharmalyte, Pharmacia, Sweden) by the method of Vecoli et al. (1983). The gels were prepared by dissolving 2 g of AgaroseIEF (Pharmacia) and 24 g of sorbitol in 187 ml of deionised water by boiling with stirring until clear. The resultant solution was dispensed in 16.5 ml aliquots into test tubes and cooled to 65 to 70 C. Using a syringe, 1.2 ml of ampholyte prewarmed to 60 C, was gently added to each tube of agarose-sorbitol, mixed, and the gel poured onto a preheated spacer mold and overlaid with the hydrophilic surface of GelBond film (FMC Corporation, Rocklaine, Maine) and a glass plate and left at room temperature for one hour to polymerize. After polymerization the molds were sealed with waterproof plastic adhesive tape (Scotch Brand Tape 471, 3M Company, St Paul, Minnesota) and the gels stored at 4°C until required.

On site IEF facilities were unavailable for this study and equipment from other sources was used when it was available. In all the focussing was performed with three different sets of apparatus. These were (1) Pharmacia power supply (E CPS 2,000/300) and Pharmacia flatbed apparatus (FBE 3,000) made available by Mr J. Presser (Forensic Science Department, Royal Hobart Hospital, Hobart), (2) Bio-Rad Model 3000/300 power supply 240 V, 50 Hz with Bio-Phoresis horizontal cell, loaned by Miss M. Degaris (Bio-Rad Laboratories Pty Ltd, NSW), and (3) Bio-Rad Model 3000/300 power supply 240 V, 50 Hz loaned by Miss M. Degaris (Bio-Rad Laboratories Pty Ltd, NSW) with LKB

Multiphor II Unit, loaned by Dr J. Sallis (Biochemistry Department, University of Tasmania).

The gels were focussed across the width at 11°C with the constant power supply increased progressively from 8 W limiting through to 22 W limiting with 2,000 V limiting and 30 mA limiting. The anode solution used was 1 M H_3PO_4, and the cathode solution was 0.5 M NaOH. The pH gradient of the gel was measured using Electron isoelectric point markers pI calibration kit range 4.7 to 10.6 (BDH Chemicals Ltd, Poole, England). Focussing continued until the pI markers were seen to be sharply focussed and the current appeared to be at a stable minimum, usually 7 to 9 mA. An average run took 55 min.

Inhibitor studies were performed by the method of Sanders et al. (1986) on the agarose gel to distinguish between type I chromosomally-mediated enzymes and other types of enzymes. Each enzyme was focussed in triplicate. One sample was not exposed to inhibitors while the duplicate and triplicate samples were exposed to lithium clavulanate or cloxacillin in the following manner. Filter paper soaked with lithium clavulanate (1,000 uM) or cloxacillin (1,000 uM) was placed on the surface of the focussed gel. After 10 sec the paper was removed, and the gel was overlaid with molten agar (MHA) containing 50 ug of nitrocephin per ml. After the agar hardened the beta-lactamases appeared as pink bands against a yellow background. By comparison of the pink band of the ~~inhibited~~ without inhibitors sample with presence or absence of pink bands for

the samples exposed to the inhibitors the inhibition profile was readily determined. The developed gel was photographed within 60 min with a Polaroid MP-4 camera model 44-32 equipped with a magenta (Kodak CC30M) filter and Polaroid 669 film.

The MISC antibiotic reference strains were used as controls for the method with MISC 103 (type IVa enzyme) and Misc 128 (type SHV-1 enzyme) used as internal controls for each plate to ensure that discrepancies did not arise from use of the different sets of IEF apparatus.

IEF studies were also performed in the absence of inhibitors to compare larger numbers of enzymes on the one gel. These were photographed by directly overlaying Ilfospeed Printing Paper (400065) with the gels and exposing them to light passed through a pale green microscope filter (type unknown) for 16 seconds.

It was not practicable to perform the necessary genetic or biochemical studies to determine beyond doubt whether each beta-lactamase was chromosomally or plasmid mediated. Classification of the genetic origin was therefore based on IEF results which were considered in conjunction with substrate profile and inhibitor studies when necessary. Thus when an isolate produced a beta-lactamase with an isoelectric point which did not correspond to any of the known plasmid mediated beta-lactamases the unknown beta-lactamase was classified as chromosomally mediated. If the beta-lactamase had an isoelectric point which was identical to a known plasmid mediated beta-lactamase it was necessary to compare

the substrate profiles and inhibitor results of the two beta-lactamases to see if they were identical.

k. Tests with Augmentin Discs

Lawn cultures were prepared on Sensitest Agar or other substitute media for disc tests (see section d of materials and methods) by directly swabbing a colony of the test organism onto the agar surface. Ampicillin and augmentin discs were then placed at least 3.5 cm apart on the inoculated plates and the plates were incubated at the appropriate temperature for the organism. After overnight incubation the plates were inspected to compare the sizes of any inhibition zones. Synergy between amoxycillin and clavulanate was inferred if the inhibition zone around the augmentin disc was larger than the inhibition zone around the ampicillin disc. This was taken to be presumptive evidence that clavulanate inhibited the beta-lactamase of the organism and that the beta-lactamase had a significant role in resistance of the organism to beta-lactam antibiotics.

l. %G+C Determination

DNA base ratios were determined for F. gleum R48, F. gleum/Group IIb R46 and Group IIb R54. DNA was extracted as detailed by Blackall et al. (1985). A Pye Unicam SP8-200 UV/VIS thermoprogrammed spectrophotometer (Pye Unicam Ltd, Cambridge, England) was used for the determination of the thermal denaturation profile. The type strain of E. coli UQM

1803, with a mol% G+C of 51.7 (Blackall *et al.*, 1985), was used as a reference strain. The mol% G+C was calculated from the formula of Mandel and Marmur (1968):

$$\%G+C(test) = \%G+C(std) + 2.08(Tm(test)-Tm(std))$$

III. RESULTS

a. Presumptive Identifications

The results of tests used to presumptively assign Flavobacterium strains to species are presented in Tables 21 to 24. Strains R43, R44 and R58 were presumptively identified as F. meningosepticum (Table 21). R43 and R44, which were isolated from the same freeze-dried culture received as NCTC 10016, produced colony types which differed in size and hue of pigmentation with R43 producing bigger but paler colonies. Microscopic inspection showed a further difference with R44 producing filaments in broth cultures.

Strains R46, R48, R49, R54, R56 and U62 were similar to the published description of F. gleum (Table 22). Mol %G+C values for R46, R48 and U62 were within the range published for F. gleum (Holmes et al., 1984b) and Group IIb but were higher than the published value for F. balustinum (Holmes et al. 1984a). Confirmation of these identifications is required as it is difficult to distinguish F. gleum from Group IIb (Table 18) and some atypical biochemical reactions were detected. R46, which produced acid from ASS sucrose, differs from F. gleum in this reaction and is therefore probably a strain of Group IIb. This is one of the few reliable tests with which to distinguish these taxa. Holmes et al. (1984b) state that F. gleum may be distinguished from representative strains of Group IIb by DNA:DNA hybridization, but confirmation at this level was not possible in this study. R48 produced acid from ASS salicin which makes it atypical of both F. gleum and Group IIb, although Holmes et al. (1984b) report one of 55 strains of Group IIb being positive for this

test. If U62 is confirmed as F. gleum it will be the first non-clinical strain of the species to be reported.

R47 and U1 were presumptively identified as Group IIb. The non-clinical U1 was similar to the description of F. balustinum but the name Group IIb was preferred because the description of F. balustinum requires revision (Holmes and Owen, 1981). It therefore seemed prudent to avoid use of this name pending such revision and determination of its %G+C.

R55 and R59 were presumptively identified as F. odoratum (Table 23) and R42 and R51 were presumptively identified as F. multivorum and F. spiritivorum (Table 24).

b. Antibiotic Susceptibility Tests

The results of the disc diffusion tests performed at 35°C and 30°C are presented in Tables 25 and 26 respectively and the MIC results of strains selected on the basis of the disc tests are listed in Tables 28 and 37. The 35°C disc susceptibility results were interpreted in two groups as follows:

Group (i): those antibiotics which are calibrated for the CDS method of Bell (1975, 1984) i.e. ampicillin, ticarcillin, moxalactam, cefotaxime, sulphamethoxazole, trimethoprim, gentamicin and norfloxacin, and

Group (ii): the remaining antibiotics, which have not been calibrated for this method i.e. aztreonam, cefamandole, cefoperazone, cefoxitin, cefsulodin, ceftazidime, cefuroxime,

TABLE 21

Some Characteristics of Strains Presumptively Identified as F. meningosepticum

Test	Type strain * NCTC 10016	R43	R44	R58
Conspicuous pigment	-	-	+	-
Colony diameter in mm	1.80	1.55	1.30	1.80
Filamentous rods	-	-	+	+
Indole production	+	+	+	+
Nitrite reduction	+	+	+	-
Urease	-	-	-	-
ONPG	+	+	+	+
Esculin hydrolysis	+	+	+	+
Starch hydrolysis	-	-	-	-
ASS glucose acid	+	+	+	+
ASS maltose acid	+	+	+	+
ASS trehalose acid	+	+	+	+
ASS salicin acid	-	-	-	-
ASS arabinose	-	-	-	-
ASS sucrose acid	-	-	-	-
DNase	+	+	+	+

* Data of Holmes (1987)

TABLE 22

Some Characteristics of Strains Presumptively Identified as F. gleum and Group IIb

	F. gleum								F. balustinum/
Test	*	U62	R46	R48	R47	R49	R54	R56	U1@(IIb)#
Conspicuous pigment	+	+	+	+	+	+	+	+	+/+
Indole production	+	+	+	+	+	+	+	+	+/+
Nitrite reduction	7/12	-	+	-	+	-	+	+	-/d
Urease	7/12	-	+	+	+	+	-	-	-/d
ONPG	9/12	+	-	+	+	+	-	+	-/d
Esculin hydrolysis	+	+	+	+	+	+	+	+	+/+
Starch hydrolysis	9/12	+	+	+	+	+	+	+	-/d
ASS glucose acid	+	+	+	+	+	(+)	+	+	+/+
ASS maltose acid	+	(+)	+	+	+	(+)	+	-	-/+
ASS trehalose acid	+	+	+	(+)	-	(+)	(+)	-	-/d
ASS salicin acid	-	-	-	+	-	-	-	-	-/-
ASS arabinose	10/12	-	+	(+)	-	-	-	-	-/d
ASS sucrose acid	-	-	+	-	-	-	-	-	-/d
DNase	10/12	+	+	+	+	+	+	+	+/d
Mol% (G+C)		37.1	36.1	38.2					
Mol% (G+C)	*36.6-38.6								#33.1/35.0-38.5

(+) Delayed positive reaction - taking at least 4 days incubation for urease and at least 6 days for ASS sugars.

\# Data of Holmes et al. (1984a), Group IIb results are shown beside F. balustinum i.e. F. balustinum/IIb

TABLE 22 (Continued)

* Data of Holmes et al. (1984) - fractions (e.g. 7/12) indicate the number of positive results and the total number of strains tested for tests in which strain variability occurs.

@ U1 was an unclassified strain which was included in Table 22 because it was biochemically similar to F. balustinum

d Variable

TABLE 23

Some Characteristics of Strains Presumptively Identified as F. odoratum

Test	F. odoratum NCTC 11036	R55	R59
Conspicuous pigment	+	+	+
Indole production	-	-	-
Nitrite reduction	+	+	+
Urease	+	+	+
ONPG	-	-	-
Esculin hydrolysis	-	-	-
Starch hydrolysis	-	-	-
ASS glucose acid	-	-	-
ASS maltose acid	-	-	-
ASS trehalose acid	-	-	-
ASS salicin acid	-	-	-
ASS arabinose	-	-	-
ASS sucrose acid	-	-	-
DNase	+	+	+

TABLE 24

Some Characteristics of Strains Presumptively Identified as F. multivorum and F. spiritivorum

Test	F. multivorum #	R42	R51	F. spiritivorum #	F. thalpophilum *
Conspicuous pigment (24 h incubation)	-	-	-	-	-
Indole production	-	-	-	-	-
Nitrite reduction	-	-	-	-	-
Urease	+	+	+	+	+
ONPG	+	+	+	+	+
Esculin hydrolysis	+	+	+	+	+
Starch hydrolysis	-	-	-	-	-
ASS glucose acid	+	+	+	+	+
ASS adonitol acid	-	-	-	-	+
ASS maltose acid	+	+	+	+	+
ASS mannitol acid	-	-	+	+	-
ASS trehalose acid	+	+	+	+	+
ASS salicin acid	+	+	+	+	+
ASS arabinose	+	+	-	d	+
ASS sucrose acid	+	+	+	+	+
DNase	d	+	+	+	d

Data of Holmes et al. (1984a)

* Data of Holmes et al. (1983)

d Variable

cephalothin, and erythromycin. Although the results of the routine 3-dimensional tests are included in Tables 25 and 26 these are reviewed separately.

It was necessary to develop interpretive criteria for the uncalibrated antibiotics (Group (ii) above). This was done by comparing MICs and annular radii for the uncalibrated antibiotics (with the assumption that MIC results constitute a reliable benchmark by which to calibrate disc tests). This approach was not possible however for cefuroxime as this drug was not included in the MIC study. It was therefore necessary to establish arbitrary criteria for cefuroxime which were based on the assumption that the absence of an inhibition zone represented resistance and a large inhibition zone (annular radius $\geq$ 10 mm) was considered to indicate susceptibility. This approach identified what were assumed to be clear-cut extremes of resistance and susceptibility only, leaving the interpretation of annular radii between 0 and 10 mm in abeyance. Intermediate results between 0 and 10 mm were not evaluable.

Attempts to calibrate other antibiotics indicated poor correlation of MICs and annular radii and as a consequence results which were not evaluable were obtained for some strains with each of the non-CDS antibiotics. This problem was greatest for tests with cephalothin, cefoperazone, cefoxitin, and ceftazidime, antibiotics for which comparatively large portions of the inhibition zone (i.e. $>$ 3 mm annular radius) were not evaluable. F. odoratum NCTC

11179 was a particular problem with cefoxitin disc testing. This strain grew to the edge of the cefoxitin disc (suggesting resistance) but was moderately susceptible by MIC testing. This result exemplifies the difficulty of providing accurate susceptibility results for Flavobacterium and suggested that any strain growing to the edge of a cefoxitin disc could not be assumed to be resistant to cefoxitin.

With only 35 Flavobacterium strains included in the MIC study it was not considered worthwhile to statistically analyse the correlation of MIC and annular radius results. In view of the complexities arising from the variability of growth and the lack of sufficient numbers of results distributed among large, intermediate and small zone sizes and high, intermediate and low MICs, it was obvious that the variation about regression lines would have been considerably greater than for the aerobic bacteria which are most frequently tested in clinical laboratories. Judging by impression, but nevertheless with some certainty, it appeared that the difficulty in accurately calibrating disc and MIC results for Flavobacterium paralleled similar difficulties which have been experienced in attempting to correlate the two techniques for anaerobes (Barry and Fay, 1974; Kwok et al., 1975). A further complication was the possibility that agar dilution testing might not necessarily be an accurate reference method by which to compare disc results. This is suggested by the detection of resistance mostly by disc testing for antibiotics such as cefotaxime, ceftazidime and trimethoprim (Table 27). The discrepancies detected when comparing disc and MIC tests therefore failed to sustain the

assertion of other workers that disc tests are unreliable and MICs are reliable because disc tests give a false impression of susceptibility.

The correlation of disc diffusion and MIC testing in this study was poor and inconsistent for all Flavobacterium taxa, not just F. meningosepticum and Group IIb, as previously reported in the literature (Maderazo et al., 1974; Von Graevenitz and Grehn, 1977; Aber et al., 1978; Winslow and Pankey, 1982; Johny et al., 1983; Bruun, 1987). The antibiotics which had poorest correlation were ceftazidime, cefoperazone, chloramphenicol, cefotaxime, cefoxitin and trimethoprim (Table 27).

Although the errors were not consistent it did appear that they were more antibiotic-dependent than method-dependent. For example in the 15 discrepancies for cefotaxime testing, resistance was detected by disc testing and not by MIC testing, whereas in 14 of 15 discrepancies with chloramphenicol the disc test failed to detect resistance as detected in the MIC test. That is, with certain antibiotics it was sometimes possible to predict the direction of a discrepancy for a specific antibiotic but it was not possible to make conclusions about either technique being prone to consistently indicate false susceptibility or false resistance.

If, to simplify discussion, it is assumed that MIC results are an accurate reference technique, 64/525 (12.2%) disc tests

failed to indicate resistance and 56/525 (10.7%) disc tests failed to detect susceptibility or moderate susceptibility. The discrepancies for the disc test were consistent (i.e. missing only resistance or missing only susceptibility) for only three antibiotics (ampicillin, ticarcillin and cefotaxime) while for a further ten antibiotics (cephalothin, cefamandole, cefoperazone, ceftazidime, cefsulodin, cefoxitin, trimethoprim, gentamicin, erythromycin and chloramphenicol) the failures were inconsistent, failing to detect both susceptibility and resistance as determined by MIC tests.

F. aquatile

F. aquatile NCIB 8694 is nutritionally fastidious and could not be tested on Sensitest agar. On cytophaga agar it failed to produce a lawn of growth after suspension in physiological saline. However direct swabbing of a colony onto cytophaga agar produced sufficient growth to detect responses to antibiotic discs. Using this technique F. aquatile exhibited confluent growth to the edges of discs containing moxalactam, cefotaxime, aztreonam, trimethoprim and gentamicin. Unlike most other species of Flavobacterium (except F. breve) F. aquatile exhibited a zone of inhibition around the cefsulodin disc. Whether or not this result could be interpreted as susceptibility to cefsulodin is uncertain because of the unstandardized technique employed. However this was a very interesting result as cefsulodin is a narrow-spectrum, essentially anti-pseudomonal antibiotic.

TABLE 25

Disc Diffusion Antibiotic Susceptibility Tests

Overnight Incubation at 35°C - Annular Radii in mm.

STRAIN CODE	ORGANISM	ANTIBIOTIC: AMP	TIC	KF	FOX	MA	CXM	MOX	CTX	CFP	CAZ	CFS	ATM	SF	W	E	C	CN	NOR
NCTC 11099	F. breve	7.5*	8.5*	4.4	12.3	4.7*	5.1	11.8	3.2	8.0*	9.3	6.7	13.8	10.7	2.5	8.6	9.4	5.5	7.2
NCTC 11162	" "	3.5	4.1	0	7.3	0	0	11.0	0	4.3	6.8	6.8	11.3	8.6	3.2	12.9	9.0	5.5	4.8
U 31	" "	13.4	14.2	5.3	13.6	6.0	5.8	12.5	3.8	11.5	12.5	9.1	0	>20.0	>20.0	16.1	13.1	4.5	13.5
NCTC 11432	F. gleum	0	0	0	6.3	0	0	7.3	0	8.0*	8.2	0	0	1.2	13.1	10.5	10.1	4.2	8.0
NCTC 10795	" "	0	0.6	0	5.5*	0	0	6.3	0	7.4*	9.2	0	0	1.0	10.7	7.0	11.8	5.0	4.8
R 48	" "	0	0	0	4.9	0	0	6.0	0	7.1*	8.0	0	0	0	11.7	9.2	8.5	3.6	5.9
R 49	" "	0	0.9	0	4.6	0	0	5.5	0	5.8	7.3	0	0	4.4	9.8	7.2	8.3	4.8	8.3
R 56	" "	0	1.0	0	6.5	0	0	6.2	0	3.1	6.2	0	0	5.1	10.8	9.5	11.8	3.2	9.1
U 62	" "	0	0.7	0	4.0	0	0	4.4	0	5.2*	9.6	0	0	2.1	12.6	10.0	10.1	6.0	7.4
NCTC 10016	F. meningosepticum	0	1.1	0	0.8	0	0	3.2	0	3.1	0	0	0	0	0	11.5	4.8	2.4	5.4
NCTC 10585	" "	0	2.0	0	6.5	1.3	0	5.9	0	3.7	0	0	0	0	0	9.3	5.3	1.6	6.5
R 43	" "	0	0.7	0	0.7	0	0	2.8	0	2.8	0	0	0	0	0	9.3	3.0	0	4.4
R 44	" "	0	1.2	0	1.2	0	0	3.6	0	3.1	0	0	0	0	0	9.9	3.2	0	5.1
R 58	" "	1.4	4.3*	1.0	9.0	3.8*	0	9.4	2.1	8.8	2.5	1.0	0	0	0	9.9	2.7	1.9	6.8
MP 816	" "	0	2.1	0	5.5	1.2	0	0	0	2.6	0	0	0	0	0	9.4	2.4	0	6.5
MP 817	" "	0	3.4	0	6.8	1.1	0	0	0	3.4	0	0	0	0	0	8.5	1.3	0	4.5
MP 818	" "	0	2.0	0	5.0	1.0	0	0	0	2.6	0	0	0	0	0	8.6	1.6	2.8	4.6
MP 819	" "	0	2.8*	0	7.1	0	0	0	0	3.0	0	0	0	0	0	12.3	3.6	3.4	8.2
MP 820	" "	0	4.4	0	1.5	0.8	0	0	0	5.5	0	0	0	4.4	1.6	8.4	13.7	2.2	5.8
MP 894	" "	0	1.0	0	0	0	0	0	0	2.5	0	0	0	1.6	4.4	10.4	8.6	0	4.6
MP 903	" "	0	5.2	0	8.9	3.1	0	7.7	0	6.0	0	0	0	0	0	8.2	10.0	0	5.1
MP 963	" "	0	4.9	0	4.3	3.0	0	7.0	0	6.5	0	0	0	0	0	7.6	5.5	0	5.9
MP 966	" "	0	3.5	0	3.8	2.8	0	1.8	1.2	3.7	2.3	0	0	0	0	7.4	3.1	0	4.5
MP 969	" "	0	5.0	0	0	0	0	0	0	5.9	0	0	0	2.0	2.0	9.0	2.6	0	4.8
MP 970	" "	0	3.7	0	4.2	3.6	0	6.8	0	6.0	0	0	0	0	0	7.7	5.4	0	4.6
MP 1702	" "	1.8	4.5	0	4.5	4.5	0	7.1	1.6	5.4	0	0	0	0	9.0	10.6	5.2	4.6	5.2
MP 1742	" "	0	2.6	0	1.0	1.0	0	4.2	0	2.9	0	0	0	0	9.8	9.9	4.8	4.6	7.2
MP 2215	" "	0	2.0	0	7.7	1.3	0	1.2	1.0	3.3	0	0	0	0	0	7.2	1.1	0	4.3
MP 2347	" "	0	5.5	0	4.5	2.0	0	0	3.4	7.9	1.6	0	0	5.5	0	8.6	7.2	6.6	5.5
MP 2348	" "	1.6	4.8	0	4.6	3.9	0	2.8	3.5	5.4	2.2	0	0	0	0	8.1	4.8	3.6	5.3
NCTC 11033	F. multivorum	2.2	6.8	2.8	1.0	3.7	3.5	6.8	2.0	2.6	7.2	0	3.4	12.2	9.6	6.2	7.5	3.5	9.3
NCTC 11343	" "	8.9	12.2	9.3	3.5	7.6	7.9	8.0	7.8	6.6	10.6	3.5	5.5	12.6	9.1	7.5	8.0	7.6	11.7
R 42	" "	0	4.4*	3.3	0	3.5	2.0	5.2	3.0	6.7	9.9	0	0	10.8	9.8	6.0	9.3	6.7	9.4
MP 1170	" "	0	4.9*	0.8	0	1.7	0	6.2	0	2.6	6.1	0	1.5	9.4	9.3	10.0	10.0	0	8.6
MP 1174	" "	0	4.6	1.4	0	2.1	0	4.4	0	2.4	4.9	0	0	8.6	8.9	8.1	9.5	0	9.8
MP 1177	" "	6.0*	9.6*	4.2	1.2	4.6	5.3	6.8	4.5	4.7	8.2	0	4.0	10.5	7.7	6.7	7.6	2.6	11.3
MP 1180	" "	4.3	2.1	3.2	0	2.5	1.7	0	1.8	0	6.9	0	0	10.3	8.1	9.0	9.8	5.0	10.3
MP 1201	" "	5.1	7.9	3.6	0.9	4.6	4.2	6.7	4.2	4.4	8.2	0	4.1	10.9	7.3	5.5	7.0	1.7	10.4
MP 1204	" "	1.0	4.9	1.0	0	2.1	1.0	4.3	0	2.4	4.6	0	0	8.9	8.2	7.8	9.1	0	9.8
MP 1205	" "	4.3	6.8	2.5	0.7	3.5	3.2	6.6	1.7	2.9	8.1	0	3.7	8.9	8.5	4.6	6.4	1.9	9.0
MP 1210	" "	0	5.7*	1.5	0	2.7	1.5	7.1	1.2	5.1*	8.6	0	0	6.7	8.2	8.1	9.3	0	8.2
MP 1213	" "	0	5.4*	1.9	0	2.4	0	4.8	0	2.9	5.9	0	0	5.3	9.3	9.6	11.4	0	8.6
MP 1231	" "	1.0	5.3*	1.1	1.2	2.6	1.0	6.1	0	3.5	6.0	0	2.4	7.6	7.6	7.7	9.9	1.6	10.3
MP 1236	" "	0	4.0	1.7	0	1.7	0	4.0	0	2.2	4.5	0	0	7.1	8.0	6.9	7.8	0	9.3

TABLE 25 (continued)

Disc Diffusion Antibiotic Susceptibility Tests

Overnight Incubation at 35°C - Annular Radii in mm.

STRAIN CODE	ORGANISM	ANTIBIOTIC: AMP	TIC	KF	FOX	MA	CXM	MOX	CTX	CFP	CAZ	CFS	ATM	SF	W	E	C	CN	NOR
MP 2324	F. multivorum	9.0	15.8	9.5	6.4	10.2	10.9	9.6	9.2	9.5	9.1	5.8	7.7	14.9	10.9	13.7	13.2	3.4	12.6
NCTC 11036	F. odoratum	5.4	4.0	0	1.6	0	0	1.5	0	0	0	0	1.8	0	0	14.3	5.1	0	5.4
NCTC 11179	" "	1.2	0	0	0	0	0	0	0	0	0	0	0	8.5	0	12.1	6.0	0	4.3
NCTC 11180	" "	6.2*	6.8*	1.8	7.1	0	2.3	5.9	0	0	1.8	3.3	8.5	2.8	0	14.3	11.1	0	6.6
R 25	" "	4.8*	2.8	0	6.2	0	0	1.4	0	0	2.2	0	6.8	0	0	9.7	7.4	0	4.6
R 28	" "	4.5*	2.2	0	4.5	0	0	3.8	0	0	0	0	7.7	3.3	0	10.6	8.9	0	6.4
R 37	" "	4.6	2.0	0	4.6	0	0	3.0	0	0	1.6	0	6.7	0	0	8.7	7.9	0	3.9
R 55	" "	2.5	1.1	0	6.2	0	0	2.1	0	0	1.8	0	5.2	0	0	8.8	5.9	0	4.6
R 59	" "	0	0	0	6.6	0	0	3.1	0	0	0	0	0	0	0	0	8.2	0	5.0
U 59	" "	12.6	10.2	6.4	8.3	1.5	6.0	8.1	0	1.5	5.6	6.1	6.8	5.1	0	11.8	12.0	8.0	5.4
NCTC 11386	F. spiritivorum	1.8	4.5*	1.4	0.7	2.8	0	1.8	0	1.5	7.5	0	0	1.6	0	6.2	6.7	3.4	7.1
NCTC 11387	" "	1.2	4.6	0	0	1.1	0	0	0	0	6.4	0	0	4.0	0	1.8	6.2	0	5.7
R 41	" "	0	3.0	2.0	1.5	3.2	0	5.5	1.2	6.2	9.0	0	0	11.4	11.8	4.8	7.8	7.6	9.1
R 51	" "	2.0	6.3*	1.8	1.8	3.1	0	2.5	0	2.0	9.8	0	0	13.2	4.5	3.4	11.2	2.2	7.8
MP 2242	" "	1.5	4.1	2.3	1.2	1.7	0	0	0.8	2.3	6.9	0	0	11.2	1.5	2.0	9.0	0	4.3
MP 997	F. thalpophilum	4.2	7.3	3.7	4.5	6.0	3.7	3.5	3.1	5.0	10.1	2.3	2.6	8.7	5.9	5.8	6.0	0	8.6
MP 1207	" "	3.1	6.6	5.7	3.8	4.5	3.6	0	3.2	4.6	10.0	0	0	10.3	2.5	6.9	6.8	1.2	10.0
MP 1232	" "	3.3	6.0	4.8	4.8	4.7	3.5	2.5	3.1	3.4	10.1	0	3.7	10.8	6.8	8.3	5.3	0	11.5
R 34	Group IIb	4.6	6.5	0	7.0	0	0	5.5	1.8	8.0	7.5	0	0	5.3	4.7	11.5	10.9	6.0	9.0
R 46	" "	0	0	0	5.3	0	0	4.1	0	6.0*	10.1	0	0	1.2	11.1	6.5	7.8	5.1	6.9
R 47	" "	0	2.0	0	5.8*	0	0	6.8	0	6.1	8.9	0	0	6.7	11.2	8.9	11.9	6.7	6.5
R 50	" "	2.7	6.2	0	7.7	0	0	5.8	1.9	7.5	6.5	0	0	5.1	8.1	7.6	7.5	3.4	6.0
R 54	" "	0	1.5	0	5.0	0	0	5.2	0	4.7	8.8	0	0	8.4	8.6	8.3	8.0	5.4	5.8
R 862	" "	0	0	0	2.6	0	0	7.6	0	8.1	9.7	2.6	0	3.3	9.3	11.6	11.0	6.1	7.0
U 58	" "	0	0	0	3.4	0	0	3.1	0	5.7*	9.7	0	0	1.2	13.7	10.5	8.0	0	6.2
MP 7	" "	0	3.3	0	4.0	0	0	1.8	0	8.4*	10.9	0	0	5.4	14.7	10.0	5.2	5.3	8.1
MP 8	" "	0	0	0	6.0*	0	0	2.5	0	6.1*	8.1	0	0	0	11.7	2.6	2.5	0.1	3.9
MP 305	" "	0	0	0	7.6*	0	0	4.5	0	7.3	8.4	0	0	1.9	12.5	5.8	2.3	3.0	6.2
MP 525	" "	0	0	0	6.1*	0	0	0	0	3.2*	5.7	0	0	1.9	9.7	1.3	4.3	0	4.7
MP 547	" "	0	1.3	0	4.9	0	0	0	0	2.0	3.7	0	0	2.8	8.0	0.8	3.4	0.7	6.4
MP 548	" "	0	0.8	0	6.2*	0	0	0	0	2.6	4.8	0	0	3.7	8.8	1.2	4.5	0.8	6.0
MP 582	" "	0	0	0	6.0	0	0	0	0	3.7	5.8	0	0	2.6	10.5	1.4	6.1	0	5.7
MP 592	" "	0	0	0	5.5*	0	0	0	0	3.0	5.2	0	0	3.1	10.1	1.1	3.6	0	3.6
MP 616	" "	0	1.3	0	6.5*	0	0	0	0	4.0	5.8	0	0	3.9	9.8	2.0	6.4	0.8	4.8
MP 628	" "	0	1.9	0	3.3*	0	0	1.3	0	5.7*	6.1	0	0	2.1	8.1	9.8	7.3	4.1	8.6
MP 639	" "	0	0.9	0	4.4	0	0	0	0	2.8	4.6	0	0	2.7	8.0	1.1	6.5	0.7	5.8
MP 1098	" "	0	0.6	0	4.4	0	0	2.1	0	6.5*	8.8	0	0	1.3	11.0	1.7	2.1	1.3	4.1
MP 1409	" "	0	1.0	0	6.2*	0	0	0	0	2.2	6.2	0	0	2.2	9.2	0	6.7	0	4.4
MP 1846	" "	0	0	0	5.3*	0	0	3.9	0	4.5*	7.5	0	0	0.1	11.6	7.2	1.9	1.9	6.2
R 5	Unidentified	0	0	0	4.2	0	0	3.1	0	4.3	5.5	0	0	5.7	5.7	11.1	9.1	9.5	9.7
R 19	"	7.7*	8.5*	4.2	11.9	4.4	3.9	14.6	5.2	8.7*	9.1	9.5	14.2	8.6	2.0	13.1	12.0	11.3	8.5
U 93	"	19.8	20.8	12.5	16.8	7.3	14.4	10.5	13.0	14.9	13.6	9.8	1.6	1.8	6.8	17.5	10.4	9.1	9.8
U 105	"	16.3	17.9	0	5.2	5.2	0	15.1	8.0	15.0	16.4	7.1	9.6	22.8	0	21.9	16.5	13.2	14.1

TABLE 25 (continued)

Disc Diffusion Antibiotic Susceptibility Tests

Overnight Incubation at 35°C - Annular Radii in mm.

STRAIN CODE	ORGANISM	ANTIBIOTIC: AMP	TIC	KF	FOX	MA	CXM	MOX	CTX	CFP	CAZ	CFS	ATM	SF	W	E	C	CN	NOR
ATCC 15997	"F. tirrenicum"	15.9	11.6	11.6	12.4	15.5	13.5	13.5	15.7	17.3	13.8	13.8	0	14.0	15.0	14.0	14.2	13.5	11.3
DSM 527	F. elegans	12.4	16.7	3.2	12.6	0*	15.3	0*	12.5	0*	12.3	2.5*	3.0*	14.5	3.3*	13.7	13.8	14.4	3.4
ATCC 17061	C. johnsonae	4.6	6.7	0	0	0	0	0	0	0	0	0	4.5	11.9	0	18.0	11.7	5.3	11.2

* zone margin distorted in 3-dimensional test

× enhancement of both growth and pigmentation in vicinity of antibiotic disc

Interpretive Criteria for Zone Sizes Listed in Table 25

	Antibiotic		Susceptible	Annular Radius in mm Not Evaluable	Resistant
	Aztreonam	ATM	> 11.2	8.6-11.2	< 8.6
	Cefamandole	MA	> 7.6	5.7-7.6	< 5.7
	Cefoperazone	CFP	> 6.6	3.1-6.6	< 3.1
	Cefoxitin	FOX	> 4.0	0-4.0	-
	Cefsulodin	CFS	> 6.2	5.8-6.2	< 5.8
	Ceftazidime	CAZ	> 10.0	4.3-10.0	< 4.3
**	Cefuroxime	CXM	> 10.0	0.1-10.0	0
	Cephalothin	KF	> 9.5	5.3-9.5	< 5.3
	Erythromycin	E	> 14.3	12.1-14.3	< 12.1
***	CDS Drugs		≥ 6.0	-	< 6.0

** More precise calibration not possible because MIC testing of this drug was not performed.

*** Ampicillin (AMP), ticarcillin (TIC), moxalactam (MOX), cefotaxime (CTX), sulphamethoxazole (SF), trimethoprim (W), chloramphenicol (C), gentamicin (CN), norfloxacin (NOR).

TABLE 26

Disc Diffusion Antibiotic Susceptibility Tests

Overnight Incubation at 30°C - Annular Radii in mm.

STRAIN CODE	ORGANISM	ANTIBIOTIC: AMP	TIC	KF	FOX	MA	CXM	MOX	CTX	CFP	CAZ	CFS	ATM	SF	W	E	C	CN	NOR
R 40	Group IIb	14.8	15.9	16.3	15.3	10.0	16.5	15.8	18.9	24.3	21.8	12.2	8.3	4.6	14.3	13.9	13.6	5.1	14.7
R 45	"	3.0	6.3	0	10.0	0	0	8.0	0	7.3	7.5	0	0	1.6	7.9	1.6	11.1	6.2	8.3
U 37	"	0	0.9	0	6.1	0	0	5.3	0	5.9	8.9	0	0	4.6	15.1	6.7	7.7	3.8	3.8
U 39	"	1.1	2.7	1.1	8.2	1.3	0	6.2	0	11.6	13.8	0	0	7.2	4.2	2.0	4.5	6.9	6.9
U 42	"	4.1	7.5	0	12.6	0	0	8.3	0	8.3	7.4	0	0	5.0	13.0	5.0	7.0	6.1	12.4
U 43	"	11.1	11.5	6.8	17.9	4.0	3.1	19.6	5.0	15.6	16.5	0	0	16.3	15.1	7.7	13.3	5.8	11.3
U 70	"	7.1	8.6	2.9	10.1	0	3.7	6.7	2.8	3.8	2.7	0	0	2.5	7.8	5.0	6.8	3.0	6.7
R 10	Unidentified	7.4	8.9	4.6	12.8	5.6	2.2	11.3	1.2	14.6	15.6	0	0	6.0	14.5	12.0	11.3	8.1	7.7
R 12	"	3.0	5.6	0	9.2	0	0	8.2	0	7.0	7.6	0	0	0.9	15.0	2.0	11.6	6.7	8.8
R 39	"	0.6	1.6	0.8	6.0	1.1	0	5.4	0	7.1	11.1	0	0	7.0	12.3	3.3	7.9	4.4	5.9
U 1	"	0	5.6	1.2	7.2	0	0	5.0	0	8.3	8.2	0	0	0	13.7	11.3	11.9	4.0	9.6
U 10	"	6.0*	9.8*	4.2	11.7	0	1.9	10.2	3.5	12.0	11.9	2.2	0	13.9	19.5	12.2	12.1	6.2	9.0
U 33	"	1.7	6.7	0	4.6	0	0	0	0	0	0	0	0	9.0	18.0	12.1	15.5	12.2	12.0
U 44	"	5.7*	8.4*	6.9	13.2	6.2	1.2	12.9	2.5	5.1	12.9	7.8	5.6	9.5	0	15.2	11.9	7.6	2.2
NCIB 8694	*F. aquatile*	12.9	11.0	10.2	13.0	9.3	11.1	0	0	8.1	6.0	7.0	0	10.0	0	12.7	15.6	0	10.1
ATCC 29551	*C. aquatilis*	0	0	0	4.5	0	0	0	0	0	0	0	0	10.0	0	12.0	9.0	0	3.8
NCIB 10150	*C. johnsonae*	3.5	5.9	0	3.2	0	0	0	0	0	0	0	0	5.2	0	10.3	10.8	3.5	9.8

* zone margin distorted in 3-dimensional test

F. breve

This was the most susceptible species tested at 35°C. The three strains varied considerably from each other in zone sizes and MICs. This finding was suggestive of heterogeneity within the species. NCTC 11162 was the most resistant strain while the environmental strain U31 was the most susceptible. All three strains of F. breve were susceptible to cefoxitin, moxalactam, cefsulodin, aztreonam, sulphamethoxazole and chloramphenicol, and resistant to cefotaxime and gentamicin by disc testing. In MIC tests all strains were susceptible to cefsulodin, cefoxitin and imipenem, and susceptible or moderately susceptible to cefoperazone, ceftazidime, cefotaxime and chloramphenicol, and resistant to gentamicin. Susceptibility to cefsulodin was a distinctive feature of the species.

The strains differed markedly in their response to ampicillin, ticarcillin, cephalothin, cefamandole, cefuroxime, cefoperazone, ceftazidime, aztreonam, trimethoprim and norfloxacin.

Discrepancies between disc and MIC results occurred with cefotaxime and trimethoprim. All strains were susceptible or moderately susceptible to cefotaxime by MIC testing but resistant by the disc diffusion method. NCTC 11162 was susceptible also to trimethoprim and erythromycin, and moderately susceptible to cefoperazone by the MIC method but resistant to trimethoprim and not evaluable to erythromycin and cefoperazone in the disc tests.

TABLE 27

Failures to Correlate Disc Diffusion and MIC Results

Antibiotic	No. of Failures*	Disc Test Failed to Detect	
		Resistance	Susceptibility or Moderate Susceptibility
Ampicillin	3	3	0
Ticarcillin	5	5	0
Cephalothin	5	3	2
Cefamandole	3	2	1
Cefoperazone	15	11	4
Ceftazidime	16	4	12
Cefsulodin	2	1	1
Cefotaxime	15	0	15
Cefoxitin	14	12	2
Trimethoprim	12	2	10
Gentamicin	8	5	3
Erythromycin	7	2	5
Chloramphenicol	15	14	1

* A failure is defined as any disc result which does not agree with an MIC result. (MIC results are assumed to be correct). There are two classes of failures - errors (i.e. disc and MIC results are contradictory), and instances where the disc test was not evaluable but the MIC test clearly indicated susceptibility, moderate susceptibility or resistance.

TABLE 28

Agar Dilution Antibiotic Susceptibility Tests
Minimum Inhibitory Concentrations in ug/ml.

STRAIN CODE	ORGANISM	ANTIBIOTIC: AMP	TIC	KF	MA	CFP	CAZ	CFS	CTX	ATM	FOX	IPM	M	CN	E	C
NCTC 11099	F. breve	4	16	32	32	8	2	4	4	1	0.5	1	16	64	16	8
NCTC 11162	" "	32	128	>128	128	32	16	16	32	4	4	2	8	64	0.5	16
U 31	" "	1	4	16	16	1	0.5	16	2	>128	0.25	0.06	0.06	16	0.25	16
NCTC 10975	F. gleum	>128	>128	>128	>128	32	4	>128	64	>128	16	2	0.25	32	32	64
NCTC 11432	" "	>128	>128	>128	>128	4	4	>128	64	>128	8	2	0.25	8	16	32
R 48	" "	>128	>128	>128	>128	32	4	>128	64	>128	16	4	0.12	16	16	64
U 62	" "	>128	>128	>128	>128	32	4	>128	64	>128	32	64	0.25	32	128	64
NCTC 10016	F. meningosepticum	>128	>128	>128	>128	32	>128	>128	64	>128	32	16	4	16	16	32
NCTC 10585	" "	>128	>128	>128	128	64	>128	>128	64	>128	16	32	2	32	32	64
R 58	" "	64	128	64	32	16	64	128	16	>128	8	16	2	64	32	64
MP 819	" "	128	>128	128	64	32	>128	>128	32	>128	16	32	2	8	8	32
MP 969	" "	>128	>128	>128	>128	32	>128	>128	64	>128	32	32	2	128	32	64
MP 2347	" "	128	>128	>128	64	16	128	>128	16	>128	16	64	2	64	32	64
NCTC 11033	F. multivorum	64	64	64	32	64	8	>128	16	64	64	32	2	16	32	32
NCTC 11343	" "	32	64	32	32	64	8	128	8	128	32	16	0.5	8	32	16
MP 1177	" "	32	64	32	32	64	8	128	8	64	32	8	1	64	32	64
MP 1210	" "	64	128	128	64	32	4	>128	16	>128	32	32	0.5	64	8	16
MP 2324	" "	16	4	16	8	8	8	32	2	32	16	0.5	0.5	32	4	8
NCTC 11036	F. odoratum	8	128	>128	>128	>128	64	>128	64	64	0.5	4	>128	128	4	32
NCTC 11179	" "	64	>128	>128	>128	>128	128	>128	>128	>128	16	16	>128	>128	4	32
NCTC 11180	" "	32	128	128	>128	>128	128	128	>128	>128	8	2	>128	>128	0.25	16
R 25	" "	64	>128	>128	>128	>128	128	>128	>128	>128	8	16	>128	>128	16	32
P 59	" "	128	>128	>128	>128	>128	>128	>128	>128	>128	8	4	>128	>128	>128	16
U 59	" "	2	8	64	128	128	64	64	32	32	2	0.25	>128	>128	16	8
NCTC 11386	F. spiritivorum	64	>128	64	64	128	16	>128	64	>128	64	16	4	128	>128	64
NCTC 11387	" "	64	>128	64	64	128	16	>128	64	>128	64	16	1	128	128	32
R 41	" "	>128	>128	64	64	64	4	>128	32	>128	64	16	0.5	16	128	32
R 51	" "	64	>128	64	64	64	16	>128	32	>128	32	8	4	64	>128	16
MP 997	F. thalpophilum	>128	>128	64	64	128	8	>128	32	128	16	16	1	128	128	64
MP 1207	" "	128	>128	64	64	128	16	>128	32	>128	16	16	1	128	128	128
MP 1232	" "	128	>128	64	32	32	8	>128	16	64	16	16	1	64	128	128
U 58	Group IIb	>128	>128	>128	>128	32	4	>128	128	>128	32	64	2	32	8	16
R 46	" "	>128	>128	>128	>128	4	1	>128	64	>128	8	64	0.25	32	128	32
R 5	Unidentified	>128	>128	>128	>128	32	16	>128	64	>128	32	>128	0.25	32	128	32
R 19	Unidentified	4	16	64	32	32	8	16	4	1	0.5	0.5	8	32	1	16
ATCC 27853	P. aeruginosa	>128	16	>128	>128	8	2	2	16	8	>128	2	>128	4	>128	128
ATCC 25922	E. coli	8	8	8	1	0.12	0.25	64	0.12	0.12	4	0.12	2	0.5	64	8
NCCLS : Susceptible		0-8	0-16	0-8	0-8	0-16	0-8	0-16**	0-8	0-8	0-8	0-4	0-8	0-4	0-0.5	0-8
INTERPRETIVE : Mod. Susceptible		16	32-64	16	16	32	16	32	16-32	16	16	8	-	8	1-4	16
STANDARDS* ; Resistant		32+	128+	32+	32+	64+	32+	64+	64+	32+	32+	16+	16+	16+	8+	32+

* Thornsberry *et al.* (1985); ** Barry *et al.* (1981)

TABLE 28 (Continued)

Symbols Used For Antibiotics

AMP - ampicillin

TIC - ticarcillin

KF - cephalothin

MA - cefamandole

CFP - cefoperazone

CAZ - ceftazidime

CFS - cefsulodin

CTX - cefotaxime

ATM - aztreonam

FOX - cefoxitin

IPM - imipenem

W - trimethoprim

CN - gentamicin

E - erythromycin

C - chloramphenicol

F. gleum

The strains of this species were more homogeneous than other species. In disc tests (Table 25) all F. gleum strains were susceptible to trimethoprim and chloramphenicol while cefoxitin and norfloxacin also appeared to be very active with only one of six strains not susceptible. All strains were resistant to ampicillin, ticarcillin, cephalothin, cefamandole, cefuroxime, cefotaxime, cefsulodin, aztreonam, erythromycin and sulphamethoxazole, and all but one strain were resistant to gentamicin. Results varied for moxalactam and most could not be evaluated for cefoperazone and ceftazidime.

In the MIC tests (Table 28) all strains were susceptible to ceftazidime and trimethoprim, and susceptible or moderately susceptible to cefoperazone, but resistant to ampicillin, ticarcillin, cephalothin, cefamandole, cefsulodin, cefotaxime, aztreonam, erythromycin and chloramphenicol. Susceptibility varied to cefoxitin, imipenem and gentamicin. Discrepancies occurred with chloramphenicol and gentamicin. The strains which were tested by both procedures were susceptible to chloramphenicol by disc testing but resistant by MIC testing and environmental strain U62 was just susceptible to gentamicin by disc testing (annular radius 6 mm) but resistant by MIC testing.

F. meningosepticum

This is a very resistant species. In both disc and MIC tests only cefoxitin inhibited a majority of strains. In MIC tests all six strains were susceptible to trimethoprim.

However 19 of 21 strains tested by the disc method were resistant to this agent.

MP969 was the most resistant strain and R58 the most susceptible. In disc tests all strains were resistant to ampicillin, ticarcillin, cephalothin, cefamandole, cefuroxime, cefotaxime, ceftazidime, cefsulodin, aztreonam and sulphamethoxazole, and most strains were resistant to moxalactam, trimethoprim, erythromycin, chloramphenicol, gentamicin and norfloxacin. Most cefoperazone disc tests could not be evaluated.

In MIC tests only trimethoprim inhibited all six strains. These were resistant to ampicillin, ticarcillin, cephalothin, cefamandole, ceftazidime, cefsulodin, aztreonam, imipenem, erythromycin and chloramphenicol. Susceptibility varied for cefoperazone, cefotaxime, cefoxitin and gentamicin. Discrepancies occurred with cefotaxime, cefoperazone, trimethoprim, chloramphenicol and gentamicin.

The ceftazidime disc test appeared to have taxonomic utility in distinguishing strains of F. meningosepticum from F. gleum and Group IIb. F. meningosepticum strains were much more resistant in this test having annular radii smaller than 2.6 mm while the other taxa had annular radii greater than 3.7 mm. It is possible that ceftazidime also has taxonomic utility in MIC tests. F. gleum and Group IIb strains had MICs $\leqslant$ 4 mcg/ml while F. meningosepticum ceftazidime MICs were $\geqslant$ 64 mcg/ml. Since only two strains of Group IIb were included in

the MIC study this evidence cannot be regarded as being as comprehensive as that derived from the disc tests.

F. multivorum

In disc tests all strains of F. multivorum were susceptible to chloramphenicol, norfloxacin and trimethoprim, and most (14/15) were susceptible to sulphamethoxazole. All strains were resistant to aztreonam and most were resistant (i.e. at least 13/15) to cephalothin, cefamandole, cefotaxime, cefsulodin, erythromycin and gentamicin. Results varied most for ampicillin, ticarcillin, cefoperazone, moxalactam, cefoxitin, and the uncalibrated zone sizes for cefuroxime also varied considerably.

In MIC tests all strains were susceptible to ceftazidime and trimethoprim, and susceptible or moderately susceptible to cefotaxime, but resistant to aztreonam. Susceptibility varied to ampicillin, ticarcillin cephalothin, cefamandole, cefoperazone, cefsulodin, cefoxitin, imipenem, gentamicin, erythromycin and chloramphenicol. Strain MP 2324 was much more susceptible than the other strains and was resistant only to aztreonam and gentamicin by both disc and MIC techniques. Apart from this strain, resistance to cefoxitin in MIC test and susceptibility to sulphamethoxazole in the disc test seemed to be reliable features by which to distinguish this species from F. meningosepticum, F. gleum and Group IIb. The only other strain which was an exception to this was MP 1213 which was marginally resistant to sulphamethoxazole. Discrepancies occurred with ampicillin, cephalothin, cefoxitin, cefamandole, cefotaxime, chloramphenicol and

gentamicin.

F. odoratum

This was a very resistant species. In both disc and MIC tests there was no agent to which all strains were susceptible. Cefoxitin was the most active agent by both techniques while chloramphenicol was equally effective only in the disc tests and imipenem was effective against four of the six strains included in the MIC tests.

In the disc tests all strains were resistant to cefotaxime, cefamandole, cefoperazone, aztreonam and trimethoprim, and most strains were resistant to ampicillin, ticarcillin, cephalothin, cefuroxime, moxalactam, ceftazidime, cefsulodin, sulphamethoxazole, gentamicin and norfloxacin. Erythromycin results were resistant or uncalibrated. If the comparatively susceptible, and therefore atypical, environmental strain U59 is excluded from consideration this species is characteristically resistant in disc tests to cephalothin, cefuroxime, moxalactam, ceftazidime, cefsulodin, cefamandole, cefotaxime, aztreonam, cefoperazone and trimethoprim, with the latter two drugs being potentially useful taxonomic markers which differentiate F. odoratum from Group IIb and all other Flavobacterium species except F. spiritivorum. Apart from variations in the zone sizes for aztreonam and sulphamethoxazole there was little evidence of heterogeneity.

In the MIC tests all strains were resistant to

cephalothin, cefamandole, cefoperazone, ceftazidime, cefsulodin, aztreonam, trimethoprim, and gentamicin. The high MICs for trimethoprim ($\geqslant$ 128 ug/ml) were clearly different from other species and Group IIb (MICs $\leqslant$ 16 ug/ml). Susceptibility varied to ampicillin, ticarcillin, cefotaxime, imipenem, erythromycin and chloramphenicol. The environmental strain U59 was however much more susceptible and clearly different from the clinical strains. Discrepancies occurred with ampicillin, cephalothin, cefoxitin, ticarcillin, cefotaxime, gentamicin and chloramphenicol.

F. spiritivorum

By both disc and MIC testing this species was the most resistant to the beta-lactam group of antibiotics and in resistance to all antibiotics was comparable to the other two very resistant species, F. meningosepticum and F. odoratum.

In disc tests all five strains were susceptible to chloramphenicol, resistant to ampicillin, cephalothin, cefamandole, cefuroxime, cefotaxime, cefsulodin, aztreonam, erythromycin and moxalactam, and unevaluable for cefoxitin. Susceptibility varied to norfloxacin, sulphamethoxazole, ticarcillin, trimethoprim and gentamicin. The zone sizes varied most for sulphamethoxazole, trimethoprim and gentamicin indicating strain heterogeneity in response to these agents.

In MIC tests all strains were susceptible to trimethoprim only, and susceptible or moderately susceptible to ceftazidime. All strains were resistant to ampicillin, ticarcillin, cephalothin, cefamandole, cefoperazone,

aztreonam, cefoxitin, gentamicin and erythromycin. Resistance varied to cefotaxime, imipenem and chloramphenicol. Discrepancies occurred with ticarcillin, cefotaxime, trimethoprim, chloramphenicol and gentamicin.

F. thalpophilum

Overall this species was similar to F. multivorum and F. spiritivorum but more susceptible, particularly to cefoxitin (by both disc and MIC tests) and ticarcillin (in disc tests only).

By disc testing the three F. thalpophilum strains were susceptible to ticarcillin, norfloxacin and sulphamethoxazole but resistant to ampicillin, cefotaxime, moxalactam, ceftazidime, cefsulodin, aztreonam, erythromycin and gentamicin. Results varied for cefamandole, chloramphenicol, trimethoprim, cephalothin and cefoxitin. By MIC testing all strains were susceptible to trimethoprim, susceptible or moderately susceptible to ceftazidime, moderately susceptible to cefotaxime and cefoxitin, and resistant to ampicillin, ticarcillin, cephalothin, cefamandole, cefsulodin, aztreonam, imipenem, gentamicin, erythromycin and chloramphenicol. Susceptibility varied to cefoperazone. Discrepancies occurred with ticarcillin, cephalothin, cefamandole, cefotaxime, trimethoprim and chloramphenicol.

Group IIb Strains

Twenty one strains of Group IIb were tested by the disc diffusion method but only two strains, U58 and R46, were

included in the MIC study because preference was given to establishing patterns for the described species. Twenty strains were susceptible to trimethoprim and 17 strains were susceptible to cefoxitin. All strains were resistant to ampicillin, cephalothin, cefamandole, cefuroxime, cefotaxime, cefsulodin, aztreonam and erythromycin. Susceptibility varied for ticarcillin and sulphamethoxazole (19/21 strains resistant), cefoperazone, ceftazidime, chloramphenicol and norfloxacin. Three strains were marginally susceptible to gentamicin. As MIC studies were not performed on these strains it was not possible to tell if this was true susceptibility or false susceptibility of the type which has been previously reported with disc tests (Johny et al., 1983).

Unclassified Flavobacterium Strains

Strain R5 was most similar to Group IIb, F. gleum and F. meningosepticum in its disc antibiogram. The annular radius for ceftazidime was 5.5 mm which was a much larger annular radius than found in F. meningosepticum strains in this study. It would therefore appear to be more nearly related to Group IIb and F. gleum. In an earlier study this strain was found to be non-saccharolytic and therefore could not be classified within the existing taxonomic framework of the genus (Thomson, 1982).

Strain R19, which was cultured from a freeze-dried vial labelled F. breve NCTC 11099, was resubmitted to NCTC and reported to be an atypical strain of Group IIb closely resembling F. breve but atypical of that species in its ability to hydrolyse both esculin and starch (Thomson, 1982).

In this study its disc and MIC antibiograms were similar to F. breve NCTC 11099 indicating that it might be a mutant of that strain.

Environmental strains U93 and U105 were very susceptible and therefore unlike other Flavobacterium strains, particularly in being susceptible to gentamicin. These two organisms had been previously referred to NCTC for an opinion where they were regarded as atypical strains of group IIb (Thomson, 1982).

Taxonomic Reference Strains Not Belonging to the Redefined Genus Flavobacterium

These strains were not received until after the MIC study had been completed and were therefore tested only by the disc diffusion method.

"F. tirrenicum" ATCC 15977

This organism was susceptible to all agents except aztreonam, to which it was resistant, and erythromycin, for which the result could not be calibrated.

F. elegans DSM 527

This flexibacter differed from typical Flavobacterium strains in being very susceptible to gentamicin. It was also susceptible to ampicillin, ticarcillin, cefoxitin, cefuroxime, cefotaxime, ceftazidime, sulphamethoxazole, and chloramphenicol. It was resistant to cephalothin, cefamandole, moxalactam, cefoperazone, cefsulodin, aztreonam,

trimethoprim, and norfloxacin. The erythromycin test was not evaluable. A conspicuous feature of this organism was that its growth and pigmentation were more conspicuous adjacent to the discs containing cefamandole, moxalactam, cefoperazone, cefsulodin, aztreonam and trimethoprim. It was not possible to determine if the increased pigmentation was merely an expression of the increased growth in the presence of antibiotics to which the organism was resistant or whether in fact the synthesis of pigment was stimulated by the antibiotics.

C. aquatilis ATCC 29551

This organism, which was tested at 30°C on blood-supplemented Sensitest Agar, appeared to be extremely resistant to beta-lactam antibiotics with only cefoxitin resulting in an inhibition zone. It also appeared to be resistant to trimethoprim and gentamicin.

C. johnsonae

Strain ATCC 17061 was susceptible to ticarcillin, sulphamethoxazole, erythromycin, chloramphenicol and norfloxacin, and resistant to all other agents in the disc tests except cefoxitin, for which the test was not evaluable. Strain NCIB 10150, which was tested at 30°C on blood-supplemented Sensitest Agar, appeared to be resistant to most beta-lactam antibiotics and trimethoprim.

Other Disc Diffusion Results at 30°C

The results of all disc tests at 30°C are presented in Table 26. There are no interpretive criteria for disc tests

at this temperature and parallel MIC studies were not performed. These tests were performed to provide a comparison of 37°C negative and 37°C positive strains. At 30°C there appeared to be considerable heterogeneity within Group IIb and amongst the unclassified strains. R40 was the most susceptible Group IIb strain and R45 and U39 were the most resistant in these tests. U33 was the most resistant unclassified strain.

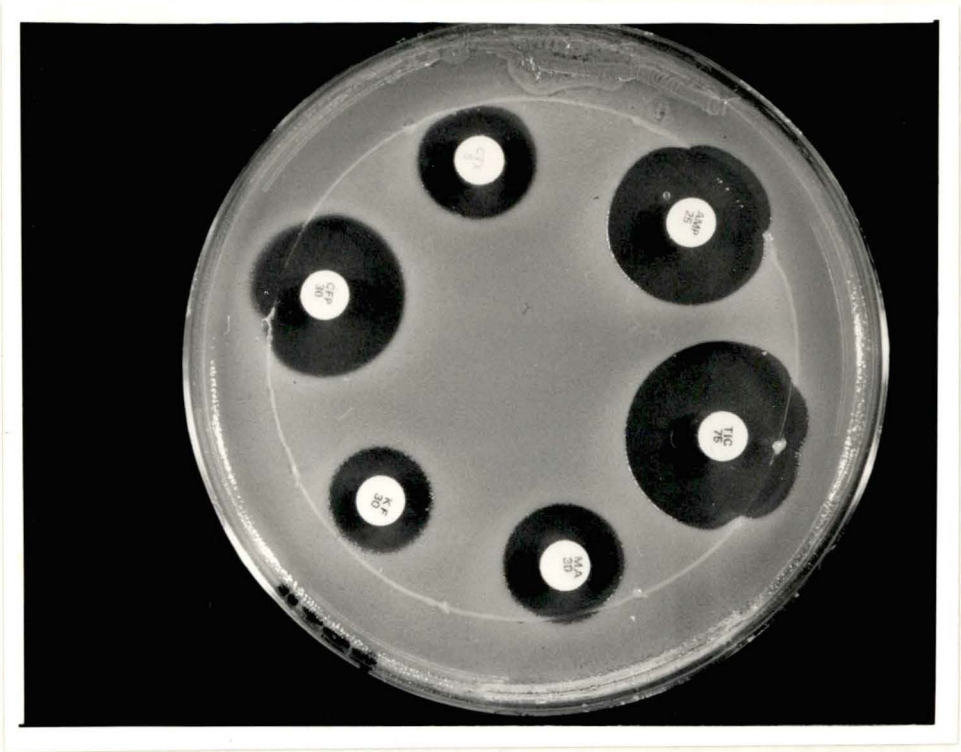
c. Routine 3-Dimensional Results

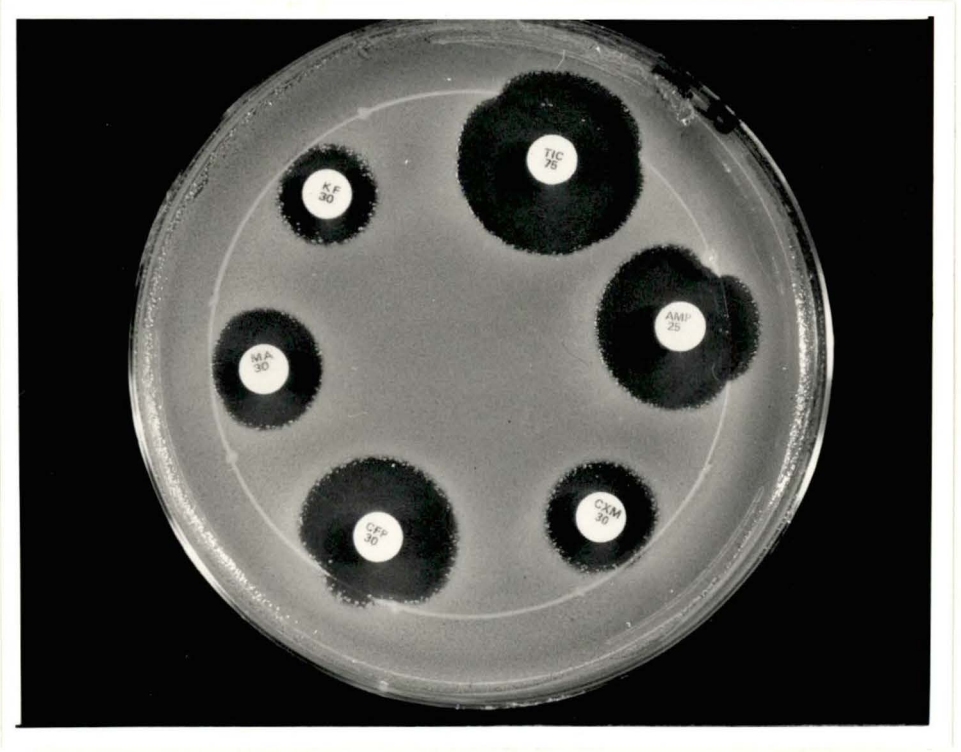
Because of the broad beta-lactam resistance of the Flavobacterium strains the 3-dimensional test did not yield as much information as expected. In many cases there was no inhibition zone around beta-lactam discs or the zone was too small to detect 3-dimensional effects. This problem is apparent in Figs 8 and 9 where F. breve NCTC 11099 and Flavobacterium R19 show distinct zone distortions for ampicillin, ticarcillin and cefoperazone but the zones for cephalothin, cefotaxime and cefuroxime are not quite large enough to readily discern enzymatic drug inactivation. Cefamandole inactivation can only just be detected. The inability of the routine 3-dimensional test to detect many drug inactivations is confirmed in the 3D+CL tests (Table 32) in which both strains clearly inactivated cephalothin, cefoxitin and cefotaxime.

Where inhibition zones were large enough to detect 3-dimensional effects there was evidence of beta-lactamase

Fig. 8. Circular 3-dimensional inoculation. Zone distortions suggest that F. breve NCTC 11099 can readily inactivate the penicillins ampicillin (AMP) and ticarcillin (TIC), and to a limited extent cefoperazone (CFP) and perhaps cefotaxime (CTX). The small zones for CTX, cefamandole (MA) and cephalothin (KF) restrict the utility of the technique.

Fig. 9. Flavobacterium R19 is capable of inactivating TIC, AMP, CFP and MA (note the flattening of the MA zone near the circular 3-dimensional inoculation). Zone sizes are too small for conclusions about KF and cefuroxime (CXM)


CFP 30
AMP 25
TIC 75
KF 30
MA 30


TIC 75
KF 30
AMP 25
MA 30
CFP 30
CXM 30

mediated inactivation of ampicillin, ticarcillin, cefamandole, cefoxitin and cefoperazone (Table 29).

d. Special 3-Dimensional Tests (Including 3D+CL Technique)

Since the utility of the 3-dimensional test was somewhat nullified by small inhibition zones in many of the routine disc tests (Tables 25 and 26), it was necessary to devise a more sensitive test to investigate the beta-lactamases of flavobacteria. To investigate the effectiveness of 3-dimensional testing in detecting a range of known beta-lactamases, each of the beta-lactamase-producing antibiotic reference strains was tested against a panel of beta-lactam antibiotics similar to that used in the disc tests. Ticarcillin was however replaced by carbenicillin which was found to be more beta-lactamase resistant and therefore a more discriminatory substrate. Three additional antibiotics were included - cephaloridine, cloxacillin and imipenem and cefsulodin was deleted. The antibiotic reference strains were tested by the more sensitive 3-dimensional technique in which either E. coli ATCC 25922 or S. aureus ATCC 25923 were used as assay organisms while the test strain was used for the 3-dimensional inoculation (Figs 10 and 11).

The results of this series of tests, which are presented in Table 30, indicated the 3-dimensional test distinguished characteristic patterns by which the reference beta-lactamases might be classified. No attempt was made to determine if the patterns were solely enzyme-determined or whether other host cell activities also contributed to the effect.

TABLE 29

Numbers of Strains Demonstrating Antibiotic Inactivation

In Routine 3-Dimensional Tests

(Results from Tables 25 and 26)

	Antibiotics *				
Taxon	AMP	TIC	MA	FOX	CFP
F. breve	1/3	1/3	1/3	0/3	1/3
F. gleum	0/6	0/6	0/6	1/6	4/6
F. meningosepticum	0/21	2/21	1/21	0/21	0/21
F. multivorum	1/15	6/15	0/15	0/15	1/15
F. odoratum	3/9	1/9	0/9	0/9	0/9
F. spiritivorum	0/5	2/5	0/5	0/5	0/5
F. thalpophilum	0/3	0/3	0/3	0/3	0/3
Group IIb	0/28	0/28	0/28	10/28	8/28
Unclassified	3/11	3/11	0/11	0/11	1/11

* AMP - ampicillin

TIC - ticarcillin

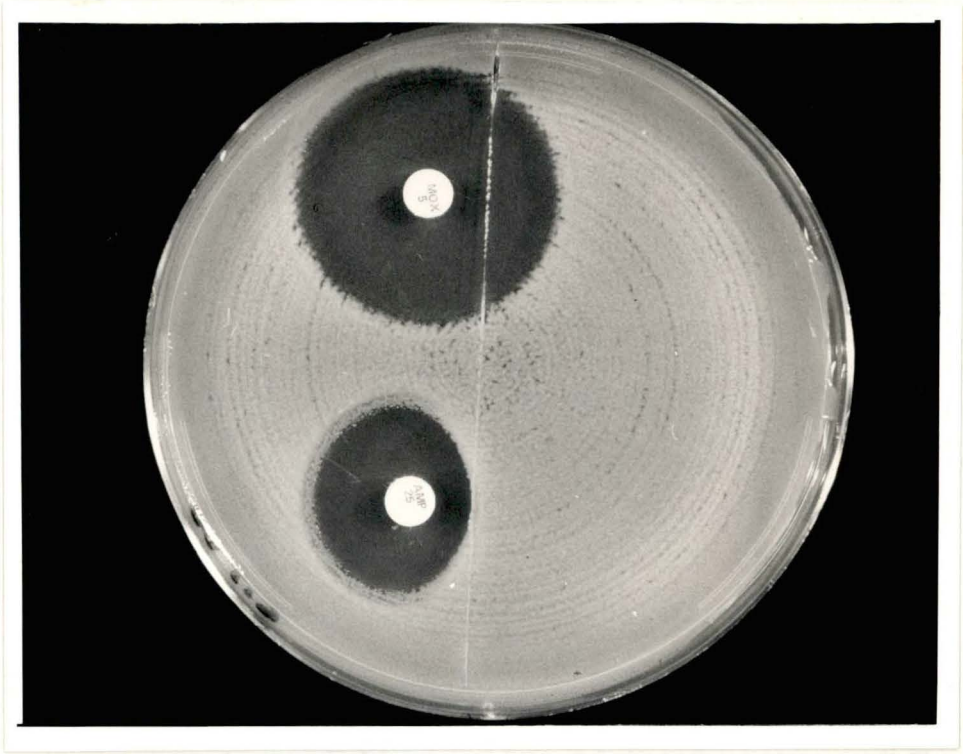
MA cefamandole

FOX - cefoxitin

CFP - cefoperazone

Fig. 10. °Special' 3-dimensional test with lawn of susceptible strain of E. coli (ATCC 25922) as assay organism and E. coli MISC 100, which produces Richmond and Sykes Class IIIa (TEM) beta-lactamase, in the straight-line 3-dimensional inoculation. The stability of moxalactam (MOX) and vulnerability of ampicillin (AMP) to the TEM beta-lactamase are apparent from the zone margins adjacent to the 3-dimensional inoculation.

Fig. 11. K. pneumoniae MISC 99, which produces a Richmond and Sykes Class IVa beta-lactamase, demonstrates powerful broad spectrum hydrolytic activity by inactivating ticarcillin (TIC) and cefuroxime (CXM). The assay organism is the same strain of E. coli as in Fig. 10.


AMP
75

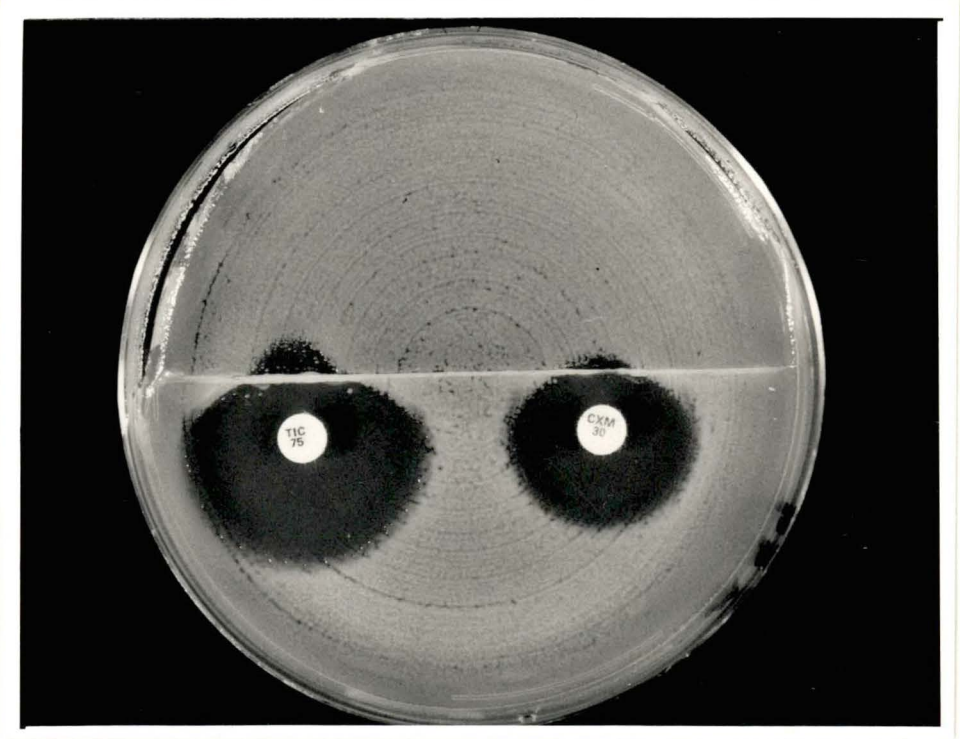

TIC
75
CXM
30

TABLE 30

Antibiotic Inactivations Detected Using 3-Dimensional Technique Modified to Increase Sensitivity: Antibiotic Reference Strains

STRAIN CODE	ENZYME: TYPE*:	ANTIBIOTIC AMP	CAR	KF	FOX	MA	CXM	MOX	CTX	CFP	CAZ	ATM	CR	OB	IPM
MISC 99	IVa	+	+	+	-	+	+	-	+	+	-	-	+	+	-
MISC 100	TEM	+	+	+	-	+	-	-	-	+	-	-	+	+	-
MISC 101	Ib	+	-	+	-	+	-	-	+	+	-	-	+	-	-
MISC 102	Ia	-	-	+	-	+	-	-	-	+	-	-	+	-	-
MISC 103	IVb	+	+	+	-	+	+	-	-	+	-	-	+	-	-
MISC 104	IIa	+	+	-	-	-	-	-	-	-	-	-	-	-	-
MISC 105	TEM	+	+	+	-	+	-	-	-	+	-	-	+	+	-
MISC 111	Ia	+	-	+	-	+	+	-	-	+	-	-	+	-	-
MISC 112	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MISC 119	TEM 1	+	+	+	-	+	-	-	-	+	-	-	+	-	-
MISC 120	TEM 2	+	+	+	-	-	-	-	-	+	-	-	+	+	-
MISC 121	OXA-1	-	-	-	-	-	-	-	-	-	-	-	+	-	-
MISC 122	OXA-2	-	-	+	-	+	-	-	+	+	-	-	+	+/-	-
MISC 123	OXA-3	-	-	+	-	+	-	-	-	+	-	-	+	+/-	-
MISC 124	PSE-1	+	+	-	-	-	-	-	-	-	-	-	-	-	-
MISC 125	PSE-2	+	-	-	-	-	-	-	-	-	-	-	-	+	-
MISC 126	PSE-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MISC 127	HMS-1	+	+	+	-	+	-	-	-	+	-	-	+	+	-
MISC 128	SHV-1	+	+	-	-	-	-	-	-	+	-	-	+	-	-
MISC 130	PSE-4	+	+	-	-	-	-	-	-	-	-	-	-	-	-
164 CD	Id	+	-	+	-	+	-	-	-	+/-	-	-	+	-	-

* Enzyme nomenclature: Roman numerals indicate Richmond and Sykes nomenclature. Terms which are generally associated with particular substrate profiles have been used for plasmid-mediated beta-lactamases

+/- Weakly positive result of unreliable reproducibility

Antibiotic Symbols

AMP - ampicillin
CAR - carbenicillin
KF - cephalothin
FOX - cefoxitin
MA - cefamandole
CXM - cefuroxime
MOX - moxalactam
CTX - cefotaxime
CFP - cefoperazone
CAZ - ceftazidime
ATM - aztreonam
CR - cephaloridine
OB - cloxacillin
IPM - imipenem

Although the more sensitive 3-dimensional technique was potentially useful for investigating the substrate profiles of beta-lactamase producing bacteria it was unsatisfactory because it yielded negative results for strain MISC 126 which produces the PSE-3 beta-lactamase. This beta-lactamase could however be detected by the clover leaf test. The clover leaf test on its own was also unsatisfactory because it failed to detect cephaloridine inactivation by the OXA-1 beta-lactamase of MISC 121 and cloxacillin inactivation by the OXA-2 beta-lactamase of MISC 122. This information was provided by the 3-dimensional test. It was therefore necessary to combine the two techniques (i.e. 3D+CL technique) to maximize the sensitivity of these investigations (Tables 31, 32, Fig. 12).

The 3D+CL technique provided distinctive profiles for both the beta-lactamase reference strains and the study strains. Strain MISC 112, the 'enzymeless' control was negative for all substrates. The penicillinase producer MISC 104 was positive only for penicillin substrates. Cephalosporinase producers MISC 101, MISC 102, MISC 111 and 164CD inactivated a range of cephems and only one penicillin, ampicillin. Richmond and Sykes Class IV broad spectrum beta-lactamase producers, MISC 99 and MISC 103, inactivated a wide range of substrates including carbenicillin and cefuroxime. Arbitrary interpretive criteria were developed from Table 31 to interpret the 3D+CL results. Penicillinase activity was regarded as demonstrable activity against either ampicillin, carbenicillin or cloxacillin. Since many of the strains showing penicillinase activity according to this criterion also showed activity against cephaloridine and/or cefoperazone

TABLE 31

Antibiotic Inactivations Detected by Combination of 3-Dimensional and Clover Leaf (3D+CL) Techniques

Antibiotic Reference Strains

STRAIN CODE	ENZYME: TYPE	ANTIBIOTIC: AMP	CAR	KF	FOX	MA	CXM	MOX	CTX	CFP	CAZ	ATM	CR	OB	IPM
MISC 99	IVa	+	+	+	-	+	+	-	+	+	-	-	+	+	-
MISC 100	TEM	+	+	+	-	+	-	-	-	+	-	-	+	+	-
MISC 101	Ib	+	-	+	+	+	-	-	+	+	-	-	+	-	+/-
MISC 102	Ia	+/-	-	+	+/-	+	-	-	+/-	+	-	-	+	-	+
MISC 103	IVb	+	+	+	-	+	+	-	-	+	-	-	+	-	+
MISC 104	IIa	+	+	-	-	-	-	-	-	-	-	-	-	-	-
MISC 105	TEM	+	+	+	-	+	-	-	+/-	+	-	-	+	+	-
MISC 111	Ia	+	-	+	+	+	+	-	+	+	-	-	+	-	+
MISC 112	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MISC 119	TEM 1	+	+	+	-	+	-	-	-	+	-	-	+	+	-
MISC 120	TEM 2	+	+	+	-	-	-	-	-	+	-	-	+	+	-
MISC 121	OXA-1	+/-	-	+	+	-	-	-	-	-	-	-	+	-	-
MISC 122	OXA-2	+	-	+	+/-	+	-	-	+	+	-	-	+	+/-	-
MISC 123	OXA-3	+	+/-	+	+/-	+	-	-	-	+	-	-	+	+/-	-
MISC 124	PSE-1	+	+	-	-	-	-	-	-	+	-	-	+/-	-	-
MISC 125	PSE-2	+	-	-	-	+/-	-	-	-	+	-	-	-	+	-
MISC 126	PSE-3	+	+	+	-	+/-	+/-	-	+	-	-	-	-	-	-
MISC 127	HMS-1	+	+	+	-	+	-	-	-	+	-	-	+	+	-
MISC 128	SHV-1	+	+	+	-	+	-	-	-	+	-	-	+	-	-
MISC 130	PSE-4	+	+	-	-	-	-	-	-	+/-	-	-	-	-	+/-
164 CD	Id	+	-	+	+/-	+	+	-	+	+	-	-	+	a	-

Antibiotic Symbols

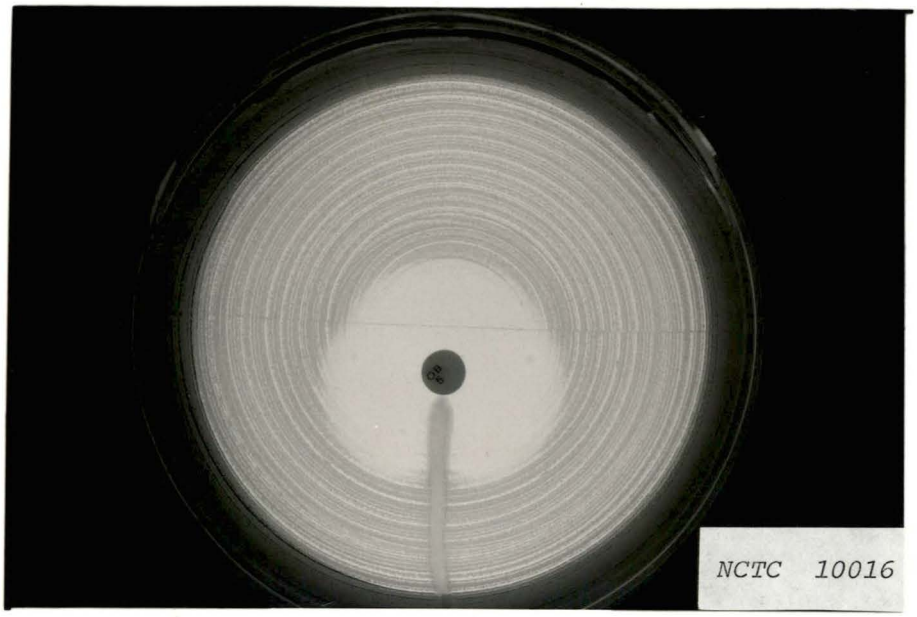
AMP - ampicillin
TIC - ticarcillin
KF - cephalothin
FOX - cefoxitin
MA - cefamandole
CXM - cefuroxime
MOX - moxalactam
CTX - cefotaxime
CFP - cefoperazone
CAZ - ceftazidime
ATM - aztreonam
CR - cephaloridine
OB - cloxacillin
IPM - imipenem

+/- weakly positive result of unreliable reproducibility

a the assay strain for the cloxacillin test was inhibited by Strain 164 CD

Fig. 12. 3D+CL test for cloxacillin. Assay strain is S. aureus ATCC 25923 and test strain in straight-line 3-dimensional inoculation (almost no surface growth visible) and clover leaf inoculation (thick line of growth on surface of agar) is F. meningosepticum NCTC 10016. Indentations are more obvious for the more heavily inoculated clover leaf component of the test the for the 3-dimensional component.

Fig. 13. 3-dimensional tests involving Group IIb MP 616 and (from left) cefuroxime (CXM) and cefoxitin (FOX), cephalothin (KF) and cefoxitin, and only cefoxitin. Zone margin distortions are obvious only in the two-disc tests.


NCTC 10016

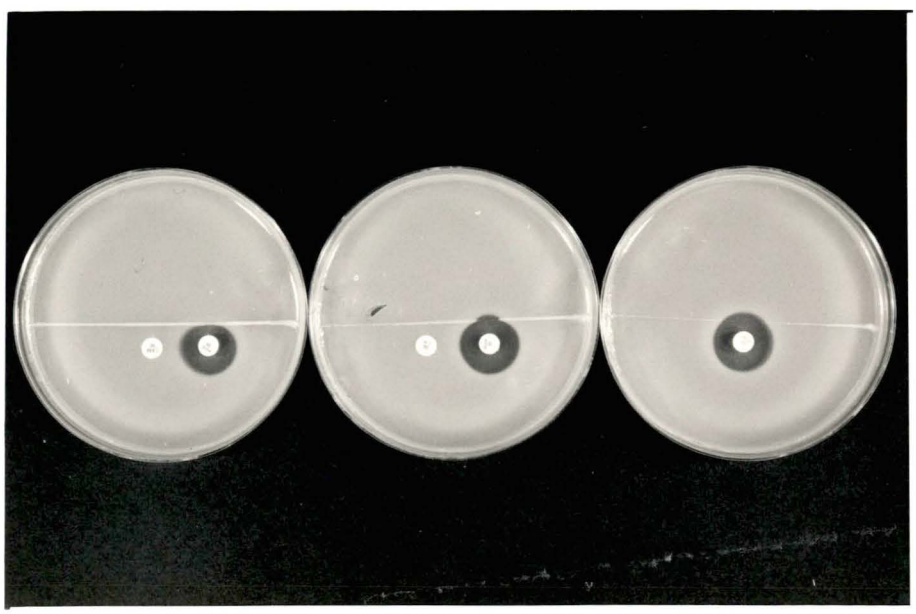


TABLE 32

3D+CL Tests for Taxonomic Reference Strains and Selected Study Strains

Organism	Substrate AMP	KF	CAR	FOX	IPM	TEM	CR	CFP	CTX	OB
F. breve										
NCTC 11099	+	+	+*	+*	+*	-	+	+/-	+	+
NCTC 11162	+	-	+	+/-	-	-	+	-	-	+
U31	+/-	+	-	-	+	-	+	-	+	+
F. gleum										
NCTC 10795	-	-	-	+	-	-	-	-	-	+
NCTC 11432	-	-	-	-	-	-	-	-	-	-
U62	+	+	-	+/-	-	-	-	-	+	+
R48	-	-	-	-	-	-	-	-	-	+
R49	-	+/-	-	+	-	-	-	-	-	+
R56	-	+	-	-	-	-	-	-	+	+
F. meningosepticum										
NCTC 10016	+	+	-	-	-	-	+	+/-	+	+
NCTC 10585	+	+	-	+	-	-	+/-	-	-	+
R58	+	+	+	+	-	-	+	-	+/-	+
MP816	+	+/-	-	+	-	-	+	-	+	+
MP819	+	+	-	-	-	-	-	-	-	+
MP894	+	+	+	-	-	-	+	-	-	+
MP969	+	+	-	+/-	-	-	+/-	-	+/-	+
MP970	+	-	-	-	-	-	+	-	-	+
MP1702	+	+	+	+	+/-	-	+	+	+	+
MP1742	+	+	+	+	-	-	+	-	+	+
MP2347	+	+	+	-	-	-	-	-	+	+
MP2348	+	+	+	+	-	-	+	-	+/-	+
F. multivorum										
NCTC 11033	-	-	-	-	-	-	-	-	-	+
NCTC 11343	-	-	-	-	-	-	-	-	-	+
MP1177	-	-	-	-	-	-	-	-	-	+
MP1210	-	-	-	-	-	-	-	-	-	+/-
MP2324	-	-	-	-	-	-	-	-	-	-
F. odoratum										
NCTC 11036			-	-	-	-	-	-	-	-
NCTC 11179	-	-	-	-	-	-	-	-	-	-
NCTC 11180	+	-	-	-	-	-	-	-	-	+
R25	+	+/-	-	+	-	-	-	-	+	+
R59	+	+/-	+/-	+	-	-	-	-	+	+
U59	-	-	-	-	-	-	-	-	-	-

TABLE 32 (Continued)

Organism	Substrate AMP	KF	CAR	FOX	IPM	TEM	CR	CFP	CTX	OB
F. spiritivorum										
NCTC 11386	-	-	-	-	-	-	-	-	+/-	+
NCTC 11387	-	-	-	-	-	-	-	-	+	+
R41	-	-	-	-	-	-	-	-	+/-	+
R51	+/-	-	-	-	-	-	-	-	+/-	+
F. thalpophilum										
MP997	+/-	+	-	-	-	-	-	-	-	-
MP1207	+/-	-	-	-	-	-	-	-	-	-
MP1232	-	-	-	-	-	-	-	-	-	-
Group IIb										
R34	-	-	-	-	-	-	-	-	+	+
R46	-	-	-	-	+/-	-	-	-	-	+
R47	-	+	-	+	-	-	-	-	+	+
R50	-	-	-	-	-	-	+/-	-	-	+/-
R54	-	-	-	-	-	-	-	-	+	+/-
R862	+	-	-	-	-	-	-	-	-	+
U58	+	+	+	-	-	-	-	-	+	+
MP7	+	-	-	-	-	-	-	-	-	+
MP8	+	+/-	-	+	-	-	+/-	+	-	+
MP305	+/-	-	-	+	+	-	-	-	-	+
MP525	+	+	-	+	-	-	+	+	-	+
MP547	+	-	+	-	+	-	+	-	+	+
MP548	+	+	-	+	+	-	+	-	+	+
MP582	-	-	-	-	-	-	+	-	-	+
MP592	+	-	+	+	-	-	+	-	+	+
MP616	-	-	-	+	-	-	-	-	+	+
MP628	+	+	+	+	-	-	+	+	-	+
MP639	+/-	+	-	-	-	-	+	-	-	+
MP1098	+	-	-	-	-	-	+	+	-	+
MP1409	+/-	+	-	+	+	-	+	+	+	+
MP1846	+/-	-	-	+	+	-	+	+	-	+/-
Unclassified										
U33	-	-	-	-	-	-	-	-	-	-
R5	-	-	-	-	-	-	-	-	+	+
R19	+	+	+*	+*	-*	-	+	-	+	+
F. aquatile										
NCIB 8694	-	-	-	-	-	-	-	-	-	-
"*F. tirrenicum*"										
ATCC 15997	-	-	-	-	-	-	-	-	-	-

TABLE 32 (Continued)

Organism	Substrate AMP	KF	CAR	FOX	IPM	TEM	CR	CFP	CTX	OB
C. aquatilis										
ATCC 29551	+	+	+	-	-	-	+	+	+	+
C. johnsonae										
NCIB 10150	+	+	+	-	-	-	+	+	+	+
ATCC 17061	+	-	-	-	-	-	+	-	+/-	+
F. elegans										
DSM 527	-	-	-	-	-	-	-	-	-	-

+/- weakly positive result of unreliable reproducibility

* test result reproduced five times

AMP	Ampicillin	TEM	Temocillin
KF	Cephalothin	CR	Cephaloridine
CAR	Carbenicillin	CFP	Cefoperazone
FOX	Cefoxitin	CTX	Cefotaxime
IPM	Imipenem	OB	Cloxacillin

these two cephalosporin substrates were regarded as insufficiently discriminatory to reliably indicate cephalosporinase activity. Cephalothin however appeared to be resistant to penicillinase activity and was regarded as a more reliable substrate with which to identify cephalosporinase activity.

Most Flavobacterium 3D+CL patterns differed from those of the antibiotic reference strains suggesting that either the genus produced different beta-lactamases or that the patterns reflected the activities of multiple enzymes. This possibility is suggested by the IEF patterns (Table 36). Cloxacillin inactivation was a distinctive feature of the genus (84% of strains positive) and some strains produced positive 3D+CL results for cefotaxime (45%), cefoxitin (39%) and positive to marginally positive results for imipenem (19%). No strains appeared to be capable of temocillin inactivation in these tests.

F. breve was heterogeneous in the 3D+CL tests and comparison of the F. breve 3D+CL patterns with those of the antibiotic reference strains suggested that the beta-lactamases of the species were unlike the reference beta-lactamases. Strain NCTC 11099 appeared to have the greatest enzymatic activity , exhibiting very broad spectrum activity against substrates such as ampicillin, carbenicillin, cloxacillin, cephalothin, cefoxitin, cefotaxime and imipenem. NCTC 11162 appeared to have predominantly penicillinase activity with positive 3D+CL results for ampicillin, carbenicillin, cloxacillin, cephaloridine and borderline activity against cefoxitin. The U31 beta-lactamase showed broad spectrum activity by the 3D+CL technique but with only

minimal penicillinase activity against ampicillin and no activity against carbenicillin compared to its stronger activity against cloxacillin. This strain also inactivated cefotaxime and imipenem.

The strains of F. gleum showed considerable heterogeneity in the 3D+CL tests and none of the 3D+CL profiles were similar to those of the reference antibiotic strains. The negative results for NCTC 11432 precluded definite conclusions about its beta-lactamase. NCTC 10795, U62 and R49 inactivated cefoxitin, although in the case of U62 the level of inactivation appeared to be at the threshold of sensitivity of the technique. U62 and R56 inactivated cefotaxime. All strains except NCTC 11432 inactivated cloxacillin but only U62 inactivated ampicillin.

Eleven of the twelve strains of F. meningosepticum exhibited broad spectrum beta-lactamase activity with nine of the strains exhibiting activity against cefotaxime and/or cefoxitin. In general the beta-lactamases of F. meningosepticum appeared to be broader in spectrum than those of F. gleum with some strains inactivating carbenicillin, cephaloridine and cefoperazone. The 3D+CL tests were all negative for these substrates for F. gleum. In the strains tested it also appeared that F. meningosepticum possessed greater activity against cephalothin than either F. gleum or Group IIb. An interesting feature of the 3D+CL tests was the clearly greater activity of the strains against cephalothin than against cefoperazone which is more vulnerable to

hydrolysis by most beta-lactamases.

Most of the F. meningosepticum 3D+CL profiles were sufficiently different from those of the reference antibiotic strains to indicate that they were distinctly different beta-lactamases. However the profiles for NCTC 10016, MP816, MP894, MP969 and MP969 could not be distinguished from those of the reference strains by 3D+CL testing. (For these strains it was necessary to consider the results of the inhibitor studies and isoelectric focussing in conjunction with the 3D+3CL results to determine that all but strain MP816 contained beta-lactamases which were different from the reference beta-lactamases. It was not possible however to exclude completely the possibility that MP816 contained the plasmid mediated OXA-2 beta-lactamase).

The most active strain appeared to be MP1702 which had positive 3D+CL results for ampicillin, carbenicillin, cloxacillin, cephalothin, cephaloridine, cefoperazone, cefoxitin, cefotaxime and an equivocal result for imipenem. This strain appeared to belong to a group of strains (R58, MP1702, MP1742, MP2347 and MP2348) capable of inactivating carbenicillin and cefoxitin and/or cefotaxime as well as other more labile substrates.

All strains of F. multivorum except MP2324 showed activity against only cloxacillin in the 3D+CL tests. In the routine 3-dimensional tests however inactivation of ticarcillin was indicated for strains R42, MP1170, MP1210, MP1213, and MP1231 while strain MP1177 appeared to inactivate both ampicillin and

ticarcillin. (The occasionally greater sensitivity of the routine 3-dimensional test, which appeared to arise from a synergistic effect of different beta-lactam antibiotics, is discussed elsewhere).

The 3D+CL tests provided three different profiles of results for the F. odoratum strains. Strains NCTC 11036, NCTC 11179 and U59 were negative for all substrates. NCTC 11180 showed apparent penicillinase activity against ampicillin and cloxacillin. Strains R25 and R59 showed broad spectrum activity which extended to cefoxitin and cefotaxime. R59 was the only strain which had a 3D+CL pattern resembling a reference strain pattern, showing similarity to the OXA-3 pattern.

The four strains of F. spiritivorum exhibited activity against cloxacillin and cefotaxime. The activity against cefotaxime was only borderline for three of the four strains. Strain R51 appeared also to exhibit borderline activity against ampicillin.

The three strains of F. thalpophilum were heterogeneous in the 3D+CL tests with MP1232 failing to exhibit beta-lactamase activity and MP997 and MP1207 exhibiting weak broad spectrum beta-lactamase activity. The 3D+CL patterns of MP997 and MP1207 were different from the patterns of the antibiotic reference strains.

The 3D+CL patterns for the 21 strains of Group IIb were

heterogeneous. All strains exhibited inactivation of cloxacillin. Two strains, R862 and MP7, exhibited apparent penicillinase activity only with positive 3D+CL results for ampicillin and cloxacillin. The other 19 strains produced broad spectrum beta-lactamases according to the 3D+CL tests, with 17 of the 19 strains showing activity against cefoxitin, cefotaxime and/or imipenem. None of the 3D+CL patterns for Group IIb resembled the patterns of the antibiotic reference strains. Four strains, U58, MP547, MP592, and MP628, appeared capable of inactivating carbenicillin and six strains, R46, MP305, MP547, MP548, MP1409 and MP1846, appeared to inactivate imipenem.

Of the unclassified strains, R19 had a similar 3D+CL pattern to F. breve NCTC 11099, strain R5 inactivated only cefotaxime and cloxacillin, and U33 was negative in all 3D+CL tests. R5 was therefore identical in these tests to Group IIb strain R34, and F. spiritivorum strains NCTC 11386, NCTC 11387 and R41.

F. aquatile, "F. tirrenicum", and F. elegans were negative in all 3D+CL tests. The cytophagas, C. aquatilis and C. johnsonae, appeared to be very similar to many of the Flavobacterium strains in the phenotypic expression of beta-lactamases with ability to hydrolyse carbenicillin, cloxacillin and cefotaxime and other more labile substrates.

It was interesting that sometimes the routine 3-dimensional test appeared to be more sensitive than the 3D+CL test. This phenomenon occurred only when more than one

antibiotic was being tested by the 3-dimensional test but only one antibiotic was being tested by the 3D+CL method. This effect is demonstrated clearly in Fig. 13 in which the cefoxitin 3-dimensional test is negative when only cefoxitin was tested against strain MP616 of Group IIb but was positive when either a cephalothin or a cefuroxime disc was placed adjacent to the cefoxitin disc. When the plates were flooded with nitrocefin solution (50 mcg/ml) a red positive reaction only occurred around the cephalothin and cefuroxime discs. This indicated that these drugs may have caused beta-lactamase leakage and thus may have synergistically contributed to a positive 3-dimensional test.

e. Hydrolysis Assays, Inhibitor Studies and Isoelectric Focussing Patterns

Table 33 lists the results obtained with the antibiotic reference strains for (1) hydrolysis assays using penicillin, cephalothin and cefoxitin as substrates, (2) cefoxitin induction studies, and (3) inhibition studies using cloxacillin and lithium clavulanate. These tests provide sufficient information to determine the broad substrate profiles of the beta-lactamases (i.e. penicillinases, cephalosporinases or broad spectrum beta-lactamases), and whether or not the enzymes belong to Class I of the Richmond and Sykes Classification of beta-lactamases (i.e. resistant to clavulanate and, usually, inducible). The cefoxitin hydrolysis assays were performed on five strains (selected on the basis of 3D+CL tests) and induction studies were performed

on three organisms known to possess Class I beta-lactamases. One of the three organisms was fully derepressed for beta-lactamase production and therefore not inducible.

To simplify determination of substrate profiles the ratio of the penicillin:cephalothin hydrolysis rates was calculated. This ratio does not have absolute meaning because of the different methodologies used to assay penicillin and cephalothin hydrolysis. However it proved to be a convenient means by which to compare different enzymes irrespective of the amount of enzyme synthesized by the host bacterium. The interpretive criteria for the ratios are provided at the bottom of Table 33.

The isoelectric points presented in Table 34 generally agreed with published pI values for these strains. The activity of the OXA-1 and OXA-2 beta-lactamases was very low and the OXA-1 beta-lactamase could not be reliably detected.

The results of hydrolysis assays using penicillin and cephalothin as substrates for selected study and taxonomic reference strains are presented in Table 35. If the criterion of inducibility is taken to be at least a three-fold increase in the hydrolysis rate for cephalothin, none of the study strains was found to produce an inducible beta-lactamase.

The hydrolysis ratios indicated that the beta-lactamases of the study and taxonomic reference strains were mainly cephalosporinases or broad spectrum beta-lactamases with predominantly cephalosporinase activity (Table 35). The beta-

TABLE 33

Results of Hydrolysis Assays and Inhibitor Studies for Beta-lactamase Reference Strains

STRAIN CODE	ORGANISM	ENZYME	INDUCED	HYDROLYSIS RATE * SUBSTRATES			# HYDROLYSIS	INHIBITED BY	
				PEN[a] (x10^{-3})	KF[b]	FOX[c]	RATIO	CLOX	CLAV
MISC 99	K. pneumoniae	IV a	-	2,810	34.6		81.2	-	+
MISC 100	E. coli	III a (TEM)	-	17,380	103.8		167.4	-	+
MISC 101	E. coli	I b	-	1,250	371.1	4.0	3.4	+	-
MISC 102	E. cloacae	I a	-	110	261.1		0.4	+	-
MISC 102	E. cloacae	I a	yes	N.T.**	1640.3				
MISC 103	K. pneumoniae	IV b	-	99.8	9.1		11.0	-	+
MISC 104	P. mirabilis	II a	-	557	4.3		129.5	-	+
MISC 105	K. pneumoniae	III a (TEM)	-	556	4.6		120.9	-	+
MISC 111	E. cloacae P99+	I a	-	356	494.4		0.7	+	-
MISC 111	E. cloacae P99+	I a	yes	N.T.	406.8	N.D.***			
MISC 112	E. cloacae P99-	lacks enzyme	-	6.8	1.2				
MISC 112	E. cloacae P99-	lacks enzyme	yes	N.T.	3.3				
MISC 119	E. coli	III (TEM 1)	-	3,550	54.9		64.7	-	+
MISC 120	E. coli	III (TEM 2)	-	12,080	420.0		28.8	-	+
MISC 121	E. coli	V (OXA-1)	-	160	3.5		45.7	N.T.	N.T.
MISC 122	E. coli	V (OXA-2)	-	33.6	3.4		9.9	-	+
MISC 123	E. coli	V (OXA-3)	-	26.2	3.1	N.D.	8.5	-	+
MISC 124	P. aeruginosa	III (PSE-1)	-	2,960	5.4	N.D.	548.1	-	+
MISC 125	P. aeruginosa	V (PSE-2)	-	50.9	3.0		17.0	-	+
MISC 126	P. aeruginosa	III (PSE-3)	-	60	2.9		20.7		
MISC 127	E. coli	III (HMS-1)	-	1,090	6.6		165.2	-	+
MISC 128	E. coli	III (SHV-1)	-	5,580	34.7		160.8	-	+
MISC 130	P. aeruginosa	III (PSE-4)	-	3,220	5.0		644.0	-	+
164 CD (1)	P. aeruginosa	I	-	3,540	20,678.3	3.2	0.2	+	-
164 CD (2)	P. aeruginosa	I	-	3,450	18,488.3		0.2		
164 CD (3)	P. aeruginosa	I	-	1,950	17,404.8		0.1		
164 CD (4)	P. aeruginosa	I	-	3,000	17,692.0		0.2		

* Hydrolysis rate in umol./min./mg. protein

** Not tested (or activity on IEF too weak to determine inhibition profile)

*** No hydrolysis detected

[a] Penicillin, [b] Cephalothin, [c] Cefoxitin

\# Hydrolysis Ratio = $\frac{\text{Penicillin Hydrolysis Rate (x10}^{-3})}{\text{Cephalothin Hydrolysis Rate (Uninduced)}}$

VALUES DERIVED FROM THE ABOVE TABLE:

ENZYME TYPE	HYDROLYSIS RATIO	SYMBOL
CEPHALOSPORINASE (CLASS I)	LESS THAN 4	C
POSSIBLE CEPHALOSPORINASE	4 to 6	?C
POSSIBLE BROAD SPECTRUM	6 to 8	?BS
BROAD SPECTRUM (CLASS III)	20 to 644	BS
BROAD SPECTRUM (CLASS IV)	11 to 82	BS
BROAD SPECTRUM (CLASS V)	8 to 46	BS
PENICILLINASE (CLASS II)	129.5	P

TABLE 34

Results of Isoelectric Focussing Studies

for Beta-lactamase Reference Strains

ENZYME SOURCE		BETA-LACTAMASE RS[a]	BETA-LACTAMASE ON[b]	pI VALUES THIS STUDY (A)[c]	pI VALUES OTHER STUDIES (A)[c]	pI VALUES OTHER STUDIES (P)[d]
MISC 99	K. pneumoniae	IVa		7.7		
MISC 100	E. coli	IIIa	TEM	5.6		
MISC 101	E. coli	Ib		8.7		
MISC 102	E. cloacae	Ia		8.5		
MISC 103	K. pneumoniae	IVb		5.5		
MISC 104	P. mirabilis	IIa		6.4		
MISC 105	K. pneumoniae	IIIa	TEM	5.6		
MISC 111	E. cloacae P99	Ia		7.9		8.2[1] *
MISC 119	E. coli	III	TEM-1	5.4	5.3[3]	5.4[2]
MISC 120	E. coli	III	TEM-2	5.6		5.6[2]
MISC 121	E. coli	V	OXA-1	N.D.[e]		7.4[1]
MISC 122	E. coli	V	OXA-2	7.5	7.6[3]	7.5[3]
				7.8	7.8[3]	7.7[3]
						8.0[5]
MISC 123	E. coli	V	OXA-3	7.1	7.2[3]	7.1[2,3]
MISC 124	P. aeruginosa	III	PSE-1	5.7	5.5[3]	5.7[2,3]
MISC 125	P. aeruginosa	V	PSE-2	6.1	6.0[3]	6.1[2,3]
MISC 126	P. aeruginosa	III	PSE-3	N.D.	6.8[3]	6.9[2,3]
MISC 127	E. coli	III	HMS-1	4.8	4.2[3]	5.2[2,3]
					4.8[3]	
MISC 128	E. coli	III	SHV-1	7.6	7.7[2]	7.6[2,3]
MISC 130	P. aeruginosa	III	PSE-4	5.2	5.2[3]	5.3[2,3]
164 CD	P. aeruginosa	I		8.3		8.4[4]
				7.6		7.5[4]

a Richmond-Sykes nomenclature

b Other name for beta-lactamase

c Agarose gel used for IEF

d Polyacrylamide gel used for IEF

e Beta-lactamase not detected

* Superscripted figures refer to the following references:

(1) Bush et al. (1985)
(2) From Matthew (1979)
(3) Vecoli et al. (1983)
(4) Gates et al. (1986)
(5) Labia et al. (1986)

lactamase preparations of F. meningosepticum and Group IIb were generally more active than those of the other species. Cefoxitin hydrolysis assays were performed on strains which appeared to inactivate the drug in 3-dimensional or 3D+CL tests.

Penicillin hydrolysis assay results were far more variable than cephalothin hydrolysis results. This is apparent from the four sets of results for F. meningosepticum R58. Whereas the cephalothin hydrolysis results are similar in magnitude the rate of hydrolysis of penicillin by enzyme preparation 1 is more than ten times greater than that of preparation 2. Several possible explanations may be offered. In the case of strain R58 the IEF results indicated the presence of multiple beta-lactamase bands (Table 36), some of which may have been responsible for penicillinase activity but have been inactive at the time preparation 4 was tested for penicillin hydrolysis. In the case of F. spiritivorum strains NCTC 11386 and NCTC 11387 grew very poorly at 35°C in MHB and required 7.5 hours of shaking incubation to produce reasonable pellets for sonication. The cell-free preparations of these two strains were less active in the penicillin hydrolysis assays than that of strain R41. Other strains which also grew poorly were F. breve U31, F. odoratum U59, NCTC 11179 and NCTC 11036, and F. gleum U62.

TABLE 35

Results of Hydrolysis Assays for Taxonomic Reference Strains and Selected Study Strains

STRAIN CODE		ORGANISM	HYDROLYSIS RATE[a] SUBSTRATES				HYDRO-LYSIS RATIO[x]	INTERPRET-ATION[y]
			P[b] ($\times 10^{-3}$)	KF[c]	KFI[d]	FOX[e]		
NCTC 11099		F. breve	28.8	4.5	3.2	4.6	6.4	?BS
NCTC 11162		" "	50.5	4.7	9.9	N.D.	10.7	BS
U 31		" "	37.8	13.1	12.0	N.T.	2.9	C
NCTC 11432		F. gleum	69.0	16.6	22.6	10.5	4.2	?C
U 62		" "	33.4	15.7	27.1	4.0	2.1	C
NCTC 10016		F. meningoseptirum	39.1	50.8	23.5	N.D.	0.8	C
NCTC 10585		" "	34.2	27.5	18.3	6.2	1.2	C
R 58	(1)	" "	295.0	32.9	11.9	N.T.	9.0	BS
	(2)		23.1	30.3	12.1	N.T.	0.8	C
	(3)		66.5	30.7	20.9	11.8	2.2	C
	(4)		112.0	25.6	17.6	N.T.	4.4	?C
MP 816		" "	72.2	61.0	N.T.	10.9	1.2	C
MP 819		" "	316.4	95.6	N.T.	N.D.	3.3	C
MP 894		" "	66.9	52.5	N.T.	7.5	1.3	C
MP 969		" "	54.1	65.1	N.T.	11.4	0.8	C
MP 970		" "	55.1	35.6	N.T.	12.7	1.5	C
MP 1702		" "	50.1	27.5	N.T.	11.9	1.8	C
MP 1742		" "	56.3	30.5	N.T.	3.5	1.8	C
MP 2348		" "	49.5	25.8	N.T.	7.9	1.9	C
NCTC 11033		F. multivorum	27.7	2.6	0.8	N.T.	10.7	BS
NCTC 11343		" "	11.8	2.7	4.2	N.T.	4.4	?C
MP 1210		" "	4.5	1.7	2.3	N.T.	2.6	C
MP 2324		" "	11.2	8.3	1.7	N.T.	1.3	C
NCTC 11036		F. odoratum	18.6	10.1	2.5	N.T.	1.8	C
NCTC 11179		" "	16.6	12.3	15.8	N.T.	1.4	C
NCTC 11180		" "	11.9	2.4	4.0	N.D.	5.0	?C
U 59		" "	7.4	2.7	3.6	N.T.	2.7	C
R 25		" "	17.8	3.8	1.7	N.T.	4.7	?C
R 59	(1)	" "	19.2	11.0	6.9	N.D.	1.7	C
	(2)		37.2	8.2	8.6		4.5	?C
NCTC 11386	(1)	F. spiritivorum	15.5	2.8	2.6	N.T.[g]	5.4	?C
	(2)		8.6	1.3	2.2	N.T.	6.6	?BS
NCTC 11387	(1)	" "	10.9	3.8	3.2	N.T.	2.9	C
	(2)		9.7	3.0	2.0	N.T.	3.2	C
R 41		" "	38.1	2.3	1.0	N.T.	15.2	BS
MP 1207		F. thalpophilum	32.1	5.4	5.6	N.T.	5.9	?C
MP 1232		" "	46.6	7.0	7.8	N.T.	6.7	?BS

TABLE 35 (continued)

STRAIN CODE	ORGANISM	HYDROLYSIS RATE[a] SUBSTRATES				HYDRO-LYSIS RATIO[x]	INTERPRET-ATION
		P[b] ($x10^{-3}$)	KF[c]	KFI[d]	FOX[e]		
U 58	Group IIb	46.3	11.5	6.6	N.T.	4.0	?C
R 34	" "	18.0	8.4	N.T.	2.7	2.1	C
R 46	" "	65.2	23.3	N.T.	20.9	2.8	C
R 47	" "	39.5	26.3	N.T.	25.6	1.5	C
R 862	" "	32.1	10.8	N.T.	6.4	3.0	C
MP 7	" "	20.5	26.8	N.T.	N.D.	0.8	C
MP 8	" "	73.9	17.8	N.T.	1.3	4.2	?C
MP 305	" "	16.8	3.0	N.T.	2.3	5.6	?C
MP 525	" "	61.4	9.8	N.T.	N.D.	6.3	?BS
MP 547	" "	51.9	6.7	N.T.	1.6	7.7	?BS
MP 548	" "	103.4	8.1	N.T.	N.D.	12.8	BS
MP 582	" "	84.8	10.8	N.T.	2.6	7.9	?BS
MP 592	" "	50.4	9.5	N.T.	10.7	5.3	?C
MP 616	" "	118.9	30.7	N.T.	72.9	3.9	C
MP 628	" "	49.9	54.1	N.T.	117.9	0.9	C
MP 639	" "	51.9	8.8	N.T.	N.D.	5.9	?C
MP 1098	" "	96.6	9.5	N.T.	3.6	10.2	BS
MP 1846	" "	79.4	8.6	N.T.	12.6	9.2	BS
R 5	Unidentified	44.9	4.6	12.0	N.T.	9.8	BS
R 19 (1)	Unidentified	36.0	6.6	4.8	N.T.	5.5	?C
(2)		44.7	5.2	9.2	N.T.	9.0	BS
ATCC 15922	*E. tirrenicum*	6.2	N.D.	N.T.	N.D.	high	?P
DSM 527	*E. elegans*	17.5	31.0	N.T.	N.D.	0.6	C
ATCC 17061	*C. johnsonae*	8.8	7.0	N.T.	3.7	1.3	C
NCIB 10150	" "	6.3	8.0	N.T.	N.D.	0.8	C

[a] hydrolysis rate in umol/min/mg protein
[b] penicillin
[c] cephalothin (uninduced enzyme preparation)
[d] cephalothin (induced enzyme preparation)
[e] cefoxitin
[f] figures in parentheses denote different beta-lactamase preparations
[g] not tested
[h] no hydrolysis detected

[x] hydrolysis ratio = $\frac{\text{penicillin hydrolysis rate } (x10^{-3})}{\text{cephalothin hydrolysis rate (uninduced)}}$

[y] INTERPRETATION OF HYDROLYSIS RATIOS BASED ON TABLE 33

(C = cephalosporinase = <4; ?C = 4 to 6; ?BS = 6 to 8;

P = penicillinase = >129.5; BS = broad spectrum = 8 to 64)

The inhibitor studies (Table 36) indicate that almost all the beta-lactamases of these strains were inhibited by lithium clavulanate but not by cloxacillin. The exceptions were the pI 8.2 band of F. spiritivorum NCTC 11387, the pI 7.4 band of F. multivorum, the pI 8.2 band of F. thalpophilum, and possibly the major band of F. breve NCTC 11162 at pI 7.8 and three minor bands at 6.8, 7.3 and 8.4.

The beta-lactamase isoelectric patterns of the strains studied showed considerable variation in isoelectric points indicating differences in the beta-lactamases of the species and sometimes differences within the species (Table 36). Some strains such as F. meningosepticum NCTC 10585 and R58, and F. gleum U62 produced multiple bands, others produced single bands, and some produced beta-lactamases which were too inactive to detect by IEF.

Because different sets of IEF apparatus in different laboratories were used and because some weakly reactive beta-lactamase bands were difficult to visualise it was necessary to focus enzyme preparations repeatedly (in some cases eight times) to obtain reliable comparisons of the pI values. Some beta-lactamase bands lost activity during storage at -23oC and could not be detected when IEF was repeated at a subsequent time. Some bands which lost detectable activity after 7 months frozen storage were the pI 5.8 band of F. spiritivorum R41, the pI 7.4 band of F. multivorum NCTC 11033 and NCTC 11343, all bands of F. thalpophilum MP1207 (major band was pI 5.8), all bands of F. breve NCTC 11162 (major bands pI 5.8,

7.85), all bands of F. odoratum R59 (major band pI 6.7). This phenomenon suggested that the beta-lactamases of at least some strains of the genus are not preserved by storage at this temperature. It also raises doubts about the values obtained in tests such as hydrolysis assays and indicates the desirability of working with freshly prepared enzyme preparations when studying the beta-lactamases of Flavobacterium. (This was not possible in this study owing to constraints of time and availability of equipment).

The possibility of beta-lactamase deterioration is also suggested by the appearance of new beta-lactamase bands after seven months frozen storage. In Fig. 16 three strains exhibit bands not detected with fresher enzyme preparations. The strains and new pI values are:

F. spiritivorum NCTC 11386: pIs 5.4, 5.5, 6.9, 7.4

F. multivorum MP1210: pIs 5.4, 5.7, 5.8, 6.5, 6.9, 7.4

F. multivorum MP2324: pIs 5.4, 5.5, 5.8, 6.5, 6.9

The new bands were interpreted to be breakdown products. The similar pI values and weak nitrocephin activity indicate beta-lactamase activity and chemical similarity. Whether this phenomenon represented breakdown of the initially detected beta-lactamase or breakdown of cellular debris to which the beta-lactamases were attached was not investigated.

Another phenomenom which requires investigation is the effect of zinc ions on IEF patterns. In Fig. 17 it can be seen that when 20 ul of 2 mM ZnSO4 was added to 1 ml of the beta-lactamase preparation of Group IIb MP1409 there was loss

of a minor beta-lactamase band. Similarly ZnSO4 was added to the preparations of F. meningosepticum NCTC 10016, MP 894, MP969, MP970, MP2348 and Group IIb MP582 but did not alter the IEF patterns.

The beta-lactamases of F. breve strains exhibited only low activity on IEF with none of the beta-lactamase bands being detected after less than 45 minutes exposure to nitrocefin and beta-lactamase activity not being detected for NCTC 11099. This low activity made it difficult to determine inhibitor results on the agarose gels. Delayed nitrocephin reactions resulted in pre-diffusion of cloxacillin and clavulanate overlays thereby decreasing the concentrations of these inhibitors in the vicinity of the beta-lactamase bands and reducing the selectivity of inhibition of this procedure.

Strains NCTC 11162 and U31 differed in their IEF beta-lactamase patterns. NCTC 11162 produced two major beta-lactamase bands at pI 5.75 and pI 7.85 while U31 produced a beta-lactamase at pI 9.3 (approximately). The latter beta-lactamase could not be sharply focussed making it difficult to establish a reliable isoelectric point. This phenomenon, which occurred with several of the beta-lactamases in this study, has been observed in other studies in which it has been attributed to unusually large beta-lactamase molecules or to the binding of the enzymes to larger molecules such as membrane fragments (Marre et al., 1982; Medeiros, 1984). In this study this type of IEF band had the appearance of a diffuse pink blob of hydrolysed nitrocephin and is therefore referred to as a 'blobby' beta-lactamase. For such diffuse

TABLE 36

Results of Isoelectric Focussing and Inhibitor Studies for Selected Flavobacterium Strains

ORGANISM AND STRAIN CODE	pI VALUES MAJOR BANDS	MINOR BANDS	INHIBITED BY CLOX	CLAV
F. breve				
NCTC 11099	N.D.[a]			
NCTC 11162	5.8		+	-
	7.85	6.8, 7.3, 8.4	-	+
U 31	(9.3)[c]	7.7, 10.05, 10.6	-	+
F. gleum				
NCTC 11432	10.6	4.9, 5.2	-	+
U 62	10.6	5.1, 5.9, 6.45, 7.45	-	+
R 49	(9.6)c	7.7	-	+
F. meningosepticum				
NCTC 10016	(9.8)	7.7	-	+
NCTC 10585	8.6	5.8, 6.1, 6.75, 8.0, 8.2	-	+
R 58	8.6	6.75, 8.0, 8.2, 10.6	-	+
MP 816	8.6	6.6, 7.1, 7.7	-	+
MP 819	7.8		-	+
MP 894	(9.8)		-	+
MP 969	8.6	5.0,5.6, 6.6, 10.6	-	+
MP 970	8.1	6.6, 7.1	-	+
MP 1702	7.9	7.7, 10.6	-	+
MP 1742	7.9	7.1, 7.6	-	+
MP 2348	(9.8)		-	+
F. multivorum				
NCTC 11033	9.3		-	+
		7.4	?	?b
NCTC 11343	9.2		-	+
MP 1210	5.8	7.4	?	?
MP 2324	7.4		+	-
F. odoratum				
NCTC 11036	7.7		-	+
NCTC 11179	7.7		-	+
NCTC 11180	5.3		-	+
U 59	N.D.			
R 25	(4.9)		-	+
R 59	6.7	4.9, 5.3, 7.7	-	+

TABLE 36 (continued)

ORGANISM AND STRAIN CODE	pI VALUES MAJOR BANDS	MINOR BANDS	INHIBITED BY CLOX	CLAV
F. spiritivorum				
NCTC 11386	5.8		+	+
	8.2		+	-
NCTC 11387	N.D.a			
R 41	5.8		-	+
F. thalpophilum				
MP 1207	5.8	5.3, 5.6	+	
		8.2		+
MP 1232	N.D.			
Group IIb				
U 58	8.2	4.9	-	+
R 34	8.8	7.1, 7.7, 8.1	-	+
R 46	8.6	9.2	-	+
R 47	9.2		-	+
R 862	8.5	5.65, 8.25, 9.1	-	+
MP 7	8.6		-	+
MP 8	10.4		-	+
MP 305	10.2	8.3	-	+
MP 525	9.2		-	+
MP 547	9.2	8.4	-	+
MP 548	9.2	7.7	-	+
MP 582	9.2		-	+
MP 592	8.8		-	+
MP 616	8.7		-	+
MP 628	8.6		-	+
MP 639	9.2		-	+
MP 1098	9.0		-	+
MP 1409	9.2	8.4	-	+
MP 1846	10.2		-	+
Unclassified				
R 5	8.6	(9.3), 10.6	-	+
R 19	N.D.			
F. elegans				
DSM 527	8.0	7.6	+	-
("*F tirrenicum*" ATCC 15922, *C. johnsonae* ATCC 17061 and NCIB 10150 N.D.)				

N.D.a: no beta-lactamase detected by IEF
?b : inhibitor result equivocal due to delayed visualisation of beta-lactamase
( )c : poorly focussed beta-lactamase

bands it was not possible to obtain precise isoelectric points.

Clavulanate inhibited the U31 beta-lactamase and the beta-lactamase of NCTC 11162 with a pI of 5.75. However inhibitor results for the NCTC 11162 beta-lactamase with a pI of 7.85 were equivocal, but seemingly more resistant to clavulanate than to cloxacillin. Delayed nitrocefin reactions such as observed with this band are not sufficiently reliable for accurate determination of inhibitor studies by this method and a more concentrated enzyme preparation than could be prepared in this study is required.

Strains NCTC 11432 and U62 of F. gleum had major bands at pI 10.6, while the third strain tested, R49, had a diffuse 'blobby' band at approximately pI 9.8.

F. meningosepticum exhibited five different major beta-lactamase bands - at pI values of 7.8 (MP819), 7.9 (MP 1702, MP1742), 8.1 (MP970), 8.6 (NCTC 10585, R58, MP816, MP969) and a 'blobby' beta-lactamase at 9.8 (NCTC 10016, MP984, MP2348).

F. multivorum was also heterogeneous by IEF with the four strains tested having different isoelectric points - NCTC 11033 (pI 9.3), NCTC 11343 (pI 9.2, probably the same as the pI 9.3 band), MP1210 (pI 5.8) and MP2324 (pI 7.4). The latter enzyme was resistant to inhibition by clavulanate. Strains NCTC 11033 and MP1210 shared a minor band at pI 7.4 which was possibly resistant to clavulanate and thus may have been the same as that beta-lactamase detected in MP2324. If so, it is

probably a chromosomal cephalosporinase of the type thought to be produced in small amounts by virtually all bacteria (Matthew and Harris, 1976).

The beta-lactamases of F. odoratum were also heterogeneous. Strains NCTC 11036 and NCTC 11179 possessed a common beta-lactamase with an isoelectric point of 7.7. The beta-lactamase of NCTC 11180 had isoelectric point of 5.3. R25 possessed a 'blobby' type of beta-lactamase which did not focus to a sharp band and had an isoelectric point of pI 4.9. This acidic isoelectric point indicated it was a different type of enzyme from the 'blobby' beta-lactamases with alkaline pIs in F. meningosepticum, F. gleum and Group IIb. R59 was the only strain of F. odoratum to exhibit multiple beta-lactamase bands having a major band at pI 6.7 and minor bands at pI values of 4.9, 5.3 and 7.7. It was interesting that the isoelectric points of these minor bands were identical to those of the other beta-lactamases detected in the other F. odoratum strains tested. No beta-lactamase bands were detected by IEF for strain U59.

The beta-lactamase preparations of F. spiritivorum were relatively inactive and the beta-lactamase of NCTC 11387 could not be detected by IEF. The two other strains included in the IEF studies, NCTC 11386 and R41, appeared to share a common beta-lactamase which was very similar to that of F. multivorum MP 1210. Both NCTC 11386 and R41 shared a beta-lactamase with an isoelectric point of 5.8, while NCTC 11386 also possessed a second beta-lactamase of pI 8.2. This latter beta-lactamase was resistant to inhibition by clavulanate but was inhibited

by cloxacillin and was very similar to the cephalosporinase of F. multivorum MP 2324. The beta-lactamases of pI 5.8 were inhibited by clavulanate but not by cloxacillin.

Two strains of F. thalpophilum were included in the IEF study, one of which (MP1232) did not produce a beta-lactamase detectable by IEF. MP1207 had a major band at pI 5.8. In addition to minor bands at pI 5.3 and 5.6 it produced a minor band at pI 8.2 which was resistant to clavulanate and was probably a chromosomal cephalosporinase. This band appeared to be similar to the clavulanate-resistant bands at pI 8.2 in F. spiritivorum NCTC 11386 and F. multivorum at pI 7.4

Group IIb exhibited greatest heterogeneity in its beta-lactamases with major bands at pI 8.2 (U58), 8.5 (R862), 8.6 (R46, MP7, MP628), 8.7 (MP616), 8.8 (R34, MP592), 9.0 (MP1098), 9.2 (MP525, MP547, MP548, MP582, MP639, MP1409), 10.2 (MP305, MP1846) and 10.4 (MP8).

The unclassified strain R5 appeared to have the same beta-lactamase at pI 8.6 as some strains of F. meningosepticum and Group IIb. F. elegans DSM 527 produced a major band (pI 8.0) and a minor band (pI 7.6) which were resistant to clavulanate. These bands were different from all Flavobacterium beta-lactamases tested in this study.

Beta-lactamases could not be detected by IEF for "F. tirrenicum" ATCC 15922 and C. johnsonae strains ATCC 17061 and NCIB 10150.

Fig. 14. Isoelectric focussing of beta-lactamases of F. multivorum by method of Sanders et al. (1986) using nitrocefin agar and inhibitor-based characterization system. Lane 1: pI markers (double blue bands are pI 4.75, 4.85, other bands are 5.65, 5.92, 6.45, 7.3, 8.3, and 10.6; lanes 2, 8, 14: reference beta-lactamases (type IVb and SHV-1); lanes 3, 9, 15: NCTC 11033; lanes 4, 10, 16: NCTC 11343; lanes 5, 11, 17: MP 1210; lanes 6, 12, 18: MP 2324. Lanes 8 to 12 were treated with cloxacillin (1,000 uM) and lanes 14 to 18 were treated with lithium clavulanate (1,000 uM) for 10 secs before being overlaid with nitrocefin agar. At the time of photographing only the pI 9.3 bands of NCTC 11033 and NCTC 11343 were visible. All other bands appeared one hour later.

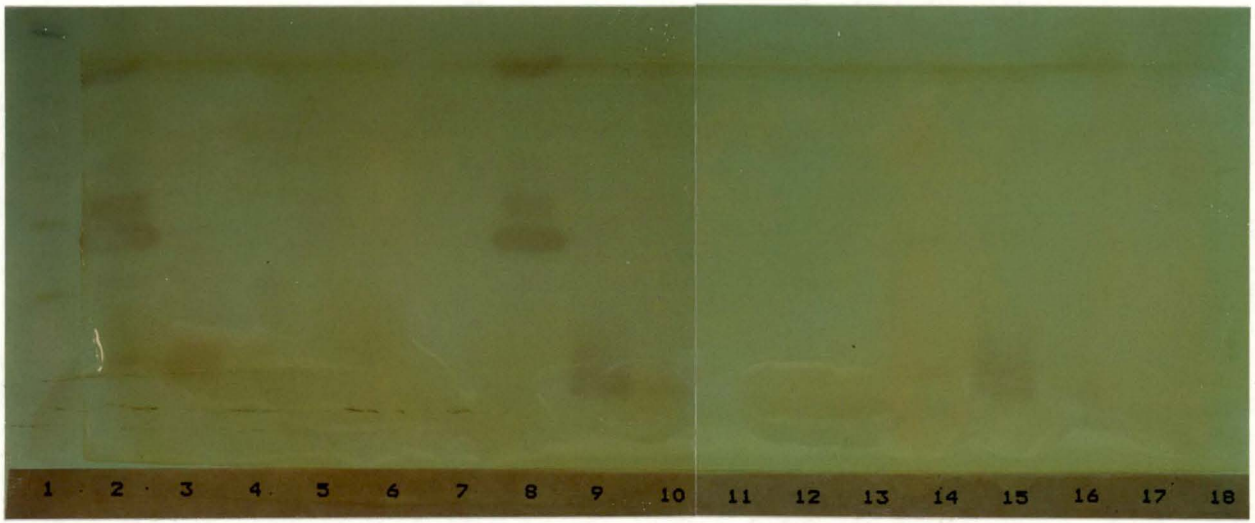

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Fig. 15. Isoelectric focussing of beta-lactamases of F. meningosepticum and unclassified strain R5. Lane 1: pI markers; lanes 2, 8, 14: reference beta-lactamases IVb and SHV1; lanes 3, 9, 15: NCTC 10016; lanes 4, 10 16: NCTC 10585; lanes 5, 11, 17: R58; lanes 6, 12, 18: R5. Inhibitors: lanes 8 to 12 treated with cloxacillin and lanes 14 to 18 treated with clavulanate as per Fig. 14. Note poorly focussed band of NCTC 10016 and identity of major band of NCTC 10585, R58 and R5 and some minor bands of NCTC 10585 and R58.

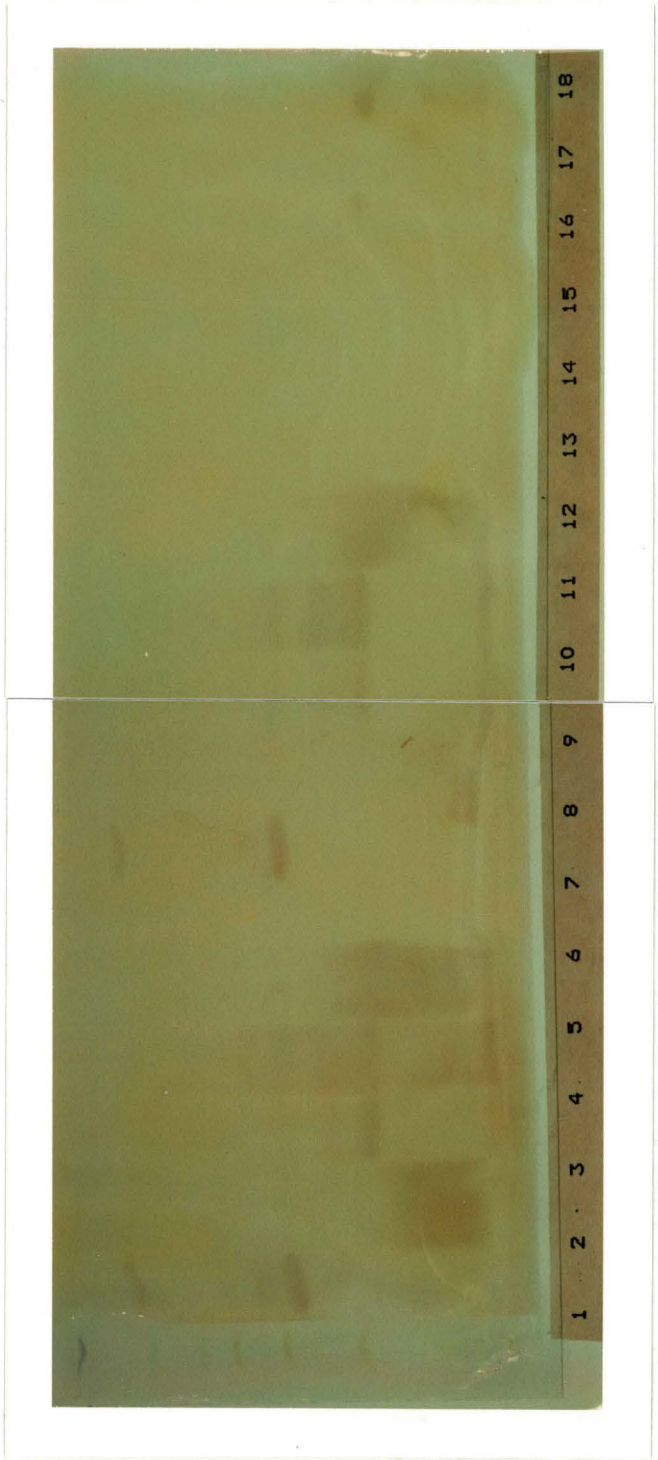

1 2 3 4 5 6 7 8 9
10 11 12 13 14 15 16 17 18

Fig. 16. Isoelectric focussing of beta-lactamases. Lanes 1 and 18: pI markers;

reference beta-lactamases:
lanes 2 (type Ia), 7 (Ib), 12 (PSE-1), 17 (OXA-3);

F. meningosepticum: lanes 3 (MP816), 4 (MP970), 11 (MP969); Group IIb: lanes 5 (MP 628), 6 (R34);

F. multivorum: lanes 8 (MP2324), 14 (MP1210);

F. spiritivorum: lanes 9 (NCTC 11386), 10 (R41);

F. thalpophilum: lane 13 (MP1207); F. breve: lane 15 (NCTC 11162); F. odoratum: lane 16 (R59).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Fig. 17. Isoelectric focussing of beta-lactamases. Lanes 1 and 18: pI markers;

reference beta-lactamases:
lanes 2 (type Ia), 15 (PSE-1); and 10 : MISC 126 (host of PSE-3) showing only host *Pseudomonas* beta-lactamase bands.

Group IIb: lanes 3 (R862), 4 (R47), 5 (MP305), 6 (MP525), 7 (MP548), 8 (MP1409), 9 (MP1409 with ZnSO4 added);

lane 11: *F. tirrenicum* ATCC 15997;

C. johnsonae: lanes 12 (ATCC 17061), 13 (NCIB 10150)

lane 14: *F. elegans* DSM 527;

F. meningosepticum: lanes 16 (NCTC 10016), 17 (NCTC 2324),

1
2
3
4
5
6
7
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18

f. Investigations of Mechanisms

Preliminary evidence about the roles of beta-lactamases and permeability in the resistance of Flavobacterium strains to beta-lactam antibiotics was obtained by combining clavulanate (2 mcg/ml) and EDTA (1 mM) with selected beta-lactam antibiotics. Three-fold lowering of MICs in the presence of clavulanate or EDTA or an increased inhibition zone for augmentin compared to ampicillin was regarded as a significant synergistic effect and presumptive evidence for beta-lactamase activity or a permeability barrier being involved in resistance to beta-lactam antibiotics.

This approach failed to implicate low permeability as a significant factor in resistance. F. breve strain U31 was inhibited by 0.5 mM EDTA, making invalid the testing of this strain at 1mM EDTA.

Clavulanate however significantly increased susceptibility of a number of strains of Flavobacterium to ampicillin, cefamandole and cefotaxime. These tests were invalidated for four strains, F.breve U31 and F. multivorum strains NCTC 11033, NCTC 11343 and MP1177, which were inhibited by 2 mcg/ml clavulanate. Although U31 appeared to be synergistically inhibited in the augmentin disc test the remaining two strains of F. breve were indifferent to the effects of clavulanate in all tests. (This was further evidence of heterogeneity in this species). All strains of F. gleum were unaffected by clavulanate. Most strains of F. meningosepticum were more susceptible in the presence of clavulanate. Only NCTC 10016

TABLE 37

Agar Dilution Antibiotic Susceptibility Tests

Effects of Clavulanate and EDTA

Minimum Inhibitory Concentrations in mcg/ml.

ORGANISM	MA	MA + CLAV	MA + EDTA	MA + CLAV+ EDTA	CTX	CTX+ CLAV	CTX+ EDTA	CTX+ CLAV+ EDTA
F. breve								
NCTC 11099	16	8	16	16	4	2	4	2
NCTC 11162	128	64	64	32	32	8	16	8
U 31 * and **	8	0.12	⩽0.06	⩽0.06	2	⩽0.06	⩽0.06	⩽0.06
F. gleum								
NCTC 10795	>128	128	128	>128	64	64	64	128
NCTC 11432	>128	128	>128	>128	64	32	64	16
R 48	>128	>128	>128	>128	64	64	64	128
U 62	>128	>128	>128	>128	64	32	128	128
F. meningosepticum								
NCTC 10016	>128	32	>128	64	128	8	128	16
NCTC 10585	>128	8	>128	32	64	8	128	8
R 58	32	4	32	8	32	8	16	8
MP 819	64	8	64	16	64	8	64	8
MP 969	128	8	>128	32	64	8	128	8
MP 2347	64	8	64	16	32	2	64	2
F. odoratum								
NCTC 11036	>128	>128	>128	>128	32	16	32	32
NCTC 11179	>128	>128	>128	>128	>128	128	128	128
NCTC 11180	>128	>128	128	>128	>128	>128	128	>128
R 25	>128	>128	>128	>128	>128	>128	>128	>128
R 59	>128	>128	>128	>128	128	128	128	128
U 59	64	64	64	64	32	16	32	32
F. multivorum								
NCTC 11033 *	32	0.12	64	0.25	16	⩽0.06	16	8
NCTC 11343 *	32	0.12	64	0.12	8	⩽0.06	4	0.12
MP 1177 *	32	0.12	64	32	4	0.25	4	2
MP 1210	64	32	64	64	8	4	8	4
MP 2324	4	0.12	4	0.5	1	0.25	1	0.5
F. spiritivorum								
NCTC 11386	64	8	32	8	32	4	16	4
NCTC 11387	128	16	64	32	32	16	32	16
R 41	128	16	64	64	16	4	16	8
R 51	32	8	32	8	32	4	16	4

TABLE 37 (continued)

ORGANISM	MA	MA + CLAV	MA + EDTA	MA + CLAV+ EDTA	CTX	CTX+ CLAV	CTX+ EDTA	CTX+ CLAV+ EDTA
F. thalpophilum								
MP 1207	32	8	32	16	16	2	16	4
MP 1232	64	0.12	64	16	16	0.12	16	4
Group IIb								
U 58	>128	>128	>128	>128	64	64	64	64
R 46	>128	>128	>128	>128	64	64	64	64
Unclassified								
R 5	>128	>128	>128	>128	>128	64	>128	>128
R 19	8	4	4	2	2	0.5	1	0.5
S. aureus								
ATCC 25923	0.5	0.25	0.5	0.12	1	0.5	1	0.25
E. coli								
ATCC 25922	0.5	1	1	1	≤0.06	≤0.06	≤0.06	≤0.06

* strain was inhibited by 2 mcg/ml clavulanate

** strain was inhibited by 1 mM EDTA

MA	Cefamandole	MA	Cefamandole
CLAV	Lithium Clavulanate	CLAV	Lithium Clavulanate
CTX	Cefotaxime	CTX	Cefotaxime

TABLE 38

Disc Tests for Amoxycillin-Clavulanate Synergy

ORGANISM AND STRAIN CODE	SYNERGY WITH AMOXYCILLIN/ CLAVULANATE	NO SYNERGY
F. breve		
	U31	NCTC 11099, NCTC 11162
F. gleum		
		NCTC 10795, NCTC 11432 U62, R48, R49, R56
F. meningosepticum		
	NCTC 10585, R58, MP816, MP819, MP969, MP970, MP1702, MP1742, MP2347, MP2348	NCTC 10016, MP894
F. multivorum		
	MP1210	NCTC 11033, NCTC 11343, MP1177, MP2324
F. odoratum		
	R59	NCTC 11036, NCTC 11179, NCTC 11180, R25, U59
F. spiritivorum		
	NCTC 11386, NCTC 11387, R41, R51	
F. thalpophilum		
	MP997, MP1232	MP1207
Group IIb		
	R34	U58, R46, R47, R862, MP7, MP8, MP305, MP525, MP547, MP548, MP582, MP592, MP616, MP628, MP639, MP1098, MP1846
Unclassified		
	U33	R5, R19
C. aquatilis ATCC 29551		*F. aquatile* NCIB 8694
C. johnsonae ATCC 17061		"*F. tirrenicum*" ATCC 15922
C. johnsonae NCIB 10150		*F. elegans* DSM 52[7]

and MP984 did not become more susceptible to ampicillin and the MIC of R58 was reduced only two-fold to cefotaxime. Three strains of F. multivorum (NCTC 11033, NCTC 11343 and MP1177) were inhibited by 2mcg/ml clavulanate and could not validly be tested in the cephem study. Strain MP2324 was however more susceptible to the cephalosporins in the presence of clavulanate but unaffected by clavulanate in the augmentin disc test. The results for strain MP1210 were the opposite - clavulanate was synergistic with ampicillin but produced no effect with the cephalosporins. No strains of F. odoratum increased in susceptibility to the cephalosporins and only strain R59 was more susceptible to ampicillin in the presence of clavulanate. All strains of F. spiritivorum were more susceptible in the presence of clavulanate although not always significantly so. Strain MP1207 of F. thalpophilum was unaffected by clavulanate whereas strains MP997 and MP1232 were synergistically inhibited. Only one strain of Group IIb, R34, became more susceptible in the presence of clavulanate, all other strains being unaffected. (Thus Group IIb and F. gleum differed from F. meningosepticum in generally being unaffected by clavulanate). Unclassified strains R5 and R19 were unaffected but U33 became more susceptible to ampicillin in the presence of clavulanate. The three strains of Cytophaga were more susceptible to ampicillin in the presence of clavulanate but F. aquatile, "F. tirrenicum" and F. elegans were unaffected.

Some unexpected results occurred when both clavulanate and EDTA were added to the cephalosporins. F. multivorum MP1177

had an MIC of 0.12 mcg/ml to cefamandole plus clavulanate. However when EDTA was added the MIC increased 8-fold to 32 mcg/ml. This suggested that EDTA interfered with either the inhibitory effect of the clavulanate or with the synergistic effect of clavulanate on cefamandole. Similar results occurred with cefotaxime for this strain of F. multivorum as well as strain NCTC 11033 and F. thalpophilum MP1232.

IV. DISCUSSION

A future revision of Flavobacterium as a taxonomically coherent genus was foreshadowed by Weeks (1974) who divided it into two distinct sections: Section I comprising nonmotile strains of low %G+C, and Section II comprising motile and nonmotile strains of high %G+C. This division separated organisms which were genetically different and therefore clearly unrelated. Prior to 1974 the lack of incisive and sensitive chemical and genetic techniques precluded recognition of the two sections within Flavobacterium.

Following Weeks' 'interim and pragmatic arrangement' (Weeks, 1974) a consensus was reached on the taxonomic limits of Flavobacterium. Hayes (1977), McMeekin and Shewan (1978), and Holmes and Owen (1979) proposed that the genus be restricted to the Section I-type organisms of Weeks (1974), and in 1980 an international symposium confirmed the acceptability of the redefined genus (Reichenbach and Weeks, 1981). The symposium was, in effect, a mandate delivered by leading workers with Flavobacterium which should have guided other microbiologists in the taxonomic handling of Flavobacterium. In 1982, in recognition of the increasing taxonomic interest in Flavobacterium the ICSB formed a subcommittee for Flavobacterium, Cytophaga and allied bacteria. Widespread recognition of the redefined genus should have been complete in 1984 when Holmes et al. (1984a) endorsed the redefined genus Flavobacterium in the traditional and authoritative 'bible' of bacteriologists, Volume 1 of Bergey's Manual of Systematic Bacteriology.

At this point it may have seemed that the major taxonomic work was done. The case for the redefined genus had been carefully researched, well argued, and widely circulated. All that remained for the completion of the work was the rejection of names of Flavobacterium species which no longer conformed to the genus description. This could have been achieved, albeit tediously, by individually transferring the species incertae sedis (Table 10) to more appropriate genera and then formally requesting the Judicial Commission of the ICSB to reject the original names.

In retrospect it can be seen that the failure to promptly perform this final piece of taxonomic housekeeping was most unfortunate and was partly responsible for perpetuating the confusion and taxonomic mismanagement which has been associated traditionally with the genus. Much of the recent taxonomic laxity stems from the increased interest in yellow-pigmented bacteria by aquatic, environmental and industrial microbiologists who publish reports of Flavobacterium strains which either do not conform to the redefined genus description or have not been identified sufficiently to warrant inclusion in the genus. For example, in 1987 the publications reviewed in Microbiology Abstracts : Section B, Bacteriology (Cambridge Scientific Abstracts, Bethesda, Md) mentioned F. heparinum (transferred to Cytophaga by Christensen, 1980) in two abstracts, "F. dehydrogenans", "F. keratolyticus", and "F. saccharophilum" (not validly published names) in one abstract each, and Flavobacterium species (no further details) in ten abstracts. These examples indicate the lack of taxonomic rigor which has continued to be associated with the genus.

It is now fourteen years since Weeks first delineated the low %G+C strains as a distinct taxonomic entity. Considering the remarkable pace at which twentieth century science has progressed, particularly in disciplines such as bacterial taxonomy where genetic and molecular approaches have rapidly advanced knowledge in spectacular and unprecedented fashion, fourteen years seems a long time. In this period genetic and chemical studies have advanced our understanding of Flavobacterium to such an extent that interest is now focussed on the relationship of the genus to the anaerobic genus Bacteroides. Such a concept was not envisaged at the time of the 1980 symposium and certainly could not have been seriously considered when Weeks grappled with the confusing taxonomy of Flavobacterium in 1974. Viewed in this context it is regrettable that the recent taxonomic publications and scientific insights should have had so little impact outside taxonomic circles.

The solution to this problem is not immediately obvious. Part of the problem is common to all sciences - i.e. the inability of the masses to keep abreast of new advances in specialised disciplines. However there is a more fundamental aspect to the problem which should not arise. It seems that a number of scientists (and editors of journals) need to be reminded that scientific names have precise meanings, and that generic and species names should not be used without checking first that they are correct. In the case of Flavobacterium it is incorrect to use the generic name without verifying that an

organism is nonmotile, nonfermentative, oxidase- and phosphatase-positive and is of low %G+C. (An approximate %G+C value can be determined conveniently by the UV test of McMeekin, 1977). If the %G+C is unknown terms such as '*Flavobacterium*-like' and 'flavobacteria' should be used instead of unsubstantiated scientific names.

Apart from the nomenclatural problems and the anachronism of some high %G+C species of *Flavobacterium* remaining in the Approved Lists (Skerman *et al.*, 1980) there are further taxonomic problems within the redefined genus which require attention. One problem is that the recent taxonomic advances have involved primarily clinical strains. This has led to an imbalance in the taxonomic framework of the redefined genus by promoting impetus in a 37°C-positive direction and has created some problems in the identification of nonclinical strains.

Within the taxonomic framework certain species appear to be genetically and phenotypically related and it is likely that distinct groups within the genus will eventually attain generic status pending the discovery of suitable phenotypic markers by which to define them (Bauwens and De Ley, 1981; Holmes and Owen, 1981; Yabuuchi *et al.*, 1983; Holmes, Owen and McMeekin, 1984). Some of the species are genetically heterogeneous and their subdivision into further species is warranted also but not yet possible because of the lack of convenient phenotypic markers which can be correlated with genetic differences.

The indistinct nature of the Flavobacterium/Cytophaga boundary is another blurred taxonomic terrain requiring additional characters to clarify relationships. Until suitable tests are found gliding motility will remain the sole determinant of the boundary. This is unsatisfactory because undue emphasis is given to a single test for which some strains yield atypical results.

The taxonomic utility of antibiotic resistance in Flavobacterium has not been evaluated systematically. There are however sound theoretical reasons for supposing that the antibiotic resistance patterns of the genus may indicate taxonomic relationships. Holmes et al (1984a) suggest that antibiotic resistance in the genus is largely controlled by chromosomal genes. If this is true antibiotic resistance patterns may be specific and stable for individual taxonomic units. This hypothesis presupposes that (1) the genes for antibiotic resistance will always be expressed, and (2) that there are significant differences between the species. Even if these requirements are met the probability of taxonomically distinct resistance patterns is not absolute as differences may be masked by other factors. For example the same resistance genes may be expressed at different levels in different strains of the same species, and other factors such as different growth rates, altered target sites and differences in permeability may lead to strain-to-strain variability in antibiotic susceptibility tests. Thus, although antibiotic resistance patterns have theoretical taxonomic potential for Flavobacterium their utility may be reduced by the interplay of other factors which mask the

phenotypic expression of the resistance genes.

There is however evidence of antibiotic resistance patterns in Flavobacterium being taxonomically useful. In one numerical taxonomic study Jooste et al. (1985) was able to delineate a cluster containing Group IIb from another cluster containing F. balustinum and F. indoltheticum only when antibiotic susceptibility tests were included in the analysis. This points towards the practical utility of antibiograms in taxonomic analysis of flavobacteria and suggests that further more detailed investigation may yield new phenotypic markers.

However a problem with this approach is the added complication that, as previously mentioned, the quality of the published antibiotic susceptibility data for Flavobacterium appears to be less accurate than comparable data for other more frequently encountered clinical taxa. Future studies must be hampered by unresolved problems such as the poor correlation between disc and MIC susceptibility testing techniques and the difficulties associated with testing strains which do not grow at 35°C.

These problems may be avoided however if tests can be used to detect the presence of specific products of chromosomal genes for resistance. Such specific products might include antibiotic inactivating enzymes. Tests for gene products would be more sensitive than antibiograms because they should yield information irrespective of temperature of incubation and whether or not resistance is phenotypically

expressed. This approach has already been applied to beta-lactamase analysis and as a taxonomic tool has been found to be sensitive to subspecies level for some organisms.

Information about beta-lactamases in Flavobacterium is limited to three reports - three enzymes of F. meningosepticum, one of F. odoratum, and one of an organism referred to as Flavobacterium strain 80 (%G+C unknown). These beta-lactamases are quite distinctive and differ from the beta-lactamases produced by the majority of clinically occurring bacteria. These initial reports therefore strongly indicate that the beta-lactamases of the genus may prove to be useful chemotaxonomic markers.

a. Susceptibility Testing

In this study it was necessary to establish interpretive criteria for the antibiotics which were not calibrated for the CDS method of disc diffusion testing. This was achieved by the matching of results of disc and MIC tests for all the uncalibrated drugs except cefuroxime, which was not included in the MIC study. For cefuroxime it was therefore necessary to provide arbitrary interpretive criteria. While the clinical significance of these arbitrary criteria is unproven their use can be justified to some extent in that:
(i) the validity of any disc test is questionable for Flavobacterium (Maderazo et al., 1974; Von Graevenitz and Grehn, 1977; Aber et al., 1978; Winslow and Pankey, 1982; Johny et al., 1983).
(ii) this is a taxonomic study and therefore comparisons of

inhibition zone sizes are relevant irrespective of their clinical implications and

(iii) the arbitrary interpretive criteria proposed for cefuroxime were cautiously and conservatively selected to minimize the likelihood of strains being incorrectly judged as either susceptible or resistant.

The study was further complicated by the inability of some strains of Flavobacterium to grow at 35°C or to grow on Sensitest agar which meant that for some strains there was no standardized method of performing antibiotic susceptibilities. However it was essential to obtain antibiotic susceptibility data to provide an impression of the wide range of organisms of the genus even if it meant that for some organisms it was not possible to directly compare test results with data from other studies. In particular it was important to obtain information about F. aquatile, the type species. Probably because of the technical difficulties presented by this organism there are no published antibiotic susceptibility data for it.

In spite of the problems encountered the antibiotic susceptibility results provided clear evidence of similarities and differences between the taxa and evidence of heterogeneity within some species and Group IIb. Both disc and MIC tests confirmed that the genus is characterized by multiple antibiotic resistance and that F. odoratum and F. meningosepticum are especially resistant and therefore likely to present very serious therapeutic problems in life-

threatening infections. In all taxa there were some strains which yielded contradictory results for disc and MIC tests. Unlike other studies the results of this study did not suggest that only disc tests give a false impression of susceptibility. Discrepancies between disc and MIC results showed no consistency and both techniques had similar failure rates in missing resistance. Disc tests appeared to miss resistance in 64 tests and MICs appeared to miss resistance in 56 tests (Table 27). However without a reliable benchmark it was not possible to judge which was the more accurate technique or if either technique was satisfactory. It is therefore not possible from these results to recommend either technique as optimal for the testing of Flavobacterium.

The antibiotics which were most frequently associated with discrepancies were ceftazidime, chloramphenicol, cefotaxime, cefoxitin and trimethoprim. This is disturbing because these are agents which have been touted as exhibiting superior activity against Flavobacterium spp. in general and F. meningosepticum in particular (Aber et al., 1978; Dhawan et al., 1980; Johny et al., 1983; Macfarlane et al., 1985; Schell et al., 1985; Gilardi, 1987). Since empiric anti-Gram-negative therapy is usually inappropriate for Flavobacterium infections it is important that optimal susceptibility testing procedures be established. Recommendations to use MICs (Johny et al., 1983) or to provide more stringent interpretive criteria for disc diffusion tests (Bruun, 1987) are based on the assumption that MIC testing provides definitive results. This has not been proven. All that previous studies have demonstrated is that with the organisms and drugs tested MICs

were more reliable in detecting resistance. With the organisms and drugs tested in this study this was not the case and it would therefore be premature and possibly dangerous to dismiss as spurious the resistance detected only in disc tests in this study.

Given the difficulty of providing accurate susceptibility tests for this genus it is fortunate that Flavobacterium infections are rare. This however is poor consolation for the individuals involved when cases occur, particularly cases of neonatal meningitis due to F. meningosepticum. In such cases there is a fatality rate of about 55% and most survivors suffer hydrocephalus (Von Graevenitz, 1981; Holmes, 1987). These are grim statistics which partly stem from a lack of ability of clinical laboratories to provide a reliable guide to therapy. It would therefore seem appropriate to take the question of improved susceptibility testing of the genus off the academic backburner and recognise it as an important and practical research priority.

Because susceptibility results tended to be method-dependent it was difficult to identify antibiotics of taxonomic or clinical utility. Nevertheless broad groupings of organisms which corresponded to described taxa were identified on the basis of their responses to antibiotics and this confirmed that antibiotic resistance is an integral and specific feature of the species and Group IIb and indicated that even more specific data, such as knowledge of resistance mechanisms, would be of value in examining the taxonomic

relationships of the genus.

b. Beta-lactamases

Since no comparable study had been undertaken previously it seemed important to detect and catalogue as many beta-lactamases as possible from representative strains of the genus Flavobacterium. For many strains the level of beta-lactamase activity was low making detection difficult by isoelectric focussing. Nevertheless many different beta-lactamases were detected within the genus.

As none of the beta-lactamases detected resembled known plasmid mediated beta-lactamases and none were inducible with cefoxitin, they were judged to be constitutive and chromosomally mediated. This assumption is supported by the scanty evidence for plasmids in Flavobacterium (Holmes, Owen and McMeekin, 1984) and is more plausible than the alternative which is that Flavobacterium contains a completely new series of plasmid mediated beta-lactamases.

3-dimensional testing was included in this study to investigate, by indirect means but using procedures available to most routine bacteriology laboratories, the beta-lactamases and underlying mechanisms of beta-lactam resistance in Flavobacterium and related species.

There were several advantages in this approach. Technically there were the advantages of speed, cheapness, convenience and versatility. From the biological viewpoint

the use of whole cells for tests meant that the organism's array of defence mechanisms was intact at the time it encountered an antibiotic and that its beta-lactamases were in pristine condition. This is not the case in conventional beta-lactamase analysis using cell-free enzyme preparations, in which the regulatory organisation of the cell envelope is lacking and the activity of the enzyme preparations may have been impaired by adverse effects during harvesting, purification, freezing and thawing. (In this study significant, but unquantified, losses of activity occurred in some beta-lactamase preparations). Thus it can be argued that techniques such as the 3-dimensional and clover leaf tests are more sensitive and biologically more meaningful than conventional methods for investigating the antibiotic-beta-lactamase encounter.

At present the more intimate details of how the bacterial cell mounts its defence against beta-lactam antibiotics are largely unknown. Where are beta-lactamases located within the periplasm and what are their concentrations in the vicinity of porins and PBPs? How many PBPs need to be attacked to cause cell death? By what route and at what rate does a particular beta-lactam antibiotic enter the periplasm? Our limited understanding of these matters cannot yet be translated into practical application so that we may determine the significance, extent and effectiveness of each of the known components of the bacterium's defence against beta-lactam antibiotics. However there is no doubt that the interplay of these factors is crucial. Conventional analysis of beta-

lactamases using [illegible] cell-free enzyme preparations which cannot take into account these factors must yield artificial and at times misleading information about an organism's response to beta-lactam antibiotics and for this reason must be recognised as of limited utility.

Tests involving whole cells must therefore more accurately portray what happens when a bacterium encounters a beta-lactam antibiotic and thus more closely mimic the in vivo therapeutic situation. This does not however mean that the conventional tests are without substantial value. A major defect of whole cell techniques arises from the inherent uncertainties associated with attempts to accurately analyse one function of a living organism while dealing with the total entity. One cellular property (e.g. a permeability barrier) may mask the effect of another (e.g. beta-lactamase activity) and it may be necessary to resort to cell-free preparations to investigate the property of interest. Another problem with whole cell tests is that it will not be obvious if the organism possesses a single or multiple resistance mechanisms (e.g. one or multiple beta-lactamases). Thus a case for both approaches can be advocated and it would seem prudent to combine them to obtain maximum information. Used in this way the simpler whole cell approach could be used as a convenient screening procedure to determine which more labour-intensive and expensive conventional investigations might be most profitable.

In this study the routine 3-dimensional tests were of limited value because many Flavobacterium strains were so

resistant that inhibition zones were either non-existent or too small to detect 3-dimensional zone distortions. Although these tests indicated possible beta-lactamase mediated antibiotic inactivations, including inactivation of the 'beta-lactamase resistant' cefoxitin, there were too many gaps in the data to draw conclusions about the genus as a whole.

The more sensitive 3-dimensional tests and clover leaf tests provided greater sensitivity but were not sufficiently comprehensive on their own. Discrepancies between the two methods may have arisen from differences in inocula, inability to reproducibly control inoculum density, and the different modes of inoculation. In the 3-dimensional test the inoculum is inserted into the agar whereas in the clover leaf test the inoculum is streaked onto the surface of the agar. The significance of the latter technical difference is however unclear in tests in which cell lysis permits beta-lactamases to diffuse through the agar.

Prior to this study there had not been an attempt to calibrate either the 3-dimensional technique or the clover leaf test against a range of well-characterized beta-lactamases. Because this study contained only small numbers of reference strains it has not been established to what degree 3D+CL profiles can vary when the same beta-lactamases are expressed in different hosts. However Table 31 indicates that the technique is a valuable screening procedure which indicates the substrate profile of an organism's beta-lactamase(s). Verification of the reliability of this technique to

demonstrate substrate inactivations could be accomplished by using the HPLC technique of Aldous et al. (1985) which was similarly used to investigate the reliability of 3-dimensional testing.

The 3D+CL technique provided much information about the beta-lactamases of the genus Flavobacterium. Comparison of the Flavobacterium profiles with those of the antibiotic reference strains confirmed the data obtained by more conventional testing procedures which indicated that Flavobacterium appears to contain distinctive, perhaps unique, beta-lactamases. A striking feature was the 3D+CL evidence indicating cloxacillin hydrolysis by almost all the Flavobacterium strains. This information, when combined with the results of conventional hydrolysis assays and the inhibitor studies, suggested that if the beta-lactamases are chromosomally mediated they probably belong to class IV of the Richmond and Sykes (1973) classification. This conclusion appears to be the same as that of Raimondi et al. (1986), who concluded that the beta-lactamase of F. meningosepticum belongs to class IV of Sykes and Matthew (1976). (Actually Sykes and Matthew did not propose a classification, so it may be assumed that Raimondi et al. were referring to the Richmond and Sykes classification). It also suggested that hydrolytic activity against cloxacillin may be a distinctive characteristic of the genus Flavobacterium. Approximately half the strains appeared to inactivate cefotaxime, less strains appeared to inactivate cefoxitin and a small number appeared to be capable of inactivating imipenem. These 3D+CL results may be regarded as presumptive evidence of

infrequently detected enzymatic activity against antibiotics which are resistant to hydrolysis by most bacterial beta-lactamases. The 3D+CL test failed to detect any strains of Flavobacterium with beta-lactamases capable of inactivating temocillin.

An interesting feature of the 3D+CL tests was the suggestion of comparatively greater penicillinase activity in Flavobacterium beta-lactamases than was apparent from the hydrolysis assays which tested cell-free enzyme preparations. This was attributed to the difference between testing intact whole cells and cell-free enzyme preparations and was indirect evidence for a selective permeability barrier which allows penicillins to penetrate more readily into the periplasm than cephalosporins. Such a barrier would explain why the predominantly cephalosporinase activity of the cell-free assays was less strongly expressed in the 3D+CL tests. The penicillins, which penetrated the outer membrane more readily were more able to encounter the Flavobacterium beta-lactamases and were thus inactivated to a far greater extent in the 3D+CL tests. This hypothesis is consistent with findings that cephalosporins tend to enter cells through porins whereas in some species (E. coli and E. cloacae) penicillins enter cells through both porin and non-porin pathways (Komatsu et al., 1981; Sawai, Hiruma et al., 1982; Yamaguchi et al., 1985). In cells with narrow restrictive porins or low amounts of outer membrane proteins penicillins can still enter the periplasm via non-porin routes. Beta-lactam antibiotics which have low intrinsic rates of penetration such as cefoperazone,

The role of the beta-lactamases in determining antibiotic resistance appeared to vary. For some strains the levels of beta-lactamase activity in hydrolysis assays did not correlate well with MIC values. Synergy studies with lithium clavulanate and beta-lactam antibiotics clearly demonstrated diminished susceptibility of some strains which may well have arisen from beta-lactamase inhibition. However since clavulanate may also exert a synergistic effect through mechanisms other than beta-lactamase inhibition (Clarke and Zemcov, 1984; Tausk and Stratton, 1986; Bakken et al., 1987) these results cannot be regarded as unequivocal evidence for a significant role of beta-lactamases in resistance. The synergy studies indicated that beta-lactamases have little or no role in beta-lactam resistance in F. aquatile, F. gleum, "F. tirrenicum", F. elegans, most strains of F. odoratum and Group IIb. The positive, and therefore somewhat ambiguous, synergy tests suggested that beta-lactamases may be important for beta-lactam resistance in F. meningosepticum, F. spiritivorum, and possibly F. multivorum, F. thalpophilum, C. aquatilis and C. johnsonae.

The negative synergy tests suggested minimal involvement of beta-lactamases in resistance. This appeared to indicate that other factors such as permeability were likely to be important determinants of resistance to beta-lactam antibiotics. However when EDTA was used in synergy studies to investigate its outer membrane-permeabilizing effects it was found to be ineffective. While this may indicate that permeability is not a significant factor in resistance in Flavobacterium an alternative explanation is that

moxalactam, cefuroxime, cefotaxime and aztreonam are selectively excluded (Jaffe et al., 1982; 1983) and these are agents to which many Flavobacterium strains were resistant in this study. This reasoning is further supported by the multiple antibiotic resistance of most strains of Flavobacterium which extends to other classes of antibiotics such as aminoglycosides, quinolones, macrolides and chloramphenicol. Multiple resistance of this nature is often caused by impermeability of the outer membrane.

The conclusion to be drawn from the apparently increased penicillinase activity detected by the 3D+CL technique is that Flavobacterium beta-lactamases perform differently when they are in their natural environment - i.e. within or immediately adjacent to the host bacterial cell where other bacterial activities can regulate the antibiotic-beta-lactamase encounter. However the results from assays using frozen or freeze dried enzyme extracts obtained from sonicated cells may differ from 3D+CL results because of enzyme denaturation and the omission of that cellular organisation which is an integral part of the beta-lactamase-antibiotic interaction of the Flavobacterium cell.

Deficiencies in conventional hydrolysis assays have been recognised for some time e.g. their inability to detect the slow hydrolysis of beta-lactamase 'stable' antibiotics by class I beta-lactamases (Vu and Nikaido, 1985).

Flavobacterium has an outer membrane arranged to conceal or protect portals of antibiotic entry similar to that thought to produce resistance to gentamicin and polymyxin B in P. cepacia (Moore and Hancock, 1986). The resistance to different classes of antibiotics by Flavobacterium is highly suggestive of a mechanism involving limited permeability. The evidence of this study therefore points towards an EDTA-resistant mechanism being implicated.

It is possible that a higher concentration of EDTA may have increased permeability. Malouin and Lamothe (1987) report that 1 mM EDTA increased cell permeability in four species of Bacteroides without inhibiting cell growth. F. breve U31 was inhibited by 0.5 mM EDTA and it was decided not to investigate the possibility that other Flavobacterium strains require a higher concentration of EDTA to increase permeability.

c. F. aquatile

The taxonomic position of F. aquatile is controversial with several lines of evidence pointing to a closer relationship with Cytophaga than with Flavobacterium (Hayes, 1977; Bauwens and De Ley, 1981; Oyaizu and Komagata, 1981; Thomson et al., 1981; Paster et al., 1985). It has been suggested that F. aquatile may eventually be accomodated in a genus which also contains "F. pectinovorum", "F. tirrenicum" and C. johnsonae (Bauwens and De Ley, 1981; Holmes and Owen, 1981; Holmes et al., 1983; Yabuuchi et al., 1983; Holmes, Owen et al., 1984).

In this study only limited information about the response of F. aquatile to antibiotics could be obtained. Technical difficulties arising from its fastidious growth requirements prevented the preparation of cell-free beta-lactamase preparations with detectable activity. Furthermore the antibiogram for this organism could not be produced using a recognised, standardized susceptibility testing procedure. However, by using cytophaga agar, a heavy inoculum and incubation at 30°C it was possible to determine that F. aquatile is quite different from the majority of Flavobacterium strains, "F. tirrenicum", F. elegans and the Cytophaga strains included in this study. The species to which F. aquatile showed greatest similarity was F. breve. Both species were much more susceptible to antibiotics than the other species of Flavobacterium and Cytophaga, and were distinctive in their susceptibility to cefsulodin. Although F. aquatile was clearly different from the majority of Flavobacterium strains there was nevertheless an underlying background of common features which suggested that it might be an atypical member of the genus. In particular it appeared to be resistant to (i.e. was not inhibited by) aztreonam, cefotaxime, gentamicin, moxalactam or trimethoprim. Resistance to these agents, in particular gentamicin, is a characteristic of many Flavobacterium strains, (and also the Cytophaga strains of this study). However F. aquatile is distanced in its relationship to these organisms by its inability to inactivate cloxacillin in the 3D+CL tests and its overall greater susceptibility.

Its resistance mechanisms were not elucidated. The 3D+CL tests were negative for all substrates, a finding which suggested that enzymatic drug inactivation was not involved in its resistance to beta-lactam antibiotics. The possibility of limited outer membrane permeability being a resistance mechanism is suggested by the absence of detectable beta-lactamase activity and because F. aquatile showed greater resistance to antibiotics which have low intrinsic rates of penetration and have been shown to be selectively excluded by other bacteria with low outer membrane permeability i.e. aztreonam, cefoperazone, cefotaxime, ceftazidime and moxalactam (Jaffe et al., 1982, 1983). Electron microscopic observations of F. aquatile indicate that it possesses a loose or disrupted outer membrane (Thomson et al., 1981). This may be an explanation for its greater overall susceptibility to antibiotics. It would be interesting if other susceptible strains of Flavobacterium, e.g. F. breve U31, F. odoratum U59 and F. multivorum MP2324, also possess similar outer membranes. This would constitute evidence for a role of the outer membrane in the antibiotic resistance of the genus.

The impact of these results on our understanding of the taxonomy of Flavobacterium is two-fold. Firstly the greater susceptibility of F. aquatile is further information adding weight to the view that its choice as type species of Flavobacterium was inappropriate. The second point is that antibiotic testing failed to support the concept that F. aquatile is more closely related to Cytophaga than to Flavobacterium. Because only three strains of Cytophaga were

studied the point cannot be made higher than that. However the impression obtained from this study was that most Flavobacterium strains and Cytophaga are more closely related to one another than they are to F. aquatile. F. aquatile therefore would seem to be an outlier of the entire Flavobacterium-Cytophaga complex rather than an organism located within the complex somewhere near the ill-defined boundary between Flavobacterium and Cytophaga.

d. F. breve

These results of antibiotic susceptibility tests, 3D+CL tests, IEF and hydrolysis assays indicated heterogeneity within F. breve and, if the beta-lactamases are chromosomally mediated, comprise additional evidence of genetic heterogeneity within the species. The phenotypic expression of this heterogeneity was detectable by both susceptibility tests and beta-lactamase analysis. Antibiotic studies were therefore most promising for investigating taxonomic relationships within F. breve and have provided new phenotypic markers. F. breve is more susceptible than other species of Flavobacterium, F. aquatile excepted, and the three strains differed markedly from each other in susceptibility to most antibiotics. Further studies of a wider range of strains of F. breve should now be undertaken to correlate differences in antibiograms and beta-lactamases with genetic heterogeneity. Because of the low activity of the beta-lactamase preparations of this species consideration should be given to developing optimal analytical procedures with greater sensitivity than

those used in this study. Sato et al. (1985) achieved 150-fold purification of a beta-lactamase extract from a strain of F. odoratum using column chromatography.

In assessing the contribution of these beta-lactamases to resistance the higher penicillinase activity of the NCTC 11162 preparation correlated with increased resistance to ampicillin and ticarcillin. This indicated a possible role of its beta-lactamase(s) in resistance to beta-lactam antibiotics. However, apart from the inestimable tests for environmental strain U31 which was susceptible to clavulanate, there was no evidence of synergy when clavulanate was added to beta-lactam antibiotics. The apparently high and very broad spectrum activity of beta-lactamases of this species detected by 3D+CL testing does not necessarily imply a role in resistance as the enzymes detected by this test may have been released during cell lysis and therefore made no contribution to the survival of the host cell. Without more detailed physiological data it is difficult to attribute a precise role in resistance to the beta-lactamases of F. breve.

Overall the beta-lactamases of F. breve appear to be Richmond and Sykes class IV beta-lactamases. In the absence of evidence to the contrary they are assumed to be chromosomally determined. In cell-free suspensions they are inhibited by clavulanate but not by cloxacillin and appear to be capable of inactivating cloxacillin. The beta-lactamases of NCTC 11099 and U31 appear to be extremely broad in spectrum of activity with hydrolytic activity against cefotaxime and imipenem and, in the case of NCTC 11099, cefoxitin. This

indicates that these enzymes are oxyiminocephalosporinases (Mitsuhashi, 1985) which belong to class IV of Richmond and Sykes (1973). Although the beta-lactamase of NCTC 11162 has borderline activity against cefoxitin by the 3D+CL technique it is not certain if it is an oxyiminocephalosporinase as this equivocal result is not supported by demonstrable activity against cefotaxime or imipenem.

F. breve NCTC 11162, F. multivorum MP1210, F. spiritivorum NCTC 11386 and F. thalpophilum MP1207 each produced a beta-lactamase with a pI of 5.8. Apart from the beta-lactamase preparation of F. multivorum MP1210 which was too inactive to be tested accurately, all of these enzymes were resistant to inhibition by clavulanate. They were therefore different from the beta-lactamase of F. odoratum GN14053 reported by Sato et al. (1985) which also had a pI of 5.8. In the 3D+CL tests the beta-lactamase of F. breve NCTC 11162 was much more active than the beta-lactamases of F. multivorum MP1210, F. spiritivorum NCTC 11386 and F. thalpophilum MP1207. This suggested that it was either a different enzyme or that all strains possessed the same type of enzyme but the cells of F. breve NCTC 11162 could be more readily made to leak beta-lactamases into their external environment. The latter hypothesis is less likely from taxonomic considerations. There is no evidence of a close relationship between F. breve and the proposed genus Sphingobacterium (F. multivorum, F.spiritivorum and F. thalpophilum) and these species represent the lower and upper extremities of the range of %G+C values of the genus (Holmes, Owen and McMeekin, 1984).

Furthermore the hydrolysis assays indicate that the beta-lactamase of F. breve NCTC 11162 is a different enzyme because it possesses greater penicillinase activity.

Strain NCTC 11162 also produced a beta-lactamase with a pI of 7.85 as a major band. Low level activity made it difficult to determine the inhibition profile but this enzyme appeared to be resistant to clavulanate and could not be induced with cefoxitin. It was unlike other beta-lactamases detected in this study. In all NCTC 11162 appeared to possess two unique beta-lactamases.

IEF results indicated that strain U31 possessed a weakly reactive, diffuse, 'blobby' beta-lactamase with pI of 9.3. Hirai et al. (1980) reported a beta-lactamase in P. cepacia GN11164 with a pI of 9.3, a similar substrate profile (hydrolysing ampicillin, cloxacillin, cefotaxime, cefuroxime and most other cephalosporins but not cefoxitin), and also resistant to inhibition by clavulanate. It differed from the beta-lactamase of F. breve in being inducible and in being inhibited by cloxacillin.

The heterogeneity of F. breve in this study contrasts with the finding of Jooste et al. (1985) that in a numerical taxonomic study 10 strains of F. breve formed a well defined and distinct cluster only when susceptibility tests were included in the analysis. Possibly this may reflect the different antibiotics tested by Jooste et al. However it is quite clear from the small number of strains in this study that F. breve contains organisms which differ greatly from one

another in their responses to antibiotics. If these differences are chromosomally coded it is important that the antibiograms and beta-lactamases of a larger collection of strains be systematically investigated to determine the taxonomic implications.

e. F. gleum

How are routine clinical laboratories to identify this organism? The published guidelines (Holmes, Owen et al., 1984) are inadequate to distinguish F. gleum from Group IIb. DNA:DNA hybridization is required for a definitive identification. In this study the only strains which could safely be regarded as F. gleum were NCTC 10795 and NCTC 11432. Four other strains were presumptively identified as F. gleum but could not be confirmed. Until a less frustrating means of identification is provided, clinical microbiologists might be advised to use terms such 'F. gleum-like' and 'F. gleum/Group IIb complex' for organisms resembling the species description of F. gleum.

Both F. gleum and the F. gleum-like strains in this study were homogeneous in their antibiograms apart from their variability to imipenem. Their antibiograms were indistinguishable from Group IIb. However in 3D+CL tests and isoelectric focussing the strains were heterogeneous. The significance of the heterogeneity cannot be evaluated until it is known whether or not the strains actually belong to F. gleum.

Strains NCTC 10795, U62 and R49 appeared to possess oxyiminocephalosporinases of Richmond and Sykes Class IV. The 3D+CL results for NCTC 11432 were negative and precluded definite conclusions about its beta-lactamase.

Isoelectric points were determined for three strains. NCTC 11432 and U62 exhibited a major beta-lactamase band at pI 10.6. Four other organisms exhibited beta-lactamase bands at pI 10.6. These were minor bands, not major bands, and were produced by F. breve U31, F. meningosepticum R58 and MP967, and unclassified strain R5. This may constitute evidence that these organisms are related. If U62 is confirmed as a strain of F. gleum the beta-lactamase at pI 10.6 might prove useful in confirming identification of this species. Routine clinical laboratories however are not normally equipped to determine the isoelectric points of beta-lactamases, so it would still be necessary to refer the strain to a reference laboratory. It may be that U62 is not a strain of F. gleum. In 3D+CL testing strain NCTC 11432 was inactive whereas U62 inactivated ampicillin, cloxacillin, cephalothin, cefotaxime and (weakly) cefoxitin. The reason for these differences was not investigated and the possibility that the two strains are fundamentally different cannot be discounted.

Synergy studies with clavulanate failed to demonstrate a role for beta-lactamases in the resistance to beta-lactam antibiotics of F. gleum or F. gleum-like strains.

Strain R49 had a major beta-lactamase band at approximately 9.6. This was one of the diffuse 'blobby' beta-lactamases similar to those found at a pI of approximately 9.8 in some strains of F. meningosepticum (NCTC 10016, MP894 and MP2348) and at 9.3 in F. breve U31. It is possible that these enzymes are the same. They could not be sharply focussed to determine a precise isoelectric point and accuracy in IEF tends to decline at extremes of pH.

f. F. meningosepticum

The high resistance of this species to antibiotics was confirmed. Cefoxitin was more active than other antibiotics but was not effective against all strains. Experience with cefoxitin in neonatal and pediatric meningitis caused by other pathogens has been disappointing (Sanders and Sanders, 1987) so the value of cefoxitin for treating neonatal meningitis caused by F. meningosepticum would not seem to be great. Although augmentin (amoxycillin-clavulanic acid) was more active than ampicillin against many strains of F. meningosepticum penetration of clavulanic acid into CSF is unreliable, sometimes failing to attain a concentration of 0.05 mcg/ml (Gould and Wise, 1987; Decazes et al., 1987). Augmentin does not appear to be a therapeutic solution either.

Therapy for this infection therefore remains a problem. Effective antibiotics are lacking, [illegible] and there are major technical problems in providing accurate susceptibility tests for F. meningosepticum. It therefore seems that until more effective agents are discovered and the laboratory problems

are overcome the outlook will continue to be bleak for infants developing meningitis due to this pathogen.

Ceftazidime exhibited potential taxonomic utility for differentiating F. meningosepticum from F. gleum and Group IIb; and trimethoprim also appeared useful in distinguishing these taxa, but only in disc tests.

Raimondi et al. (1986) studied the beta-lactamases of seven strains of F. meningosepticum and reported three broad spectrum beta-lactamases, with predominantly penicillinase activity, and isoelectric points of 7.65, 7.8 and 7.9. The beta-lactamase of strain NCTC 10585 was constitutive and all beta-lactamases were inhibited by clavulanic acid, cloxacillin and pCMB and were classified as belonging to class IV of Sykes and Matthew (1976).

In this study the IEF results confirmed the reported heterogeneity of beta-lactamases by Raimondi et al. (1986). However the strain which was common to both studies, NCTC 10585, had a pI of 8.6 for its major band in this study whereas Raimondi et al. (1986) reported a range of pI values of 7.65 to 7.9 for the major bands of F. meningosepticum and the beta-lactamase of NCTC 10585 (pI not stated) would have been in this lower pI range. Most of the other isoelectric points in this study (range pI 7.8 to 9.8) differed from those reported by Raimondi et al. (1986).

Four strains had beta-lactamases with an isoelectric point of 8.6. These were NCTC 10585, R58, MP816 and MP969. These strains were generally similar in their MICs to beta-lactam antibiotics, hydrolysis ratios, in rate of cefoxitin hydrolysis and 3D+CL profiles. Clavulanate synergy tests indicated that this beta-lactamase was involved in resistance to beta-lactam antibiotics. Three strains of Group IIb also appeared to produce this beta-lactamase (R46, MP7 and MP628) but, from clavulanate synergy studies, did not use it in resistance to beta-lactam antibiotics.

Strains NCTC 10016, MP894 and MP2348 shared a 'blobby' beta-lactamase with a pI of approximately 9.8. These strains were similar in most tests except NCTC 10016 hydrolysed cefoxitin at a much lower rate. This beta-lactamase was distinctively different in appearance on an IEF gel from the beta-lactamases of other strains of F. meningosepticum and served to delineate this group of strains. The only other beta-lactamase resembling this one was that of F. gleum R49 with a pI of approximately 9.6.

Strains MP1702 and MP1742 were very similar in most respects and shared a beta-lactamase with a pI of 7.9. This value was the highest pI reported by Raimondi et al. (1986). The beta-lactamase of strain MP819 had a pI of 7.8, a value also reported for F. meningosepticum by Raimondi et al., and MP970 possessed a beta-lactamase with a pI of 8.1.

Most strains exhibited comparatively high levels of beta-lactamase activity and all beta-lactamases were inhibited by

clavulanate but not by cloxacillin. This latter finding was in disagreement with that of Raimondi et al. (1986) who reported the inhibition of beta-lactamases by cloxacillin. This discrepancy may reflect differences in methodology. Hydrolysis assays in this study showed the enzymes to be primarily cephalosporinases, whereas Raimondi et al. concluded that the beta-lactamases of F. meningosepticum are broad spectrum enzymes with predominantly penicillinase activity. The determination of substrate profile, like inhibitor studies, is method-dependent. The orthodox approach to penicillin and cephalosporin hydrolysis assays is to use different, and optimal, techniques for penam and cephem substrates (Richmond and Sykes, 1973). Raimondi et al. performed both penicillin and cephalosporin hydrolysis using the same technique, the spectrophotometric technique of Samuni (1975), whereas in this study a spectrophotometric technique was used for cephem hydrolysis assays and a microiodometric technique for the penicillin hydrolysis assays. The different techniques used may have contributed to the different results obtained in the two studies.

The beta-lactamases of nine of the 11 strains of F. meningosepticum hydrolysed cefoxitin (Table 35). The two strains for which activity was not detected were NCTC 10016 and MP 819. This finding adds support to previous evidence that NCTC 10016 differs from the majority of F. meningosepticum strains (McMeekin et al., 1972; Sottile et al., 1973; Owen and Snell, 1976; Callies and Mannheim, 1980; Fautz et al., 1981; Thomson, 1982; Ursing and Bruun, 1987). The ability to

hydrolyse cefoxitin did not however necessarily confer resistance to this agent as only two of the nine strains, MP 894 and MP 969, were resistant.

In contrast to the results of the hydrolysis assays and more in keeping with the interpretation of Raimondi et al. (1986), eleven of the twelve strains tested by the 3D+CL technique exhibited broad spectrum beta-lactamase activity. The substrate profiles obtained by this method, coupled with other parameters, suggested that all strains of F. meningosepticum produced Richmond and Sykes Class IV beta-lactamases and in nine of the strains the beta-lactamases were oxyiminocephalosporinases. In general the beta-lactamases of F. meningosepticum appeared to be broader in spectrum than those of F. gleum and F. gleum-like strains, with some strains of F. meningosepticum conspicuously inactivating carbenicillin, cephaloridine and cefoperazone. F. meningosepticum also seemed to possess greater activity against cephalothin, a finding which was not apparent from the susceptibility tests and hydrolysis assays. Further strains need to be investigated to determine whether or not this is a real difference as it may have taxonomic significance.

The greater apparent penicillinase activity indicated by the 3D+CL tests may arise from a selective outer membrane which impeded entry of cephems into the periplasm.

The 3D+CL technique showed good correlation with the hydrolysis assays for cefoxitin hydrolysis by NCTC 10585, R58, MP1702, MP1742 and MP2348, and in detecting no cefoxitin

hydrolysis by NCTC 10016 and MP819. However it failed to detect cefoxitin hydrolysis by the beta-lactamases of MP816, MP894, MP969 and MP970. This may indicate that in cells of these four strains the capability to inactivate cefoxitin is not expressed, perhaps because of a permeability barrier, or that for these strains there was no lysis of cells or permeabilizing effect which released beta-lactamase into the external environment. Another possibility is error in the 3D+CL technique.

Another interesting 3D+CL result was the suggestion of greater enzymatic activity of the strains against cephalothin compared to cefoperazone, an antibiotic which is usually more vulnerable to beta-lactamase hydrolysis. This effect might be due to differences in porin permeabilities for cephalosporins. Yoshimura and Nikaido (1985) report that the diffusion rates of beta-lactam antibiotics through porin channels in E. coli, and other bacteria, are determined by factors such as molecular size, charge, the presence of exceptionally bulky side chains and hydrophobicity. Both cephalothin and cefoperazone are monoanionic compounds but cefoperazone, with a molecular weight of 644 and a substituted ureido group on the alpha-carbon of the position-7 side chain, has a particularly slow penetration rate. Cephalothin, with a molecular weight of only 395, is thus able to penetrate faster and therefore encounter the beta-lactamases of F. meningosepticum at a significantly greater rate than cefoperazone and thereby produce 3D+CL results in which cephalothin is apparently inactivated faster than

cefoperazone. This hypothesis infers that the 3D+CL test detects periplasmic beta-lactamase activity as well as extracellular activity.

These investigations confirm that strain NCTC 10016 differed from the majority of strains of F. meningosepticum and is therefore an unrepresentative choice for type strain of the species.

The apparent sharing of a common beta-lactamase by some strains of F. meningosepticum and the F. gleum/Group IIb complex is interesting. Apart from the difference in hue of pigmentation these taxa are very similar. Has undue emphasis been placed on the paler pigmentation of F. meningosepticum, and if so, how valid is the current species description? The beta-lactamase with a pI of 8.6 needs further investigation as if it is chromosomally mediated it may have very important taxonomic implications. Other evidence which points away from an intimate relationship between these strains of F. meningosepticum and the F. gleum/Group IIb complex is their distinctly different results in the synergy tests with clavulanate and their differences in susceptibility to ceftazidime. These results, which are however subject to the inaccuracies introduced by using whole cells in tests, suggest that the currently accepted taxonomic arrangement is correct and that these are distinctly different taxonomic groups. It is difficult to weigh these opposing lines of evidence. Because of the genetic heterogeneity of F. meningosepticum (Sottile et al., 1973; Owen and Snell, 1976; Callies and Mannheim, 1980; Ursing and Bruun, 1987) it is recognised that

the present classification does not contain full and correct information, and certainly greater weight should be given to information about a specific gene product such as a chromosomal beta-lactamase than to results of whole cell tests. It is therefore important that this beta-lactamase and the strains which produce it be investigated in greater depth.

g. *Flavobacterium* Species Belonging to the Proposed Genus *Sphingobacterium* (*F. multivorum*, *F. spiritivorum* and *F. thalpophilum*)

These three species exhibited similar patterns of antibiotic resistance with *F. spiritivorum* being generally more resistant, particularly in the disc tests, and *F. thalpophilum* being more susceptible, particularly to cefoxitin and (in disc tests) to ticarcillin.

Although Table 15 indicates that strains of *F. multivorum* have been previously reported to be susceptible to erythromycin, the 15 strains tested in this study were resistant apart from strain MP 2324 which was an exceptionally susceptible strain for this species. This strain would be an ideal one from which to derive antibiotic resistant mutants by subculturing onto antibiotic gradient plates. By comparing the outer membrane proteins, PBPs and antibiotic-inactivating enzymes of the mutants and MP2324 it might be possible to elucidate the resistance mechanisms of this species, and perhaps the genus. Such information could be invaluable, not only from a taxonomic viewpoint, but also because it might

lead to the development of antibiotics which can evade the formidable resistance mechanisms of Flavobacterium and other bacteria.

The beta-lactamases of the three species all exhibited low levels of activity in hydrolysis assays, 3D+CL tests and isoelectric focussing. Although there was evidence of heterogeneity within the species this group of organisms appeared to constitute a cohesive taxonomic unit in which there were common beta-lactamases (e.g. pI values of 5.8, 7.4 and 8.2). This suggested that arguments for the proposed genus Sphingobacterium, containing these three species and "S. mizutae", are soundly based. However the delineation of species boundaries within the genus may require future modification after further strains are studied and the extent and taxonomic impact of the intra-species heterogeneity is evaluated.

The principal beta-lactamases of the proposed genus appear to belong to Richmond and Sykes Class IV. These were difficult enzymes with which to work because of their low levels of activity. To detect these enzymes by IEF it was necessary to concentrate preparations to the extent where low level chromosomal cephalosporinases (pI values 7.4, 8.2) which were resistant to clavulanate were sometimes detected. In the case of the highly susceptible F. multivorum MP2324 the only beta-lactamase detected was its cephalosporinase with minimal and basal activity. The lack of a major beta-lactamase, as found in most other strains, probably explains the increased susceptibility of this strain to beta-lactam antibiotics.

The low levels of beta-lactamase production of these organisms suggests that a mechanism other than beta-lactamases is involved in resistance to beta-lactam antibiotics but does not necessarily mean that beta-lactamases have no role in resistance.

The beta-lactamases detected in F. multivorum are possibly related to the beta-lactamases of other organisms. The three strains tested by IEF produced a beta-lactamase with a pI of 7.4. The inhibition profile could be detected unequivocally for strain MP2324 only, but it did seem that all strains produced the same enzyme, an enzyme with predominantly cephalosporinase activity which is inhibited by cloxacillin. Sawai et al. (1968) reported a similar enzyme in B. fragilis 1604E - a constitutively produced chromosomally mediated cephalosporinase which is inhibited by cloxacillin. If these enzymes are identical it would constitute strong evidence for a relationship between these two sphingolipid-producing species.

The beta-lactamase of F. multivorum with a pI of 9.2 needs to be compared to the beta-lactamase of Group IIb strains MP525, MP547, MP548, MP582, MP639, and MP1409. Group IIb is a large, diverse collection of organisms and, although unsuspected from phenotypic characters, the possibility of some strains being related to F. multivorum cannot be dismissed without sufficient evaluation.

h. F. odoratum

The range of G+C values for F. odoratum is 4.7 mol% making this species the most genetically diverse species of the genus. It is even more diverse than the heterogeneous Group IIb (range 3.5 mol%). Holmes, Owen and McMeekin (1984) suggest that the genetic diversity of F. odoratum is sufficient to warrant subdivision into two or three species. However such revision is not possible at present because phenotypic characters which correlate with genetic differences are lacking.

This species was very resistant to antibiotics with only cefoxitin, chloramphenicol (in disc tests only), and imipenem showing moderate activity and no single agent effective against all strains. Susceptibility tests showed environmental strain U59 to be much more susceptible to antibiotics and therefore atypical. This strain, like F. multivorum MP2324, offers potential as a susceptible control strain for research into resistance mechanisms.

Susceptibility testing appeared to yield taxonomically useful information as F. odoratum and F. meningosepticum differed from all other flavobacteria in having high MICs to ceftazidime ($\geqslant$ 64 ug/ml) and differed from each other in trimethoprim MICs - F. meningosepticum being susceptible (MIC $\leqslant$ 4 ug/ml) and F. odoratum being highly resistant (MIC $\geqslant$ 128 ug/ml).

Only low levels of beta-lactamase were detected with strain U59 having no activity detectable by IEF. The other five strains produced beta-lactamases with predominantly cephalosporinase activity. In the 3D+CL testing NCTC 11180 appeared to possess greater penicillinase activity than was apparent in the hydrolysis assays. This result may have been due to low outer membrane permeability for cephalosporins leading to only the penicillinase activity of its beta-lactamase being expressed. Diminished permeability may also explain the negative 3D+CL tests for strains NCTC 11036 and NCTC 11179. R59 was the only strain to be synergistically inhibited by clavulanate, and only in combination with amoxycillin (i.e. augmentin). This suggested that its beta-lactamase may be involved in resistance to penicillins but not to cephems. If so, this is indirect evidence of a selectively permeable outer membrane which excludes cephems but permits entry of penicillins to the periplasm.

Strains NCTC 11036 and NCTC 11179 had a single beta-lactamase band with an isoelectric point of 7.7, and R59 had a minor band with the same pI. This was also the pI of a minor band in each of F. meningosepticum NCTC 10016, F. gleum R49, F. breve U31 and Group IIb strains R34 and MP548. Perhaps with more sensitive methods this beta-lactamase might be found to occur widely throughout Flavobacterium and thereby constitute a genus-specific beta-lactamase. Raimondi et al. (1986) report a beta-lactamase of F. meningosepticum with a pI of 7.65. Although this value is numerically similar to 7.7 the discrepant ranges for pI values of F. meningosepticum in the two studies prevents direct comparison of these beta-

lactamases. However the possibility of a beta-lactamase specific for the genus Flavobacterium should be explored and is further reason to seek more sensitive techniques by which to investigate the beta-lactamases of the genus.

Strain NCTC 11180 produced a beta-lactamase with an isoelectric point of 5.3 and R59 had a minor beta-lactamase band with this pI. In the light of the genetic evidence of a relationship between Bacteroides and Flavobacterium it is interesting that Olsson-Liljequist et al. (1980) report a cephalosporinase of B. uniformis with a pI of 5.3. This enzyme has broad cephalosporinase activity and hydrolyses cefuroxime and cefoxitin. It therefore seems to be similar to many of the Flavobacterium beta-lactamases detected in this study.

B. uniformis was previously regarded as a subspecies of B. fragilis and thus produces sphingolipids (Holdeman et al., 1984) and has more biochemical characteristics in common with the proposed genus Sphingobacterium than with F. odoratum. (B. uniformis is positive for esculin hydrolysis, indole production, and acid production from arabinose,glucose, lactose, maltose, raffinose and sucrose). F. odoratum, on the other hand, is biochemically more similar to B. fragilis and B. vulgatus. However a relationship between F. odoratum and B. fragilis or B. vulgatus is not strongly suggested as these organisms share negative, rather than positive, biochemical tests and both Bacteroides species produce sphingolipids whereas F. odoratum does not.

Further, but tenuous, evidence of a relationship with Bacteroides is the report of Eley and Greenwood (1986) that five strains of B. fragilis have beta-lactamases with a pI of 5.3. However these beta-lactamases appear to be different as they were inhibited by both cloxacillin and clavulanic acid, whereas that of NCTC 11180 is inhibited by only clavulanic acid.

The G+C ranges for the above-mentioned species of Bacteroides are similar to those found in the upper G+C range for Flavobacterium - B. uniformis (45-48 mol%), B. fragilis (41-44 mol%) and B. vulgatus (40-42 mol%) (Holdeman et al., 1984). This is further evidence of a relationship between these species and the proposed genus Sphingobacterium (range 39.6-45 mol%) rather than a relationship with F. odoratum (31.4-36.1 mol%).

F. odoratum R59 produced a beta-lactamase with a pI of 6.7. In a review of anaerobic bacteria Nord (1986) cites 21 reported isolates of B. ovatus and four isolates of B. thetaiotaomicron which also produce beta-lactamases with a pI of 6.7. (B. thetaiotaomicron is another species which produces sphingolipids and has a G+C range (40-43 mol %) similar to the proposed genus Sphingobacterium.) Bello et al. (1987) report inactivation of cefotaxime, cefoperazone and ceftazidime by the beta-lactamases of A. calcoaceticus strain 4 and P. putida strain 5 but insufficient details of these beta-lactamases are provided to compare these beta-lactamases with that of F. odoratum R59.

F. odoratum R25 also produced a beta-lactamase which had a diffuse band at pI 4.9. Strain R59 also produced a minor band at this pI, as did F. gleum NCTC 11432 and Group IIb U58. However the minor bands were sharply focussed in contrast to that of R25. Whether or not the sharply focussed minor bands were the same enzyme as the diffuse band of F. odoratum R25 is unknown.

The beta-lactamase of F. odoratum R25 could also be compared to beta-lactamases of Bacteroides. Eley and Greenwood (1986) report a beta-lactamase of B. fragilis 107 with a pI of 4.9. Like that of F. odoratum R25 this enzyme is poorly inhibited by cloxacillin but strongly inhibited by clavulanic acid. It causes slow hydrolysis of cefoxitin, and moxalactam but does not hydrolyse imipenem. These results are similar to the 3D+CL results of R25. Nord (1986) cites six additional references in which B. fragilis is reported to produce a beta-lactamase with a pI of 4.9, plus two references for B. vulgatus and one reference for B. distasonis.

Lacroix et al. (1984) report a beta-lactamase of B. bivius which is inhibited by clavulanic acid but not by cloxacillin and on IEF produces a 'streak band' with a pI of approximately 4.8 to 5.0. This beta-lactamase slowly inactivates cefoxitin, ceftizoxime and moxalactam and is regarded as belonging to the Group II beta-lactamases of Bacteroides of Timewell et al. (1981). These are broad spectrum beta-lactamases which occur in B. asaccharolyticus, B. melaninogenicus, B. bivius and B.

ovatus. The diffuse 'blobby' band of the beta-lactamase of F. odoratum R25 seems to be very similar to the 'streak band' of the beta-lactamase of B. bivius. This similarity, and the additional species of Bacteroides just mentioned, help to focus attention on the more important organisms to investigate when studying the relationship between Flavobacterium and Bacteroides.

The G+C values of two of these species are in the same range as Flavobacterium species - B. bivius (40 mol %) and B. melaninogenicus (36-40 mol%). The G+C range of B. asaccharolyticus (50-51 mol%) however is slightly above that of Flavobacterium and B. asaccharolyticus is also more susceptible to antibiotics than the other above-mentioned species of Bacteroides.

The significance of isoelectric points of the minor bands of strain R59 being identical to those of the major beta-lactamase bands of the other strains of F. odoratum needs to be determined. This finding indicates a relationship between strikingly different strains of F. odoratum. It may mean that the beta-lactamases of F. odoratum can serve as taxonomic markers of subspecies within the species, or, should the species be recognised as a separate genus, as markers of species within the genus.

The beta-lactamases of F. odoratum differed between strains and differed from the F. odoratum beta-lactamase described by Sato et al. (1985) which was not inhibited by clavulanic acid. In this study the different beta-lactamases

were not expressed phenotypically in routine susceptibility tests but differences between the strains could be detected in the 3D+CL tests in which there were three profiles. Strains NCTC 11036, NCTC 11179 and U59 were negative for all substrates. NCTC 11180 showed apparent penicillinase activity against ampicillin and cloxacillin which, in conjunction with the other parameters, suggested it produces a Richmond and Sykes Class IV beta-lactamase. Strains R25 and R59 possessed broad spectrum activity extending to cefoxitin and cefotaxime and therefore probably produce an oxyiminocephalosporinase of Richmond and Sykes Class IV. This type of enzymatic activity was not however expressed in cefoxitin hydrolysis assays. This suggests that the 3D+CL tests were a more sensitive test of substrate profile than hydrolysis assays.

The next stage in this research should be an attempt to correlate the different beta-lactamases with the genetic heterogeneity of the species. This would necessitate studying a larger number of strains. However if a correlation can be made, the 3D+CL tests for ampicillin, cefotaxime and cloxacillin (and possibly additional substrates) constitute very simple and convenient characterization tests by which to delineate different taxonomic groups within F. odoratum.

i. Group IIb

The search for taxonomic groups within Group IIb has not, in general, been a rewarding task (Price and Pickett, 1981; Shewan and McMeekin, 1983; Yabuuchi et al., 1986). However

Owen and Holmes (1978) and Holmes, Owen et al. (1984) delineated F. balustinum (a species consisting of a single strain) and F. gleum (a species which requires DNA:DNA hybridization analysis to identify it) from within Group IIb. The latter workers suggest that further species descriptions based on strains of Group IIb are imminent.

In this study it was not possible to define discrete subgroups within Group IIb. In disc susceptibility tests Group IIb was heterogeneous, generally resistant, with no single agent effective against all strains. Trimethoprim and cefoxitin were the most effective agents, and moxalactam resistance was greater than expected from the literature.

The level of beta-lactamase activity was generally high and comparable to that of F. meningosepticum. The beta-lactamases of Group IIb, like those of F. meningosepticum, were heterogeneous by both IEF and 3D+CL testing. All Group IIb beta-lactamases were inhibited by clavulanate but not by cloxacillin. In 3D+CL testing all strains inactivated cloxacillin, a finding which in conjunction with the routine beta-lactamase analyses suggested that Group IIb produces Richmond and Sykes class IV beta-lactamases. Two strains, R862 and MP7, exhibited only penicillinase activity. The remaining strains however produced broad spectrum beta-lactamases, with 17 of 19 strains exhibiting activity in 3D+CL tests against cefoxitin, cefotaxime and/or imipenem, and thus appeared to produce oxyiminocephalosporinases. Clavulanate synergy tests were negative for all strains except R34 suggesting that the beta-lactamases of Group IIb are not a

major determinant of resistance to beta-lactam antibiotics.

There were nine different isoelectric points for the major bands of the beta-lactamases of Group IIb. Although some bands were associated with groups of strains, particularly pI values 8.6 and 9.2, with one exception it was not possible to find common phenotypic characters with which to delineate subgroups. The exception was strains MP305 and MP 1846 which produced a beta-lactamase with a pI of 10.2, had similar 3D+CL profiles with both inactivating cefoxitin and imipenem, and had similar antibiograms, differing only in susceptibility to erythromycin.

One Group IIb beta-lactamase was identical in its isoelectric point to other Flavobacterium beta-lactamases in this study. Strains R46, MP7 and MP628 produced a beta-lactamase of pI 8.6. Other organisms producing beta-lactamases with this pI were F. meningosepticum strains NCTC 10585, R58, MP816, MP969, and the asaccharolytic unclassified strain R5. In view of the phenotypic differences among these organisms it is difficult to establish the level of relationship and its implications for the current taxonomic framework. Perhaps, as indicated by Bruun (1982, 1983), Thomson (1982) and Hayward and Sly (1984), Flavobacterium does not comprise taxonomically discrete species, but rather consists of a collection of organisms in which phenotypic patterns merge into each other but certain patterns occur more frequently and correspond to the described taxa. The significance of a beta-lactamase which occurs independently of

species boundaries is consistent with this hypothesis.

j. Taxonomic Implications for the Flavobacterium/Cytophaga Complex

In this study representative strains of Flavobacterium produced beta-lactamases which appeared to belong to Richmond and Sykes Class IV. Many of the beta-lactamases hydrolysed at least one of the supposedly beta-lactamase-stable antibiotics cefoxitin, cefotaxime and imipenem and were therefore regarded as oxyiminocephalosporinases. Although the beta-lactamase preparations of Cytophaga strains were too inactive for isoelectric focussing, the antibiograms and 3D+CL patterns for these strains suggested that C. aquatilis and C. johnsonae also produced oxyiminocephalosporinases and were therefore more closely related to the majority of Flavobacterium strains in their beta-lactamases and antibiograms ~~strains~~ than F. aquatile, the type species of the genus. Further studies should focus more closely on the beta-lactamases of these genera to determine whether or not they share particular chromosomal beta-lactamases. The finding of a common beta-lactamase in strains of Flavobacterium and Cytophaga would suggest a closer relationship than previously suggested and would indicate that the 'ill-defined' boundary between the genera is an artificial one.

Some of the beta-lactamases of F. odoratum and F. multivorum appeared to resemble beta-lactamases reported in Bacteroides species. If these genera share the same beta-lactamases it might mean that radical reconstructive taxonomic

reforms would need to be considered. It is therefore important that the implications of this finding be thoroughly investigated.

Within Flavobacterium the analysis of beta-lactamases appeared to be a taxonomic tool of great sensitivity. There was considerable heterogeneity within the various species and Group IIb. Marre et al. (1982) report that IEF of chromosomal beta-lactamases is specific to the subspecies level in L. pneumophila. Since subspecies have not been defined for Flavobacterium, perhaps beta-lactamase analysis might be helpful in defining subspecies. There is however wider genetic and phenotypic variation within Flavobacterium than is usually associated with the subspecies level and therefore beta-lactamase analysis could also be employed to investigate the wider taxonomic problems of the genus.

One of the problems which has not been satisfactorily addressed is the significance of atypical strains. Bruun (1982, 1983), Thomson (1982) and Hayward and Sly (1984) were unable to assign atypical strains to recognised taxa. The heterogeneity of beta-lactamases in Flavobacterium parallels the biochemical heterogeneity encountered by these workers. Bruun (1982) compared Flavobacterium to "the green fluorescent pseudomonads where very many different biochemical patterns occur gradually merging into each other, but with some occurring more frequently than others; a situation which makes subdivision into stable, recognizable species extremely difficult". The validity of this analogy is strengthened by

the heterogeneity of the beta-lactamases encountered in this study.

The occurrence of beta-lactamases which appear to cross species boundaries could be considered as evidence that the present species descriptions are unsoundly based. This would explain why it is so difficult to accomodate 'atypical' strains in the current taxonomic framework of the genus. Whether or not the species should be redefined, and whether or not there actually are discrete species within Flavobacterium are moot points. However in view of the more major taxonomic issues at the genus level these questions should probably be placed on the taxonomic backburner for the time being. More important questions involve the relationships of the genus to Cytophaga and Bacteroides. Until these matters have been dealt with satisfactorily the taxonomic framework of the redefined genus should be left as it is.

How then does the heterogeneity of the beta-lactamases and other antibiotic tests impact on our broader understanding of the taxonomy of the genus?

Four genetic and phenotypic groups containing species of Flavobacterium have been delineated as prospective genera (Bauwens and De Ley, 1981; Holmes and Owen, 1981; Holmes et al., 1983; Yabuuchi et al., 1983; Holmes, Owen et al., 1984). The first of these, which contains F. aquatile, "F. pectinovorum", "F. tirrenicum" and C. johnsonae, is the least satisfactory arrangement when examined by the antibiotic data of this study. The antibiograms and 3D+CL tests suggested

that F. aquatile, "F. tirrenicum" and C. johnsonae differed substantially from each other and that C. johnsonae was more closely related to the majority of Flavobacterium strains than to the organisms of this group.

The other groups however appear to be taxonomically more coherent. These are:

Group 2: F. odoratum

Group 3: F. balustinum, F. breve, F. gleum, F. meningosepticum and Group IIb

Group 4: F. multivorum, F. spiritivorum, F. thalpophilum.

With one possible exception, the results of this study supported this division of species into groups. The assignment of F. breve to Group 3 would appear less clear cut when considered in the light of its interactions with antibiotics. This species was much more susceptible to antibiotics than the other species of Group 3 and Group II. Susceptibility to cefsulodin in particular differentiated F. breve from the other members of the group. Furthermore the beta-lactamases of F. breve generally appeared to be different from those of F. meningosepticum and the F. gleum/Group IIb complex. By IEF the only similarity was the minor band of strain U31 which had the same pI as the major bands of F. gleum NCTC 11432 and F. gleum-like strain U62, and the minor bands of four strains of F. meningosepticum (R58, MP816, MP969 and MP1702) and the unclassified strain R5.

Overall it appears that antibiotic investigations, and particularly beta-lactamase analysis, have considerable

potential for evaluating taxonomic relationships within the Flavobacterium/Cytophaga complex. The 3D+CL test proved to be a sensitive, convenient and comparatively rapid procedure which can be used as a screen for unusual beta-lactamases and to establish which substrates should be included in conventional hydrolysis assays. Since IEF of beta-lactamases is beyond the capabilities of most routine laboratories it is important that the results obtained by IEF be correlated with more convenient tests such as antibiograms and 3D+CL tests.

The taxonomic utility of antibiograms is a further reason to find ways to overcome the poor correlation between disc and MIC susceptibility procedures for Flavobacterium. The technical problems, which seem to be greater for this genus than for most other clinically occurring genera, may arise from the different ways in which the organism-antibiotic encounter occurs in each method. The differences may perhaps be exacerbated by factors such as unusual resistance mechanisms (e.g. oxyiminocephalosporinases, EDTA-resistant permeability barriers) and suboptimal growth of strains on routine susceptibility media at 37°C. However the clinical need for a reliable susceptibility test far exceeds the taxonomic need since the high level of multiple antibiotic resistance of the genus makes clinicians more dependent than ever on the laboratory for an accurate guide to therapy.

V. CONCLUSIONS

1. Antibiotic susceptibility testing was useful in delineating F. aquatile, F. breve, F. meningosepticum and F. odoratum.

2. Representative strains of Flavobacterium and Cytophaga appeared to produce Richmond and Sykes Class IV beta-lactamases. Many of these beta-lactamases were unusual in their ability to hydrolyse at least one of the purportedly beta-lactamase-stable antibiotics cefoxitin, cefotaxime and imipenem. This indicated that these enzymes could be further classified as oxyiminocephalosporinases.

3. Most species of Flavobacterium were heterogeneous in antibiotic susceptibility patterns and in the types of beta-lactamases they produced. Since Class IV beta-lactamases are chromosomally mediated this suggested that the beta-lactamases might prove to be phenotypic markers which can be correlated with the genetic variability which has been previously reported in some species.

4. The antibiotic susceptibility pattern of F. aquatile, the type species of the genus, was very different from the patterns of most other strains of Flavobacterium. This was further evidence that it is an inappropriate choice for type species.

5. The beta-lactamase of F. meningosepticum NCTC 10016, the type strain of the species, was different from the beta-lactamases of most other strains of F. meningosepticum. This was further evidence that NCTC 10016 is an unrepresentative type strain for the species.

6. Similarities in the susceptibility patterns and beta-lactamases of F. multivorum, F.spiritivorum and F. thalpophilum was further evidence that these species are related to each other and are different from other Flavobacterium species and added support for the proposal that these species be accomodated in a separate genus.

7. Antibiotic susceptibility patterns and beta-lactamase analysis were not useful in delineating subgroups within Group IIb.

8. Clavulanate synergy studies indicated that beta-lactamases may be involved in the resistance to beta-lactam antibiotics of F. meningosepticum, F. multivorum, F. spiritivorum, F. thalpophilum, C. aquatilis and C. johnsonae, but not significantly involved in the resistance of F. aquatile, F. gleum, most F. odoratum and Group IIb strains, "F. tirrenicum" and F. elegans.

9. By combining the 3-dimensional and clover leaf tests it was possible to screen strains rapidly for unusual beta-lactamases. This approach is cheaper, faster, more versatile and more suited to routine microbiology laboratories than more conventional methods for investigating the substrate profiles of beta-lactamases.

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VII ADDENDUM

Since completion of this thesis the author received a reprint of:

FUJII, T., SATO, K., YOKOTA, E., MAEJIMA, T., INOUE, M. and MITSUHASHI, S. (1988) Properties of a broad spectrum beta-lactamase from Flavobacterium meningosepticum GN14059. J. Antibiot. XLI: 81-85.

These workers report production of a broad spectrum beta-lactamase by F. meningosepticum GN14059. The enzyme has an isoelectric point of 5.1, significant hydrolytic activity against various beta-lactam antibiotics including oxyiminocephalosporins and aztreonam, and is inhibited by clavulanic acid, sulbactam, imipenem and cephamycins. Fujii et al. classify the enzyme as a unique CXase type I.

Apart from the more acidic isoelectric point, this beta-lactamase appears to be similar to the beta-lactamases of F. meningosepticum reported by Raimondi et al. (1986) and the enzymes encountered in this study. The lower isoelectric point suggests that the heterogeneity of beta-lactamases within the species is even greater than suggested by the results of this study. It is also interesting that the pI value of 5.1 for the beta-lactamase of strain GN14059 is similar to the pI values obtained in this study for the beta-lactamase of F. odoratum R25 (4.9) and the minor beta-lactamase bands of F. odoratum R59 (4.9, 5.3), F. meningosepticum MP969 (5.0), F. gleum NCTC 11432 (4.9, 5.2) and Group IIb U58 (4.9).

VIII. APPENDICES

APPENDIX 1

Abbreviations - Names of Microorganisms

Genus	Species Abbreviations
Achromobacter	A. xylosoxidans
Acinetobacter	A. calcoaceticus
Aeromonas	A. salmonicida
Alcaligenes	A. denitrificans
	A. faecalis
Bacillus	B. cereus
	B. stearothermophilus
	B. subtilis
Bacteroides	B. asaccharolyticus
	B. bivius
	B. distasonis
	B. fragilis
	B. melaninogenicus
	B. ovatus
	B. thetaiotamicron
	B. uniformis
	B. vulgatus
Cardiobacterium	C. hominis
Chlamydia	C. trachomatis
Citrobacter	C. diversus
	C. freundii
Cytophaga	C. aquatilis
	C. johnsonae

APPENDIX 1 (Continued)

Genus	Species Abbreviations
	C. lytica
	C. marinoflava
	C. salmonicolor
Enterobacter	E. cloacae
Escherichia	E. coli
Flavobacterium	F. aquatile
	F. balustinum
	F. breve
	F. gleum
	F. indologenes
	F. meningosepticum
	F. multivorum
	F. odoratum
	"F. pectinovorum"
	F. spiritivorum
	F. thalpophilum
	"F. tirrenicum"
Flexibacter	F. elegans
Haemophilus	H. influenzae
Klebsiella	K. aerogenes
	K. oxytoca
	K. pneumoniae
Legionella	L. dumoffi
	L. gormanii
	L. longbeachae
	L. micdadei

APPENDIX 1 (Continued)

Genus	Species Abbreviations
	L. pneumophila
Morganella	M. morganii
Neisseria	N. gonorrhoeae
	N. meningitidis
Proteus	P. mirabilis
	P. vulgaris
Providencia	P. rettgeri
	P. stuartii
Pseudomonas	P. aeruginosa
	P. cepacia
	P. maltophilia
	P. pseudomallei
	P. putida
	P. thomasii
Rhodopseudomonas	R. capsulata
	R. sphaeroides
Salmonella	S. typhimurium
Serratia	S. marcescens
"Sphingobacterium"	"S. mizutae"
Staphylococcus	S. aureus
Streptococcus	S. faecalis
	S. faecium
	S. pneumoniae
	S. uberis
Yersinia	Y. enterocolitica

APPENDIX 2

Other Abbreviations

AIDS	acquired immunodeficiency syndrome
ATCC	American Type Culture Collection
CDC	Centres for Disease Control
CSF	cerebrospinal fluid
CXase	oxyiminocephalosporin beta-lactamase
DHP-I	dihydropeptidase-I
EDTA	ethylenediaminetetraacetate
HPLC	high pressure liquid chromatograph
IEF	isoelectric focussing
M	Molar
mA	milliampere
mM	millimolar
mcg (or ug)	microgram
MHA	Mueller-Hinton agar
MHB	Mueuller-Hinton broth
MIC	minimum inhibitory concentration
MW	molecular weight
NCCLS	National Committee for Clinical Laboratory Standards
NCTC	National Collection of Type Cultures
OD	optical density
Omp	outer membrane protein
PBP	penicillin binding protein
pCMB	p-chloromercuribenzoate
pI	isoelectric point
SDS-PAGE	Sodium dodecyl-sulphate polyacrylamide gel electrophoresis

CDS Calibrated Dichotomous Scheme

Other Abbreviations

ug	microgram
ul	microlitre
uM	micromolar