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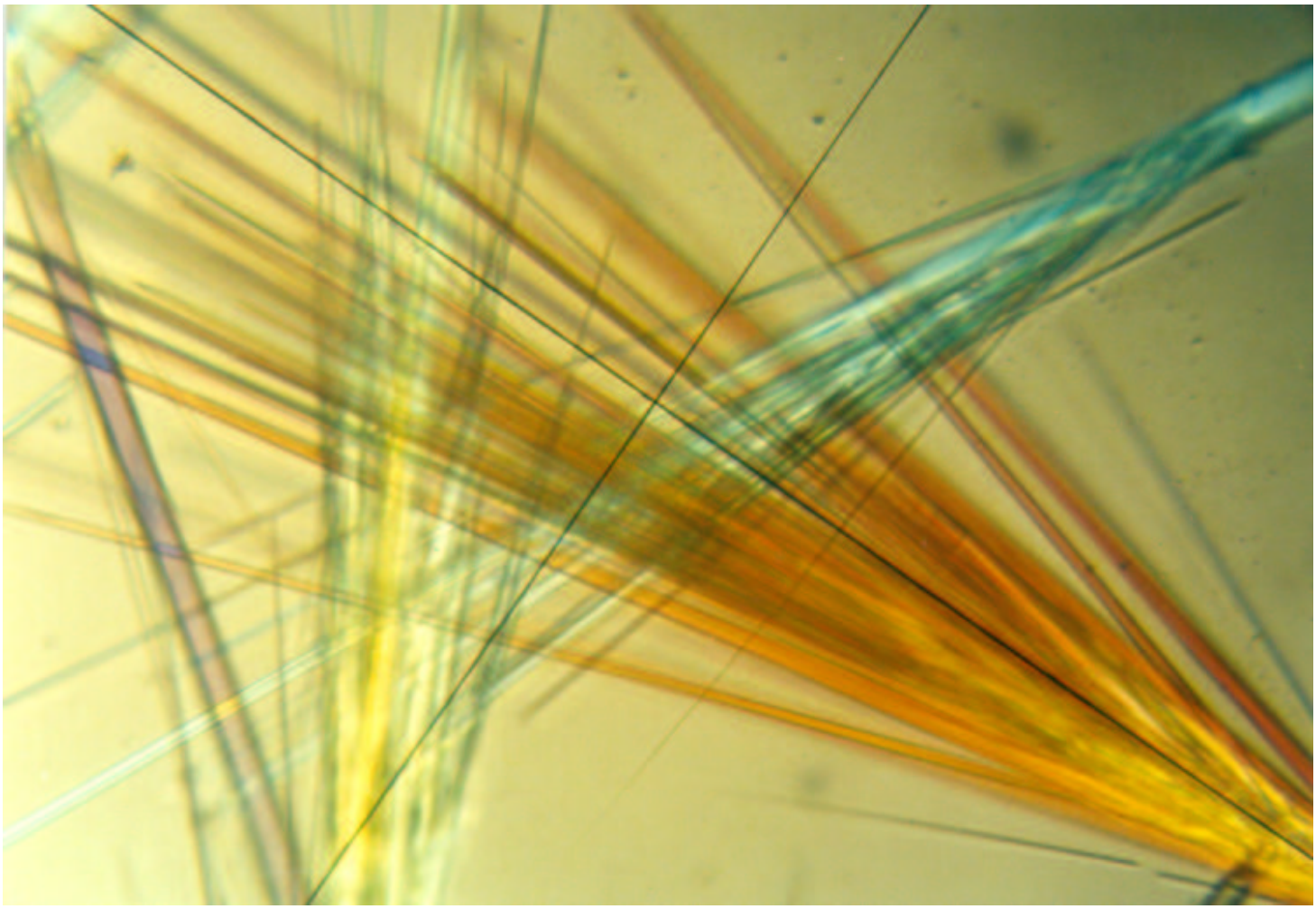
CENTRE FOR PROTEIN ENGINEERING



ACTIVITY REPORT 1995–1999

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Cover illustration:
Crystals of the class A β-lactamase from *Mycobacterium fortuitum*.

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INTRODUCTION

During the period 1995–1999, a large part of the activities of the Centre for Protein Engineering has been devoted to a continuation of the research projects described in the first Activity Report (1990–1994). They mainly concerned the proteins involved in the polymerization of peptidoglycan and the β-lactamases.

Polymerization of peptidoglycan is catalysed by penicillin-binding proteins (PBPs) in conjunction with other proteins [39; Vollmer *et al.*, 1999]. Using lipid II, the undecaprenyl-P-P-disaccharide-pentapeptide as precursor, class A bifunctional PBPs catalyse glycan chain elongation and peptide crosslinking between two adjacent glycan strands. The cell morphogenetic class B PBPs presumably use this new material to assemble peptidoglycan for cell elongation and cell division. In *Escherichia coli*, PBP2 and RodA are required in wall expansion whereas PBP3 is involved in cell septation with other cell division proteins.

Class A PBPs are anchored into the membrane by a pseudo-signal peptide. They combine the glycosyl transferase and the penicillin-sensitive acyl transferase (or DD-transpeptidase) activities in a single polypeptide, which folds at the outer face of the inner membrane. Class B PBPs have a similar modular organization. However, they have no glycosyl transferase activity. They combine a non-catalytic morphogenetic determinant and a penicillin-sensitive acyl transferase activity in a single polypeptide [101].

Throughout the cell cycle, the wall peptidoglycan undergoes constant changes. Monofunctional PBPs hydrolyse D-Ala-D-Ala bonds or interpeptide bonds. These DD-carboxypeptidases thus control the extent of peptide crosslinking and allow the insertion of new materials.

The production of β-lactamases is a defensive mechanism that bacteria have developed to protect their wall peptidoglycan-synthesizing machineries against the toxic effect of penicillin.

Serine β-lactamases, monofunctional PBPs and the penicillin-binding module (PB) of multimodular PBPs fulfill different functions. However, they operate on R_1-CO-X-R_2 carbonyl donors by the same proton abstraction-donation mechanism and their catalytic sites exhibit a highly conserved topology. On the basis of structural data and careful sequence alignments, three conserved

elements have been identified which contribute to the formation of the active site: 1) a Ser*-Xaa-Xaa-Lys tetrad where Ser* is the nucleophilic serine, situated at the N-terminus of a rather long helix and Xaa represents a non-conserved residue; 2) a Ser-Xaa-Asn or Tyr-Xaa-Asn triad on a loop forming one side of the catalytic cavity; 3) a Lys(His)-Thr(Ser)-Gly triad, on a piece of β-strand, forming the other side of the cavity. The serine β-lactamases and the PBPs are thus members of a large family of penicilloyl serine transferases several of which are involved in bacterial resistance to β-lactams [56].

The continuous emergence of new β-lactamases, including an unexpected number of carbapenem-hydrolysing class B metallo (Zn) enzymes has been accompanied by the appearance of low affinity multimodular PBPs, mainly in Enterococci, Staphylococci, Pneumococci and *Neisseria* sp. Moreover, vancomycin resistance has become a major problem in Enterococci and the accumulation of plasmid-borne resistance factors has resulted in the selection of strains resistant to all clinically useful antibiotics. Thus, it is more than ever necessary to understand the enzymatic mechanisms involved in the resistance phenomena to allow the design of more efficient compounds and to initiate a search for new bacterial targets.

The spreading of resistance factors has followed a variety of pathways but the same trends have been observed in the cases of both β-lactamases and low affinity PBPs.

1) The acquisition of new genes, most often plasmid- or transposon-borne. Some strains were thus isolated, which could produce up to 5 different β-lactamases. A similar mechanism has also been responsible for the appearance of vancomycin-resistant Enterococci.
2) The deregulation of the control system, resulting in the production of large amounts of β-lactamase or penicillin-resistant transpeptidase.
3) The selection of point mutations which significantly modify the substrate profile of β-lactamases.
4) The appearance of mosaic genes, encoding low affinity PBPs, and formed by recombinations of the host gene with that from a resistant, related organism.

The study of structure-function relationships in β-lactamases and DD-peptidases and of the mechanisms responsible for the control of the synthesis of these enzymes has in consequence remained one of our major centres of interest. However, in the search for new potential bacterial targets, the glycosyl transferase reaction, which precedes the transpeptidation in the synthesis of bacterial peptidoglycan, and the mechanism of bacterial cell division have also retained our attention.

Our interest for D-stereospecific peptidases has resulted in the isolation and characterization of two novel aminopeptidases.

In parallel, we have developed studies of the factors responsible for the stability of proteins and for the efficiency of the folding process. Other significant activities have been devoted to the induction of xylanase production in *Streptomyces*, to the study of a novel, highly active xylanase produced by a *Streptomyces* strain and to the construction of *Bacillus* and *Streptomyces* secretion vectors.

SCIENTIFIC REPORT

1. Protein-ligand interactions (substrates or inhibitors)

1.1. β-Lactamases

A new penem derivative BRL42715 (Fig. 1) was found to be a very good inactivator of all tested active-site serine β-lactamases (classes A, C and D), but was hydrolysed by the class B metallo enzymes. By contrast, it was a very poor inactivator of the *Streptomyces* R61 and *Actinomadura* R39 DD-peptidases [1].

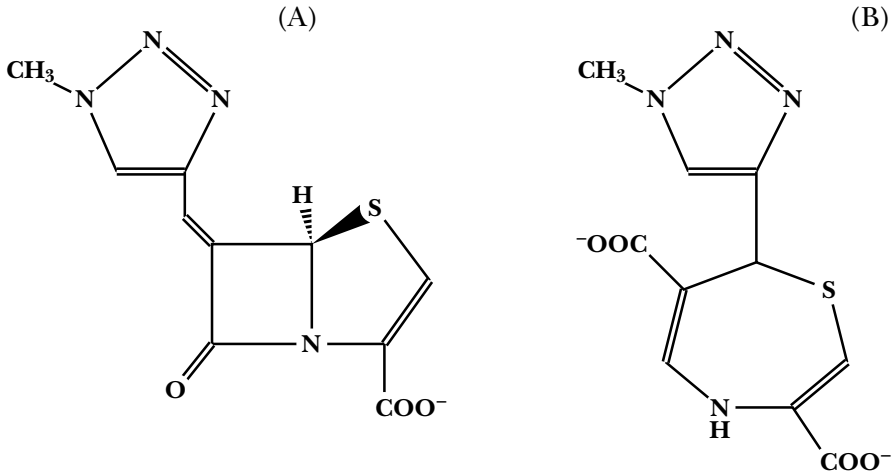


Fig. 1. Structure of BRL42715 [(A) sodium (5*R*)-(Z)-6-(1-methyl-1,2,3-triazol-4-ylmethylene] and of the chromogenic dihydrothiazepine obtained from base- or enzyme-catalyzed hydrolysis of this compound (B).

In contrast to the generally accepted idea, several β-lactamases hydrolyse peptides of general structure R-D-Ala-D-Ala or R-Gly-D-Ala. Although the activity remains very low, the enzymatic rate enhancement factors are not negligible, due to the high intrinsic stability of the peptide bond. Class C β-lactamases exhibit the highest peptidase activity [118].

As shown before [Adam *et al.*, 1991; 19], thiolesters can be substrates of both DD-peptidases and β-lactamases. Surprisingly, some of the enzymes can hydrolyse compounds of general structure R-D-Ala-S-*CHR′-COO^- where the asymmetric carbon *C presents an L configuration, whereas the stereospecificity of all the enzymes is D with the peptide substrates [10].

1.1.1. Class A β-lactamases: catalytic mechanism

1.1.1.1. The TEM β-lactamase

On the basis of the results obtained during the previous five-year period (1990-1994), where the function of many residues was probed by site-directed mutagenesis [2], a possible catalytic mechanism was proposed [Lamotte-Brasseur *et al.*, 1991; 2, 16]. This mechanism, resulting from molecular modelling studies based on the catalytic properties of the mutants and on the refined structures obtained for various class A enzymes [31], highlights the pivotal role of a conserved water molecule. This water molecule, which participates in an intricate hydrogen-bonding network within the active site, would serve as a relay in the transfer of the proton between the Ser70 hydroxy group and the Glu166 carboxylate, the latter acting as the general base.

A second hypothesis [Strynadka *et al.*, 1992] assumes a non-symmetrical mechanism, with two different general bases, Lys73 and Glu166, involved in acylation and deacylation, respectively. Additional evidence, however, has been gained in favour of the first mechanism.

a) The E166N mutant

The second hypothesis mainly rests on the structural properties of the E166N mutant of the TEM-1 β-lactamase [Strynadka *et al.*, 1992], which forms long-lived acyl-enzymes [Adachi *et al.*, 1991]. This observation led to the argument that Glu166 is expendable for acylation. A careful re-examination of the catalytic properties of the E166N mutant of the TEM-1 β-lactamase [53] unambiguously indicated, however, that if the mutation truly causes conversion of the penicillin-hydrolysing TEM-1 β-lactamase into a penicillin-binding protein (due to an extremely large decrease of the k_3 value, i.e., $\sim 10^9$ fold for benzylpenicillin), the k_2/K' value, which characterizes the efficiency of acylation, was also significantly impaired ($\sim 10^3$ fold for benzylpenicillin) at least for the good substrates of the wild-type enzyme. These kinetic data indicate that both acylation and deacylation rates appear to be decreased by the mutation, and are thus consistent with the mechanism proposed by Lamotte-Brasseur *et al.* (1991), where both catalytic steps involve Glu166 acting as a general base *via* a conserved water molecule.

b) The pK_a of Lys73

The pH-dependence of the chemical modification of ε-amino groups and the NMR titration of the ^{13}C-labelled lysine residues in the TEM-1 β-lactamase [24] clearly indicated that the pK_a value ($\geqslant 10$) of the Lys73 side chain is not significantly decreased in the wild-type enzyme, making it a very unlikely candidate for proton abstraction in catalysis.

These experimental findings have been further strengthened by a calculation of the pK_a values of key residues in the active site of four β-lactamases of known structures [69]. Thus, despite the apparent different electrostatic environments in the various enzymes, the pK_a values of both Lys73 and Lys234 were found to range between 11.1 and 11.7. Interestingly, when the carboxylate side chain of residue 166 (which is very close to the ε-amino group of Lys73) is substituted by a neutral side chain, the computed pK_a values show only a marginal decrease relatively to the wild-type enzymes, suggesting that these mutations are not sufficient to allow the deprotonation of Lys73 at neutral pH [69].

It has been argued that a dramatic reduction of the pK_a value of Lys73 occurs when the substrate binds to the active site [Swarén *et al.*, 1995; Zawadzke *et al.*, 1996]. Continuum electrostatic calculations performed in the presence of different types of β-lactam antibiotics indicate, however, that both Lys73 and Lys234 display pK_a values yielding protonated ammonium side chains at neutral pH [120].

Thus it seems that the present evidence substantiates the view that Glu166 is the genuine general base catalyst in both formation and hydrolysis of the acyl-enzyme intermediate formed with class A β-lactamases, at least with the best substrates. Detailed discussions of the many available data can be found in references [83, 118].

1.1.1.2. The *Bacillus licheniformis* BS3 β-lactamase

The BS3 β-lactamase isolated from a thermophilic strain of *Bacillus licheniformis* BS3 differs from the *Bacillus licheniformis* 749C β-lactamase by only seven mutations [35]. The protein was overproduced in *E. coli* and purified [63]. It has been crystallized under two monoclinic forms, the first one containing a tetramer in the asymmetric unit and diffracting to 2.5 Å resolution and the second one containing a dimer in the asymmetric unit and diffracting to 1.65 Å resolution. X-ray diffraction data were collected at high resolution on the DW32 and D41A beamlines at the *Laboratoire pour l'Utilisation du Rayonnement Électromagnétique* (LURE-DCI, Orsay, France). The structure has been solved by molecular replacement (AMORE software) using the *B. licheniformis* 749C β-lactamase as starting model.

The final BS3 structure has been refined with a conventional R factor of 19.7 % and R_{free} factor of 23.5 % (XPLOR 3.851 software). The dimer contains 4020 protein atoms and 152 water molecules.

Crystalline complexes have been prepared with cefoxitin, the monobactam SQ26324 and the suicide substrates sulbactam and β-iodopenicillanic acid

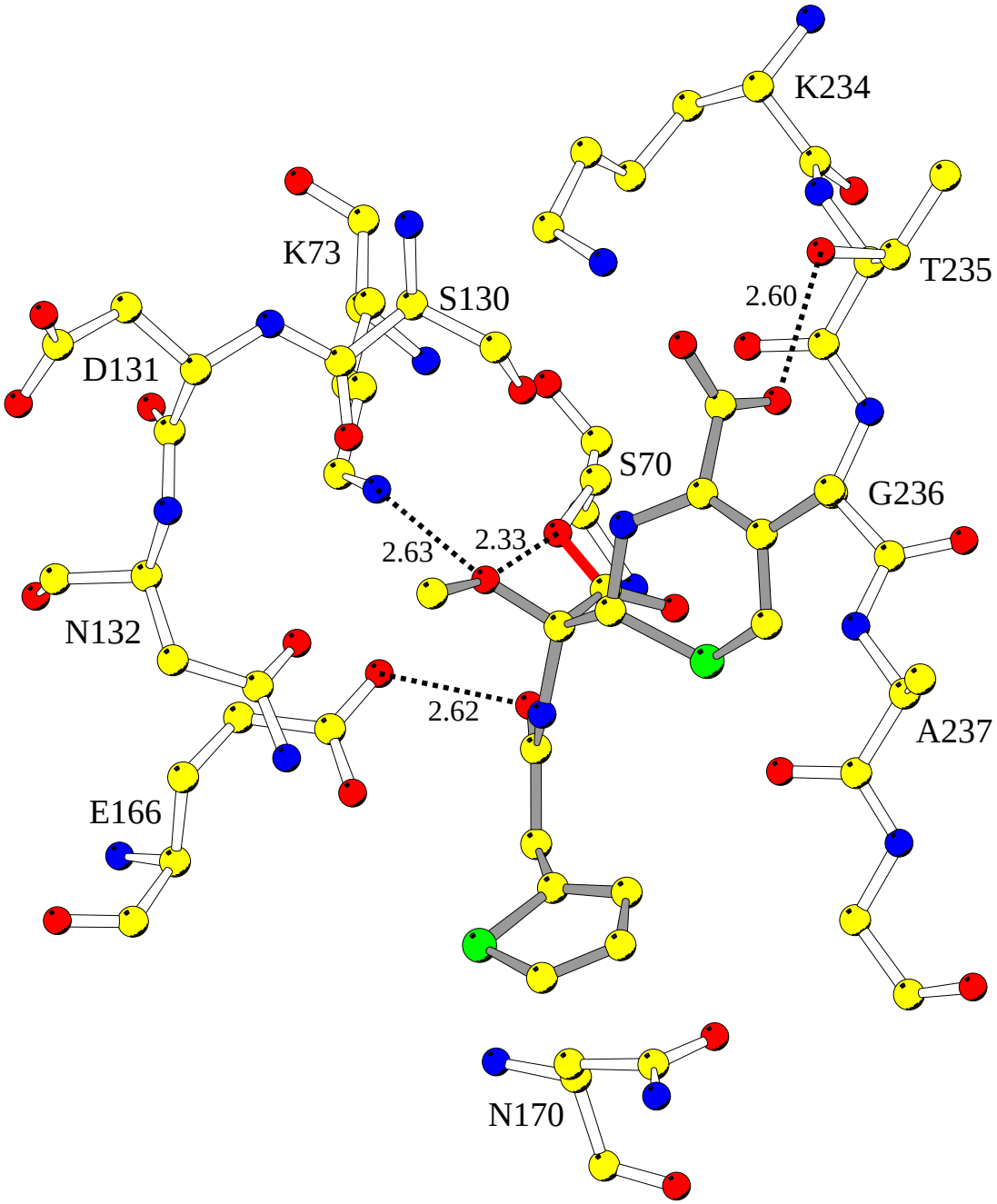


Fig. 2. Structure of the acyl-enzyme intermediate in β-lactam hydrolysis: the active site serine residue of the class A β-lactamase from *Bacillus licheniformis* BS3 is acylated by cefoxitin (represented in balls and sticks and colored by atom type). The figure has been generated by the *Molscript* program [Kraulis, P.J. (1991). *J. Applied Cryst.*, **24**, 946–950].

(BIP) and the data were collected under the same conditions as for the free enzyme. The complex with the cefoxitin shows a clear additional density in the active site, assigned to a cefoxitin molecule whose β-lactam ring has been opened. This result allows the observation of the acyl-enzyme. The structure of the acyl-enzyme with cefoxitin has been refined to 1.7 Å ($R = 21.6\,\%$, $R_{\rm free} = 25.6\,\%$), and compared to that of the free enzyme. Figure 2 shows the active-site serine 70 acylated by cefoxitin and the hydrogen bonding network stabilizing the cefoxitin molecule. The complex with BIP also shows an additional density in the active site. The active serine is acylated and the additional density can be assigned to a rearrranged BIP molecule, as described by Cohen & Pratt (1980) and Frère *et al.* (1982). The structure is in the course of refinement.

The characteristics of this enzyme and of its crystal form do meet the necessary conditions for using the LADI diffractometer and should allow the first neutron diffraction study of a class A β-lactamase. These characteristics are the large amount of protein available, the possibility to grow large prismatic crystals up to 1.0 mm^3, unit cell parameters giving a volume of roughly 300,000 $Å^3$ and finally the availibility of 3D structures determined by X-ray crystallography for the native enzyme and complexes at a resolution of 1.7 Å.

Crystals were equilibrated against a D_2O mother liquor before exposure (in order to help reduce incoherent background) by vapour diffusion and by direct soaking and preliminary experiments have shown that the crystals are diffracting beyond 3.0 Å resolution. Further studies are expected to provide more details on the protonation state of key residues in the active site, and help identify the general base in the class A β-lactamases catalytic mechanism in a definitive manner.

1.1.1.3. The *Mycobacterium fortuitum* β-lactamase

The class A β-lactamase of *Mycobacterium fortuitum* (MFO) is a so-called broad-spectrum β-lactamase as it hydrolyzes both cephalosporins and penicillins with similar efficiencies. Benzylpenicillin, ampicillin, cefuroxime, cephaloridine, carbenicillin and cefotaxime are rather good substrates although the catalytic efficiency of MFO for benzylpenicillin hydrolysis ($k_{\rm cat}/K_{\rm M} = 1000\,{\rm mM}^{-1}\,{\rm s}^{-1}$) is lower than that of the best class A β-lactamases. Cefoxitin, imipenem and oxacillin act as poor transient activators and ceftazidime is not recognized by the enzyme.

Using a strain of *Mycobacterium fallax* naturally devoid of β-lactamase, it was shown that production of the *M. fortuitum* β-lactamase significantly increased the MIC of *M. fallax* for good substrates of the enzyme. These results emphasize the importance of enzymatic degradation as a major factor in the resistance of Mycobacteria to penicillin [61].

The *M. fortuitum* β-lactamase has been crystallized under two forms (see cover figure). Phases were calculated by molecular replacement, using the structure of the class A β-lactamase from *Streptomyces albus* G as probe model. Data collected on the orthorhombic form at the LURE synchrotron allowed the refinement of the structure to 2.4 Å resolution, with a conventional R factor of 16.1 % and an R_{free} factor of 27.7 %. The final structure contains 1969 protein atoms and 142 water molecules. The atomic coordinates of the refined structure have been deposited in the Protein Data Bank, Brookhaven, USA, with the entry code **1mfo**. The structure of the MFO β-lactamase is similar to those of other class A β-lactamases (Fig. 3).

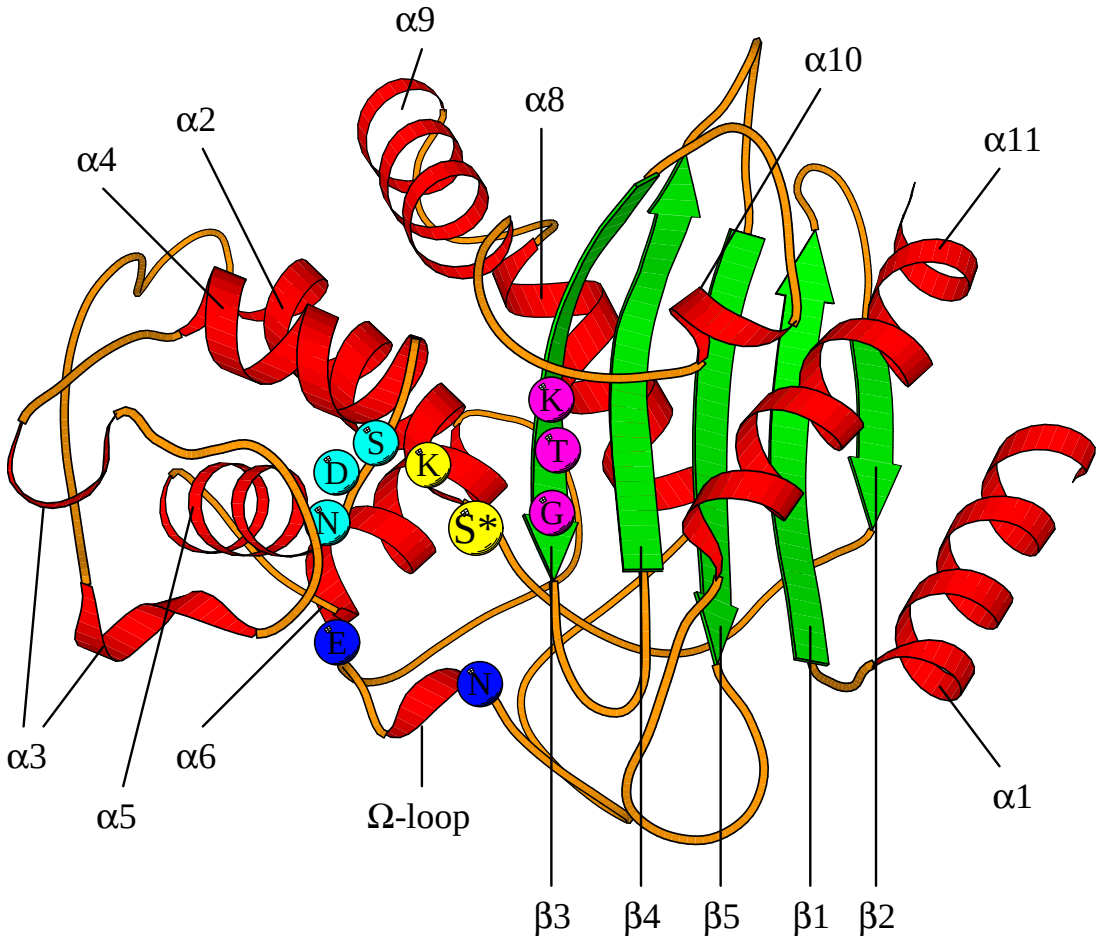


Fig. 3. Ribbon representation of the three-dimensional structure of the class A β-lactamase from *Escherichia coli* TEM1 (PDB code 1XPB). The α-helices are drawn in red and the β-strands as green arrows. The amino-acid residues defining the active site of the enzyme are represented as colored dots. The figure has been generated by the *Molscript* program [Kraulis, P.J. (1991). *J. Applied Cryst.*, **24**, 946–950].

The class A β-lactamase of *M. fortuitum* shares 42 % of identical residues and two residues near the active site, Ser237 and Arg276, which can play a key role in the substrate specificity, with the extended spectrum β-lactamase (ESBL) Toho-1. These characteristics are also found in the MEN-1 ESBL or the broad spectrum class A β-lactamase from *Yersinia enterocolitica* and differentiate them from the other class A β-lactamases. The Ser237 and Arg276 residues can influence the substrate specificity since their side chains interact with the carboxylate of penicillins and cephalosporins.

The MFO-benzylpenicillin acyl-enzyme was constructed by modelling. The carboxylate of the substrate can interact with three residues (Thr235, Ser237 and Arg276) whereas only two residues (Ser235 and Arg244) are available for similar interactions in the case of TEM-1. This results in a different orientation of the carboxylate and might explain the positive influence of a hydroxylated residue in position 237 in hydrolyzing cephems rather than penams. Transition state models of the acylation step have been generated at the quantum level. The insertion of these models into the active site suggests that this hydroxyl group could hinder the movement of one of the methyl group of the penicillin thiazolidine ring during the course of the acylation process. This can explain the decrease of penicillinase activity. However, these molecular mechanics models are only indicative of the binding mode of the substrate carboxylate in the active site of MFO.

1.1.1.4. Carbapenem-hydrolysing β-lactamases belonging to class A

Imipenem and related carbapenem antibiotics (e.g., meropenem) display a broad antibacterial activity, combined with a high resistance to most β-lactamases. Due to these remarkable properties, these compounds are very efficient antibiotics, often used as last resort drugs for patients in intensive care units. However, β-lactamase-mediated resistance to carbapenems has recently been reported. These carbapenem-hydrolysing enzymes (carbapenemases) belong to two major groups: the first group corresponds to class B metallo-β-lactamases (see below), which require a Zn^{++} ion for their activity, whereas the second one presently consists of three class A β-lactamases from *Enterobacter cloacae* (NmcA and IMI-1) and *Serratia marcescens* (Sme-1), sharing about 70 % sequence identity [Rasmussen and Bush, 1997].

The three-dimensional models of NmcA and Sme-1 were constructed by homology modelling [51]. These models suggested the presence of a disulphide bridge between Cys69 and Cys238, which creates a new covalent bond between the two domains and thus significantly modifies the active site geometry. This distinct feature was later confirmed by the X-ray structure of the NmcA β-lactamase obtained at 0.164 nm resolution [89]. This unique property

has been proposed to be responsible, at least partially, for the high catalytic efficiency of these enzymes against imipenem [51].

1.1.1.5. Other class A enzymes

The stability of various natural mutants of the TEM β-lactamase was studied in detail [11].

The *Mycobacterium smegmatis* β-lactamase was purified to protein homogeneity [58].

The *Streptomyces clavuligerus* class A β-lactamase was purified. It exhibits a low enzymatic activity. This is probably due to the fact that the usual Ser-Asp-Asn sequence of the second conserved element is replaced by Ser-Asn-Gly. Interestingly, the best substrate of the enzyme is cefoxitin, which is usually very poorly hydrolysed by class A β-lactamases [71].

A new chromosome-encoded, extended-spectrum β-lactamase was purified from *Chryseobacterium meningosepticum* [130].

1.1.2. Class C β-lactamases

The *Enterobacter cloacae* class C β-lactamase is inhibited by phosphonamidate derivatives of glycine, D- or L-phenylalanine, β-alanine and β-phenyl-β-alanine. The second-order inactivation rate constants range between 50 and 250 $M^{-1}\,s^{-1}$ [22].

1.1.3. Class D β-lactamases

1.1.3.1. β-lactamase from *Salmonella typhimurium* OXA2

A first crystal form has been obtained in ammonium sulfate (orthorhombic unit cell, with $a = 78.6$ Å, $b = 113.2$ Å and $c = 133.7$ Å). Diffraction tests have been performed on the X11 beamline of the DESY synchrotron at EMBL-Hamburg. The resolution limit was 2.8 Å and a partial data set has been collected. These crystals are unstable in the high-flux synchrotron X-ray beam. The use of cryogenic temperature will be necessary for full data collection.

A second, slightly different crystal form has been obtained in sodium formate (orthorhombic unit cell C222, with $a = 78$ Å, $b = 106$ Å, $c = 128$ Å). New data sets have been collected, with a better completeness and a higher resolution at the ESRF in Grenoble (Dr. C. Berthet) using a marresearch IP as detector, a rotating anode as X-ray source and under cryogenic conditions, and at LURE, beamline D41A (July 99), using a marresearch IP as detector and under cryogenic conditions.

A 83 % complete native data set to a resolution of 2.24 Å (with 78 % $> 2\sigma$), and two heavy atom derivatives data sets (Ir, Hg and Pt salts) have been collected.

1.1.3.2. β-lactamase from *Pseudomonas aeruginosa* PSE2

Crystals have been obtained in several conditions with ammonium sulfate as precipitating agent. They belong to the orthorhombic space group $P2_12_12_1$, with the unit cell parameters $a = 48$ Å, $b = 96$ Å, $c = 125$ Å.

Diffraction tests and data collection have been performed in cryogenic conditions on the D41A beamline of LURE. A 99 % complete native data set to a resolution of 2.4 Å (and 87 % for the resolution shell 2.4–2.24 Å) has been collected.

For the phase calculation, we intend to combine information obtained by several methods. The first one is the classical MIR-MIRAS method (Multiple Isomorphous Replacement with Anomalous Scattering). In contrast to OXA2 which does not contain any cysteine, PSE2 contains 3 cysteine residues. Different mercuric derivatives and a set of different heavy atom derivatives have been prepared and several X-ray data sets collected. Another possibility for phasing is based on the MAD method (Multiwavelength anomalous dispersion) with the production of a selenomethionine-labelled protein. PSE2 contains 5 methionine residues. The conditions for the production of the selenomethionine-labelled protein have been optimized and the protein produced and crystallized in the same conditions as the native enzyme. These crystals will be used for MAD data collections on the BM30, BM14 beamlines at the ESRF in Grenoble.

1.1.4. Class B β-lactamases

Metallo-β-lactamases were identified 40 years ago in a *Bacillus cereus* strain. They are now known to be expressed by more than 20 different strains including *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Chryseobacterium meningosepticum*, *Chryseobacterium indologenes*, *Fluoribacter gormanii*, *Bacteroides fragilis*, *Aeromonas hydrophila*. The majority of these bacteria are human pathogens. The genes coding for the metallo-β-lactamases are generally located on the chromosome. Recently, plasmid-mediated class B β-lactamases have been found in clinical isolates from Japan and Italy. These findings underline the possibility of horizontal transfer of β-lactam resistance between nosocomial strains [118].

The primary structures of 20 metallo-β-lactamases are now available. Members of the metallo-β-lactamase family exhibit a low degree of sequence identity (less than 43 %). These enzymes can be divided in three groups namely Ba [*B. cereus* (BcII), *B. fragilis* (CfiA), IMP-1, VIM-1, *C. menigosepticum* (BlaB), *C. indologenes* (IND1)], Bb [*A. hydrophila* (CphA) and *A. veroneii* (ImiS)] and Bc [*S. maltophilia* (BlaS and L1), *C. indologenes* (GOB1) and *F. gormanii* (FEZ-1)].

In the last years, the three-dimensional structures of the *B. cereus* metallo-β-lactamases [17, 81, 111; Fabiane *et al.*, 1998], *B. fragilis* [67, 81; Concha *et al.*, 1996] and *S. maltophilia* [Ullah *et al.*, 1998] have been solved at high resolution by X-ray crystallography.

This allowed a direct comparison of the three-dimensional structures of the class Ba and Bc metallo-β-lactamases. Despite their low degree of sequence similarity, the overall folds of these enzymes are similar. They all have a β/β sandwich structure comprising two β sheets in the core and five α-helices on the external faces. The amino-terminal and carboxy-terminal parts of the molecule, each containing a β-sheet and two α-helices can be roughly superimposed by a two fold rotation around a central axis, suggesting that they may have arisen from a gene duplication event. The structure of the *B. fragilis* enzyme is almost identical to that of *B. cereus* except for some differences in the loops.

The metallo-β-lactamases contain two zinc binding sites. The *B. cereus* and *B. fragilis* enzymes function in their mono-Zn(II) form [102, 127]. The presence of the second zinc ion slightly increases the activity of the β-lactamase. By contrast, the CphA enzyme exhibits a maximum activity in its mono-Zn(II) form and the presence of a second Zn(II) ion inhibits the metallo-β-lactamase [68].

The dinuclear zinc centre of each protein is located at the bottom of a wide shallow groove between the two β-sheets. The active sites are easily accessible and can accommodate a variety of β-lactam molecules which provides the structural basis for their broad substrate specificity. The coordinations of the dinuclear centres of the *B. cereus* and *B. fragilis* metallo-β-lactamases are similar. They contain a tetrahedrally coordinated (Zn1) and a trigonal bipyramidally coordinated (Zn2) zinc ions bridged by a water molecule/hydroxide ion. Zn1 is coordinated by a His triad and the bridging water molecule and has a fairly similar orientation in the two structures. Zn2 is ligated by the bridging water/hydroxide ion and conserved His and Cys residues in equatorial positions and a conserved Asp. The Zn-Zn distance slightly differs in the BcII (3.7–4.4 Å) and CfiA (3.5 Å) structures.

1.1.4.1. Zn β-lactamase of *Bacillus cereus* 569/H

The affinity for the two zinc ions was studied. The first site (Zn1) is formed by His residues 86, 88 and 149. His210, Cys168 and Asp90 are interacting with the second zinc (Zn2). Earlier binding studies at low pH (< 6) revealed rather different dissociation constant values for the first and second Zn ions which were $< 1\,\mu M$ (K_1) and in the mM range (K_2), respectively. At pH 7.5, our results showed that the *B. cereus* enzyme binds a second equivalent of zinc with

a dissociation constant below 10 μM (K_2). Equilibrium dialysis in citrate buffer at pH 5.6 provided a K_1 of 10 μM and no evidence of a second zinc ion [127].

The enzyme showed a two-fold increase of the kcat value for both nitrocefin and imipenem upon binding of a second zinc ion. In the mononuclear enzyme, the metal is delocalised between the two sites. PAC experiments [137] revealed the presence of two populations in the mononuclear enzyme, in which the metal is present either in the Cys or in the three-His sites. Moreover, the analysis of EXAFS spectra revealed a contribution of a zinc-coordinated sulphur for the mononuclear enzyme. The structure of this metallo-enzyme has been analysed by NMR [135]. Four residues (His86, 88, 149 and 210) are involved in the two zinc binding sites. Histidine 86 has been replaced by a serine or an alanine residue. The modified enzymes were still able to bind two zinc ions but the k_{cat}/K_M values for benzylpenicillin, nitrocefin, cephaloridine and imipenem were modified. The activity of the different mutants towards cefotaxime was similar to that of the wild type with a two-fold decrease of the k_{cat}/K_M values ($k_{cat}/K_M = 6000$ and 13000 $M^{-1}\,s^{-1}$ for the H86S and wild type β-lactamases respectively). The H88S mutant is unstable and remains to be studied. Substitutions of His149 or His210 by serine residues yield poorly active enzymes. The activity of the mutants against all the tested compounds was modified. For example, k_{cat}/K_M value for imipenem was 60 fold lower for the H149S β-lactamase ($k_{cat}/K_M = 4$ $mM^{-1}\,s^{-1}$) than for the wild type ($k_{cat}/K_M = 240$ $mM^{-1}\,s^{-1}$). In addition, the two mutants were unable to bind more than one zinc ion even in presence of 100 μM Zn(II). In consequence, the catalytic properties of H149S and H210S were not modified by increasing zinc ion concentrations.

The conserved Cys168 residue plays an essential role in the monozinc form of the enzyme. Substitution of Cys168 by a serine or an alanine also modifies the kinetic behaviour of the enzyme. The modified protein can bind two zinc ions with affinities similar to those of the wild type. The kinetic analysis of the C168A mutant showed that the catalytic efficiency of the mononuclear form is strongly reduced but not that of the binuclear form. The monozinc form was totally inactive against benzylpenicillin. The k_{cat} value for the hydrolysis of this β-lactam was lower than $10^{-3}\,s^{-1}$. Addition of zinc ions drastically modified the activity of the mutant. In the presence of 100 μM zinc, the k_{cat} value of C168A was equal to 140 s^{-1}. With the wild-type enzyme, the k_{cat} value was only increased two-fold [127].

Replacement of Asp90 by Asn leads to a completely inactive enzyme which can bind two zinc ions. These results may indicate that Asp90 is mainly involved in the activation of the water molecule.

Cadmium species of the different enzymes were studied by NMR. Studies of the ^{15}N labelled enzyme allowed the assignment of one of the signals to His236 [135].

1.1.4.2. X-ray structure of the β-lactamase from *Bacillus cereus* II 5/B/6 at two different pH values

The β-lactamase from *Bacillus cereus* II 5/B/6 differs from that of *Bacillus cereus* 569/H by 17 mutations.

The protein has been crystallized at pH 5.0 and 8.0, as tetragonal prisms, with one molecule in the asymmetric unit. Data have been collected at 1.9 Å resolution at the LURE synchrotron, on both crystal types. Both structures have been solved by molecular replacement, using the *Bacillus cereus* 569/H structure as probe model.

The structure at pH 8.0 has been refined to 1.9 Å resolution with a conventional R factor of 23.4 %. It contains 1661 protein atoms and 108 water molecules. In contrast with the structure of *Bacillus cereus* 569/H, only one zinc atom is found in the active site. The side chain of residue His210, which is one of the ligand for the second zinc ion in the *Bacillus cereus* 569/H structure, exhibits a quite different orientation in the 5/B/6 structure, unsuitable for a possible interaction with a second zinc ion.

The structure at pH 5.0 has been refined to a resolution of 2.0 Å with a conventional R factor of 22.49 %. In those conditions, the protein is devoid of zinc ion since the 88–96 loop which bears the His88 and Asp90, Zn1 and Zn2 ligands, respectively, has moved and the active-site geometry is completely different.

1.1.4.3. Zn β-lactamase of *Bacteroides fragilis* CfiA [102]

Dialysis of the *B. fragilis* enzyme against cacodylate buffer pH 6.5 led to zinc release. The kinetic results obtained for a partially Zn depleted species can be explained by a release of one zinc ion yielding the mononuclear species at pH 6.

The two metal sites exhibit different kinetics of metal exchange. The dialysis of the *B. fragilis* enzyme against Cd-containing buffer yielded a hydrid enzyme in which one equivalent of Zn(II) is replaced by Cd(II). The replacement of the second equivalent is much slower. PAC experiments demonstrated the selective occupancy of the three His and Cys sites by Zn(II) and Cd(II) respectively in the Zn, Cd species. The binuclear enzyme was 25 % more active than the mononuclear enzyme with nitrocefin as substrate whereas with benzylpenicillin and imipenem maximal activity persists for the mononuclear enzyme. Furthermore, the mononuclear enzyme was about three fold more

active than the binuclear enzyme with cefoxitin. Strikingly, the catalytic efficiency of the binuclear centre is improved with cefoxitin if one of the zinc ion is replaced by Cd.

1.1.4.4. Zn β-lactamase of *Aeromonas hydrophila* AE036 (CphA)

The dissociation constant of the first binding site for Zn(II) is 6–7 pM at pH 6.5–7.5. The monozinc enzyme exhibits a maximum activity against imipenem in the same pH range. The binding of a second zinc ion in the lower affinity site results in a decrease of activity with a K_i value of 46 μM at pH 7.5. EXAFS spectroscopy has shown that in the high affinity site the zinc is coordinated by two histidines, an O/N donor ligand and a cysteine at 2.3 Å both at pH 6.5 and 7.5. The CphA enzyme also binds other metal ions such as Cd, Co and Cu at neutral pH. Cd and Co are weakly bound at the first binding site with dissociation constants of 60 pM and 220 nM at pH 6.5 respectively. Cu(II) ions are also bound at both sites with similar K_d values. The UV-Vis spectra of the mono Co- and mono Cu- enzymes exhibit the S-to-Co and S-to-Cu LMCT bands respectively. The intensities of the d-d transitions of the Co-enzyme suggest that the high affinity site is most likely four coordinated. The electronic spectra of the Co_2 and Cu_2 enzymes were significantly different from those of the mononuclear form. After modification of the cysteine by iodoacetamide, the enzyme was totally inactive but could still bind two Zn equivalent per mole of enzyme with Kd values for Zn(II) increased to the μM range [147].

1.1.4.5. Zn β-lactamase of *Pseudomonas aeruginosa* IMP-1

The genetic environment of the bla_{IMP} gene, which encodes the IMP-1 metallo-β-lactamase, was investigated in a Japanese *P. aeruginosa* clinical isolate (101/1477) that produced the enzyme [122, 123]. In this isolate, bla_{IMP} was carried on a 36 kb plasmid similarly to the identical alleles found in *S. marcescens* and *K. pneumoniae* clinical isolates. The gene was located on a mobile gene cassette inserted into an integron. The entire structure of this integron, named In31, was determined. In31 is a class I element belonging to the same group of defective transposon derivatives that originated from Tn21-like ancestors such as In0, In2, and In5. The general structure of In31 appeared to be most closely related to that of In5 from pSCH884, suggesting a recent common phylogeny for these two elements. In In31, the bla_{IMP} cassette is the first of an array of five gene cassettes that also includes an *aacA4* cassette and three original cassettes that have never been described in other integrons. The novel cassettes carry, respectively, (i) a new chloramphenicol acetyltransferase-encoding allele of *catB* family, (ii) a *qac* allele encoding a new member of the

small multidrug resistance family of proteins, and (iii) an open reading frame encoding a protein of unknown function. All the resistance genes carried on cassettes inserted in In31 were found to be functional in decreasing the *in vitro* susceptibilities of host strains to the corresponding antimicrobial agents.

The IMP-1 β-lactamase was overproduced in *Escherichia coli* and purified to homogeneity with a final yield of 35 mg/litre of culture [123]. The structural and functional properties of the enzyme purified from *E. coli* were identical to those of the enzyme produced by *P. aeruginosa*. The IMP-1 metallo-β-lactamase exhibits a broad spectrum activity profile that includes activity against penicillins, cephalosporins, cephamycins, oxacephamycins and carbapenems. Only monobactams escape its action. The enzyme was inhibited by metal chelators, of which 1,10-o-phenanthroline and dipicolinic acid were the most efficient. Two zinc binding sites were found. The zinc content of the *P. aeruginosa* 101/1477 metallo-β-lactamase was not pH dependent.

1.1.4.6. Interaction with suicide substrates of active-site serine β-lactamases

The metallo β-lactamases hydrolysed clavulanic acid, sulbactam, tazobactam and β-iodopenicillanic acid with various efficiencies. α-Iodopenicillanic acid was not a better substrate than the β-isomer [116].

1.2. Penicillin-binding proteins (PBPs)

1.2.1. The monofunctional PBPs/DD-peptidases

A theoretical approach to the reversible inhibition of enzymatic systems involving covalent intermediates was developed [54].

1.2.1.1. The PBP/DD-peptidase of *Streptomyces* K15

The *Streptomyces* K15 PBP/DD-peptidase catalyses hydrolysis of peptide, thiolester or ester bonds of carbonyl donors and their aminolysis in the presence of suitable amino acceptors. The protein shows a higher affinity for acceptors than the PBP/DD-peptidases of *Streptomyces* R61 or *Actinomadura* R39. In consequence, it behaves as a strict DD-transpeptidase under certain conditions. Kinetic data suggest that the acceptor behaves as a simple alternate nucleophile at the level of the acyl-enzyme in the case of thiolester substrates, but binds to the enzyme-carbonyl donor Michaelis complex and thus influences the rate of enzyme acylation in the case of peptide donors [5].

The enzyme structure has been determined at a resolution of 2.0 Å and refined to an R factor of 18.6 % ($R_{\mathrm{free}} = 24.3\,\%$) and contains 1927 protein atoms and 149 solvent molecules [128]. The atomic coordinates have been deposited in the Protein Data Bank under the code **1skf**. The fold adopted by

the 262-amino acid polypeptide generates a two-domain structure that is close to those of class A β-lactamases. However, the K15 enzyme has two particular features. It lacks the amino-terminal α-helix found in the other penicilloyl serine transferases, and exhibits an additional, superficial four-stranded β-sheet. The structure analysis with the WinnMGM software underlines an unusual hydrophilic/hydrophobic surface distribution. In the *S.* K15 enzyme, a hydrophobic continuum is generated by the 20-residue insert (K177–K197) that is part of the additional four-stranded β-sheet, and the N-terminal segment and the C-terminal α-helix. This quite large and flat hydrophobic surface might serve to anchor the enzyme into the plasma membrane. The overall topology of the catalytic pocket of the *S.* K15 enzyme is also similar to that of class A β-lactamases, but the Ω-loop which bears the essential catalytic Glu166 residue in the class A β-lactamases, is deeply modified, and adopts a conformation similar to those found in the *Streptomyces* R61 DD-carboxypeptidase and the class C β-lactamases, with no acidic residue equivalent to Glu166.

The role of various amino acid residues potentially involved in the catalytic mechanism of the K15 DD-peptidase was analysed by site-directed mutagenesis. Lys38 in the first conserved motif (S_{35}XXK where S is the active-site serine) and Ser96 in the second conserved motif (S_{96}GC) play essential roles in hydrolysis, transfer and penicillin binding reactions. They are involved in the proton relay system. Lys38 might be the general base. Arg248 (equivalent to Arg244 of class A β-lactamases) and Lys213 (in the third KTG conserved motif) are probably involved in the orientation of the substrates or the inhibitors in the active site. Asn198 has a structural role and is also involved in the transpeptidation reaction [N. Rhazi, Ph. D. thesis]. Several mutants have been produced and crystallized. The structures of six of them have been determined to very high resolution. Figure 4 illustrates the quality of the electron density of the Ser216Ala mutant structure. The atomic coordinates have been deposited in the Protein Data Bank under the codes **1eqs**, **1es2**, **1es3**, **1es4**, **1es5** and **1esi** for the Lys38His, Ser96Ala, Cys98Ala, Cys98Asn, Ser216Ala and Arg248Leu mutants, respectively. No drastic modification of the active-site geometry is observed but the solvent content undergoes some modification especially in the S96A and S216A mutants where a water molecule occupies the position of the missing hydroxyl group of the Ser side chain. In the wild-type enzyme, the Ser96 hydroxyl group is hydrogen-bonded to the side chains of both Lys38 and Lys213. In the S96A mutant, the water molecule replacing the hydroxyl group establishes similar hydrogen bonds, which suggests that it could take over the role of the hydroxyl group of Ser96 in some aspects of catalysis. In the S216A mutant, the additional water molecule lies in a position equivalent to

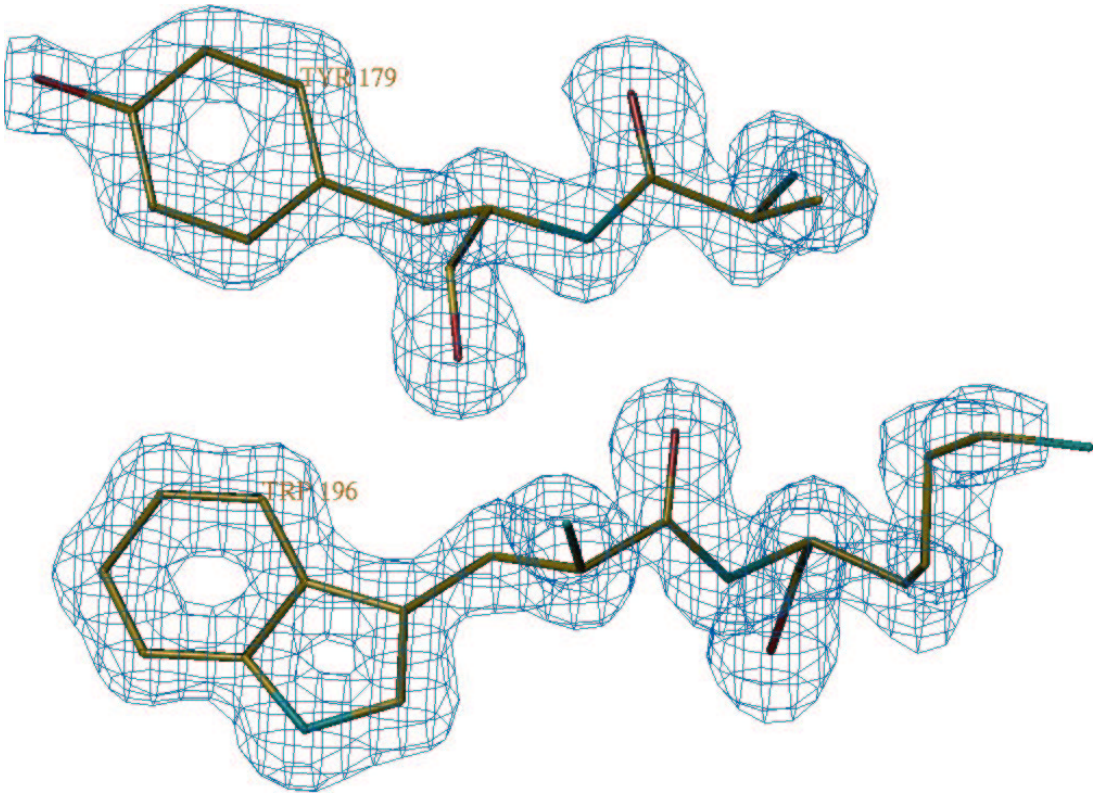


Fig. 4. Electron density for amino acid Tyrosine 179 and Tryptophane 196 residues of the catalytic mutant Ser216Ala of the DD-transpeptidase/penicillin-binding protein from *Streptomyces K15* (PDB code 1ES5). The electron density map has been calculated at a resolution of 1.4 Å, contoured at 1.4 σ level, and generated with the *TURBO-FRODO* package [Roussel, A. and Cambillau, C.(1989). *Silicon Graphics Geometry Partner Directory*, pp. 77–78, Silicon Graphics, Mountain Vieuw, CA, USA.].

that observed for the conserved water molecule found in the oxyanion hole of class A and class C β-lactamases.

Analysis of various mutations in the *S.* K15 and *Streptomyces* R61 DD-peptidases and the *E. coli* PBP5/DD-peptidase shows the importance of the lysine residue in the first conserved motif and the Ser or Tyr in the second conserved motif of all the enzymes. By contrast, in the *Actinomadura* R39 enzyme, Ser298 of the second conserved motif seems to be less important than Asn300 of the same motif and Lys410 of the third conserved motif (see below).

1.2.1.2. Class C monofunctional PBP/DD-peptidase

a) Bacillus subtilis *PBP4a/DD-peptidase*

The primary structure of the *B. subtilis* PBP4a is similar to those of *E. coli* PBP4 and *Actinomadura* R39 DD-peptidase (28 % and 46 % of identities, respectively). It possesses an additional domain located between motifs 1 and 2,

and is therefore a new member of the low-Mr PBPs/DD-peptidases class C. PBP4a catalyses the hydrolysis of peptide and thiolester substrates. By contrast to the *E. coli* PBP4 and *A.* R39 DD-peptidase, PBP4a shows a moderate affinity for benzylpenicillin ($k_2/K = 1{,}400\ \mathrm{M}^{-1}\,\mathrm{s}^{-1}$) [unpublished data].

b) Escherichia coli *PBP4/DD-peptidase*

E. coli PBP4 catalyses the cleavage of peptide, thiolester and ester carbonyl donors and the aminolysis of thiolester substrates in the presence of adequate acceptors. It shows a high affinity ($k_2/K = 114{,}000\ \mathrm{M}^{-1}\,\mathrm{s}^{-1}$) for benzylpenicillin. Kinetic data suggest that the acceptor behaves as an alternate nucleophile at the level of the acyl-enzyme [unpublished data]. The pathway of the transfer and hydrolysis reactions is similar to that proposed for the *S.* K15 enzyme and *E. coli* multimodular PBP3 [5, 66].

c) Actinomadura *R39 DD-peptidase*

The role of some residues in the conserved structural elements 2 and 3 of the *A.* R39 DD-peptidase has been studied by site-directed mutagenesis. Replacement of Ser298 of motif 2 (Ser298D/N) by Ala or Gly significantly decreases the $k_{\mathrm{cat}}/K_{\mathrm{M}}$ value for the peptide substrate, but only by a factor of 15 and has little effect on the other catalytic properties. Mutations of Asn300 of the same motif and of Lys410 of the third motif KTG yield very unstable proteins. However, the N300S mutant can be purified as a fusion protein with thioredoxin that exhibits decreased rates of acylation by the peptide substrate and various cephalosporins. Similar fusion proteins obtained with the N300A, K410H and K410N mutants are unstable and their catalytic and penicillin-binding properties are very strongly affected. In transpeptidation reactions, the presence of the acceptor influences the $k_{\mathrm{cat}}/K_{\mathrm{M}}$ values, which suggests a catalytic pathway more complex than a simple partition of the acyl-enzyme between hydrolysis and aminolysis of thiolester substrates [65].

1.2.2. The multimodular penicillin-binding proteins

1.2.2.1. Hierarchical analysis of multimodular PBPs

Sixty-three multimodular PBPs have been analysed by hierarchically combining pairwise comparisons of two sequences, either a sequence and a preexisting alignment or two preexisting alignments. The results of the analysis are consistent with those described previously. The 63 PBPs fall into two classes A and B. In class A PBPs, the N-terminal glycosyl transferase modules form a continuum of diverging sequences. The C-terminal penicillin-binding (PB) modules fall into five subclasses. In class B PBPs which are involved in cell morphogenesis, the N-terminal (N) modules and the PB modules cluster in a concerted manner. A PB module of subclass B2 or B3 is linked to an N-terminal

module of subclass B2 or B3 in Gram-negative bacteria. Gram-positive bacteria are divided in two subclasses B4 or B5 and some of them are grouped in an additional subclass B1 which shows a low affinity for β-lactams. Class B PBPs are an assortment of orthologs and paralogs [101].

1.2.2.2. Class A PBPs

a) PBP1b of E. coli

The glycosyltransferase (GT) located in the N-terminal module of PBP1b of *E. coli* catalyses glycan polymerization with a high efficiency (k_{cat}/K_M = 39,000 $M^{-1} s^{-1}$) from the lipid II precursor (undecaprenyl-P-P-disaccharide-pentapeptide). This module contains five conserved motifs which are also present in the monofunctional glycosyltransferases. The Glu233 in motif 1 is essential to the catalysis. It is proposed that the Glu233 γCOOH donates its proton to the oxygen atom of the scissile phosphate bond of the lipid II leading to the formation of an oxocarbonium ion which then undergoes attack by the 4-OH group of the N-acetylglucosamine of another lipid II molecule. Asp234 in motif 1 and/or Glu290 in motif 3 could be involved in the activation of the 4-OH group of the N-acetylglucosamine. They could also be involved in the stabilization of the oxocarbonium ion. Lys303 and Tyr310 in motif 4 seem to be important elements for the folding of the protein. The GT module of PBP1b, the lysozymes and the catalytic domain of the lytic glycosyltransferase Slt70 have similar catalytic machineries and might be members of the same superfamily [132].

The acyl-transferase, C-terminal penicillin-binding module of PBP1b possesses the catalytic centre-defining motifs of the penicillin serine transferases. It catalyses the formation of crosslinked tetrapeptide-pentapeptide and tetrapeptide-tetrapeptide dimers and the formation of uncrosslinked tetrapeptide monomers. It also catalyses the aminolysis and the hydrolysis of thiolester substrates but lacks activity on short D-Ala-D-Ala-terminated peptides, an observation which suggests that the transpeptidase activity requires the attachment of the pentapeptide to the glycan chains made by the glycosyl transferase.

The glycosyl transferase activity of PBP1b is acyl transferase-independent whereas the functioning of the acyl transferase is glycosyl transferase-dependent. PBP1b thus catalyses the glycan polymerization and the peptide crosslinking in a sequential manner. Attempts to produce truncated forms of PBP1b were unsuccessful, a result which suggests that the multimodular protein behaves as an integrated folding entity [132].

b) PBPs 1 and 1 of* Mycobacterium leprae

Mycobacterium leprae contains two class A PBPs, PBPs 1 and 1*. PBP1* catalyses hydrolysis of thiolester substrates but is devoid of glycosyl transferase activity with *E. coli* lipid II precursor as substrate. It has a high affinity for penicillins ($k_2/K = 50{,}000\ M^{-1}\,s^{-1}$) [60]. By contrast, PBP1 has no activity on thiolester substrates or lipid II and shows a low affinity for penicillin [37].

c) PBP1 of Streptococcus mitis

The sequence of the class A PBP1 of a penicillin-resistant clinical strain of *S. mitis* was established and shown to have a mosaic structure very analogous to that of PBP1a of resistant clones of *Streptococcus pneumoniae*.

d) The ponA *gene encoding the PBP1 of* Enterococcus faecalis *JH2-2*

The *ponA* gene encoding the PBP1 of *Enterococcus faecalis* JH2-2 was cloned and sequenced. The deduced PBP1 amino acid sequence exhibited 53 % identity and 71 % similarity with that of the PBP1a of *S. pneumoniae*. Alignment of the *E. faecalis* PBP1 and *E. coli* PBP1b sequences showed that the five conserved motifs in the N-terminal domain contained identical key amino acid residues. This would imply that they have the same functions in both proteins.

e) PBPs of Chlamydia

Chlamydia is a peptidoglycanless bacterium because it does not have the genetic information for the synthesis of class A PBPs (or monofunctional transglycosylases). It was proposed that *Chlamydia* utilizes one or several N-acetylmuramoyl-L-alanine amidases and two different class B PBPs to manufacture a covalently closed glycanless wall polypeptide made of peptide repeats whose synthesis is penicillin-sensitive [131].

1.2.2.3. Class B PBPs

a) PBP3 of E. coli

The soluble membrane anchor-free G57-V577 PBP3 of *E. coli* catalyses the hydrolysis and, in the presence of suitable acceptors such as D-Ala, the aminolysis of thiolester substrates of the type

$$C_6H_5–CO–NH–CH(R_1)–CO–S–CH(R_2)–COOH$$

suggesting that the protein is designed to catalyse transpeptidase reactions. D-Ala behaves as a single alternate nucleophile leading to the formation of a ternary acyl-enzyme-D-Ala complex that is only productive for the formation of a peptide bond. In contrast, the truncated PBP3 is devoid of glycan polymerization activity with the *E. coli* lipid II intermediate as substrate, which suggests that the N-terminal module is not a glycosyl transferase [66].

Two crystal forms of the soluble membrane anchor-free G57-V577 PBP3 have been obtained. The first crystal form grows as long and

thin needles ($500 \times 5 \times 5\,\mu m^3$) and the second as bipyramidal prisms ($400 \times 100 \times 100\,\mu m^3$). Both crystal forms have been used for X-ray data collection under cryogenic conditions at the ESRF in Grenoble. Both crystals belong to the tetragonal space group with different unit-cells, putatively containing 12 and 64 molecules for the needles and prisms, respectively, with diffraction limits of 3.0 Å and 4.0 Å. Heavy atom derivatives are being prepared to solve the phase problem.

In order to facilitate the crystallization process and enhance the crystal quality, cocrystallization experiments with a Fab fragment of a monoclonal anti-PBP3 antibody have been performed. The crystallization process was accelerated and different crystallization conditions were found. Unfortunately, the diffraction power of the different crystal forms so far investigated was not enhanced.

The structure of the monoclonal anti-PBP3 Fab fragment must be determined before solving the structure of the complex. Crystals of anti-PBP3 Fab fragment have been obtained. One crystalline form has been measured on beamline ID14/3 at ESRF (data complete to 3.0 Å , $R_{sym} = 5.4\,\%$) and the structure determination by molecular replacement is under way.

b) Low affinity PBPs in Enterococci

In each wild-type enterococcal species, at least one low affinity class B PBP is found, the size of which is about 75 kD [92]. In several clinical and laboratory *E. faecium* clones of intermediate resistance, the low affinity PBP5 is overproduced in the membranes and the PBP5 affinity is rarely modified when compared to that of wild-type cells. However, in highly resistant clones of the same species, the affinity of PBP5 is reduced [27].

Overproduction of the low affinity PBP4 and PBP5 is also observed in resistant laboratory mutants of *E. faecalis* and *E. hirae*, respectively.

Finally, the clinical *E. hirae* S185 strain isolated from pig intestine possesses a second low affinity PBP designated as PBP3r and determined by a plasmid-borne gene (*pbp3r*). This gene is positioned in the vicinity of a transposon-like element which bears three antibiotic resistance markers (erythromycin, spectinomycin and streptomycin) [86 and unpublished results].

The genes encoding the low affinity PBPs of *E. faecium*, *E. hirae* S185 and *E. faecalis* JH2-2 were cloned, sequenced and compared to that of *E. hirae* PBP5 [27, 86 and unpublished results]. All have a 110-residue polypeptide insertion between the membrane anchor and the N-terminal module. No definite function is attributed to this insert except that it seems to be required for a normal folding of the protein. When deletions are made in the *E. hirae pbp5* region encoding that polypeptide, no functional PBP5 derivative can be detected [38].

Soluble derivatives of *E. faecium* and *E. hirae* PBP5s were overproduced in *E. coli* after each of the encoding genes was truncated at its 5′ end for that part coding for the membrane anchor. Both derivatives were synthesized in large amounts and purified to obtain pure material for crystallization attempts [unpublished results].

Small crystals of the *Enterococcus faecium* PBP5, often twinned, are easily obtained. The crystals belong to the monoclinic space group with either 12 or 16 molecules of PBP5 per unit-cell. Data collection was performed to a resolution of 3.5 to 2.5 Å, on beamlines BM14 or ID14/2 at ESRF. A selenomethionine-labelled protein has been produced, purified and crystallized in the same conditions. Several highly redundant MAD data sets have been collected at ESRF on beamlines BM14 or ID14/4. The data have been investigated by the use of different softwares (MOSFLM, XDS and DENZO) and are currently used for the phasing procedure as developed for the SAD and MAD methods in the SOLVE and S 'n B softwares. The structure determination is underway.

Two soluble derivatives of PBP5 from *Enterococcus hirae* have been used for crystallization assays. The first soluble derivative bearing a hexahistidine at the N-terminal end of the protein (sPBP5NH) gave unreproducible, non-diffracting crystals. By eliminating these additional His residues, the second soluble form was more easily crystallized.

2. Protein-protein and protein-DNA interactions in complex machineries

2.1. Cell division machinery

The class B multimodular PBP3 of *E. coli* is specifically involved in septal peptidoglycan synthesis with the cell division proteins FtsA, K, L, N, Q, W, Z and ZipA. All these proteins localize to the division site in an interdependent fashion and presumably form a complex called divisome, that encompasses the cytoplasm, the inner membrane and the periplasm [70, 91].

PBP3 is composed of an N-terminal membrane anchor-containing module that is essential for cell septation, a non-penicillin-binding (n-PB) module and a C-terminal penicillin-binding (PB) module. The n-PB module contains three conserved motifs and is devoid of glycosyl transferase activity. It is required for the folding of the penicillin-binding module [41].

The non-catalytic module of PBP3 might provide specific regions for interactions with the cell division proteins (FtsL, N, Q, W, …). Association of these proteins to the PBP3 N-terminal module might modulate its peptidoglycan cross-linking activity at the division site.

Analysis of the hydrophobic profile of PBP3 allows the prediction of protein-protein interaction regions the roles of which was analysed by site-directed mutagenesis. A model of PBP3 has been constructed using the 3D structure of the class B PBP2x of *S. pneumoniae* [Pares *et al.*, 1996; Marrec-Fairley *et al.*, in press]. It was possible to identify three regions of the non-catalytic module of PBP3, each performing distinct and specific functions. The membrane anchor and the G57-Q66 segment B contain the information responsible for the proper positioning of PBP3 at the cell septation site. The I74-L82 segment C and the H160-G172 segment E overlap motifs 1 and 2, respectively. Together with motif 3, they are located at or close to the junction between both modules. They are involved in the acquisition of the proper conformation by PBP3. The E201-V217 segment F is exposed at the surface of the non-catalytic module and is essential for the proper functioning of PBP3 within the fully complemented divisome [41; Marrec-Fairley *et al.*, in press].

FtsW possesses 8 transmembrane segments and two loops, one of which is located in the periplasm, and the other in the cytoplasm. Protein-protein interaction sites are predicted in each loop. Mutations in these sites block the cell septation (unpublished data). It is proposed that FtsW interacts with PBP3 to synthesize septal peptidoglycan and with FtsZ, a tubulin, which forms an intracellular cytoskeletal ring at the division site [Lutkenhaus and Addinall, 1997].

A chromosomal 10355-bp segment of *E. hirae* S185 contains nine *orfs* which occur in the same order as the MraW-, FtsL-, PBP3-, MraY-, MurD-, MurG-, FtsQ-, FtsA- and FtsZ-encoding genes of the division and cell wall clusters of *E. coli* and *B. subtilis*. The *E. hirae* DNA segment lacks the genes which encode the Ddl, MurC, MurE and MurF ligases and the integral membrane protein FtsW of *E. coli*. The encoded *E. hirae* and *E. coli* proteins share 25 % to 50 % identity except FtsL and FtsQ (~ 14 % identity). FtsW is localized in another region upstream of the low affinity PBP5-encoding gene [99].

When cells of *E. hirae* are inhibited by β-lactams, most if not all the functions of the PBPs, including PBP3s which is essential for cell division, are taken over by PBP5. It is thus presumed that PBP5 is integrated in the machinery of the divisome and participates in protein-protein interactions. Six sites were predicted in the N-terminal domain of PBP5 to constitute protein-binding sites: T74 to A83, K108 to S111, K141 to N142, E170 to K182, L200 to K206 and K309 to N315. The first three are located in the polypeptide inserted between the membrane anchor and the class B PBP conserved motif 1. Site 4 overlaps with and site 5 follows motif 1. Site 6 overlaps the class B PBP conserved motif 3.

Single and double mutations (K172N and R173N) induced by site-directed mutagenesis in the *pbp5* region coding for motif 1 destabilize PBP5 to such a point that it could not be detected in *E. coli* transformants. This would indicate that motif 1 is absolutely required for PBP5 folding or stability. Mutations in site 5 do not seem to modify the biochemical and microbiological properties of PBP5.

2.2. The protein secretion system in *Streptomyces*

The chosen organism was *Streptomyces coelicolor* A3(2), genetically the most studied strain of the genus. Genes coding for proteins involved in the protein secretion pathway were cloned (*secA*, *secY*, *secD/F*, *dnaK/grpE/dnaJ*) or received from other laboratories (*secE*, *groEL/ES*). All their sequences were determined and deposited in the EMBL gene bank. The isolated genes have been localized in the *S. coelicolor* genome in collaboration with Helen Kieser (Norwich, UK) [Redenbach *et al.*, 1996].

2.2.1. Chaperone proteins [A. Brans, Ph. D. thesis]

The *dnaK/grpE/dnaJ* operon coded for proteins of 66 kDa (DnaK), 23.7 kDa (GrpE) and 42 kDa (DnaJ). The similarity with homologous proteins previously characterized was very high for DnaK (75–85 %) and DnaJ (57–69 %), lower for GrpE (50–58 %) [33].

S1 mapping analysis of the region upstream of *dnaK* revealed the presence of a promoter responding to heat shock [59]. No CIRCE sequence was found in this region, but another palindromic sequence, very close to the complementary strand of CIRCE, was observed and called CIRCE II. CIRCE II is probably responsible for the heat shock response of the *dnaK* promoter.

Attempts to overproduce the chaperones GroEL, GroES, DnaK, GrpE and DnaJ, *via* cloning on multicopy plasmids, have failed due to the instability of the recombinant vectors in *S. coelicolor* or *S. lividans* TK24.

It was also attempted to inactivate the *dnaK* operon, but without success, a result which suggests that some functions of the proteins coded by this operon are essential.

2.2.2. The *sec* pathway [A. Loriaux, Ph. D. thesis]

secY encodes a 437-residue protein, sharing 46 and 42 percents of identities with SecY of *E. coli* and *B. subtilis*, respectively. Hydrophobic profiles of these proteins are practically superimposable. It can be supposed that they adopt the same topology in the cytoplasmic membrane, i.e., 10 transmembrane segments, with the C- and N-termini in the cytoplasm.

secE encodes a 94-amino acid protein. Its similarity with other SecE proteins (which differ in size) is rather low. SecE proteins of several *Streptomyces* strains exhibit identical hydrophobic profiles, close to that of *B. subtilis* SecE. In this case, the topology includes only one hydrophobic transmembrane segment, instead of three in *E. coli*.

secD and *secF* encodes 603- and 373-amino acid proteins, respectively. Based on their hydrophobic profiles, these two proteins should be similarly integrated in the membrane, with 6 transmembrane segments, a large cytoplasmic loop and their C- and N-termini exposed in the cytoplasm.

secA contains 2844 nucleotides. Compared to the *S. lividans* secA, the *S. coelicolor* protein shows 8 silent mutations, but a strictly identical amino acid sequence.

Many experiments were designed in order to isolate the *Streptomyces* protein homologous to SecG, an essential component of the *E. coli* translocase. All of them failed.

It is clear that the protein secretion machinery in *Streptomyces* is very close to those in *E. coli* and *B. subtilis*.

Transcriptional analyses were carried out on the *secA* gene and showed that this gene has its own promoter.

The final aim of this research was to moderately overproduce all the proteins involved in the secretion pathway, and to obtain a hypersecretory cell. In order to follow this overproduction, we tried to prepare antibodies directed against each of the components of the translocase and against SecA. The preparations were difficult to obtain and the few positive results were disappointing. This line of research is presently discontinued.

2.3. Low affinity PBP regulation in *Enterococcus* sp.

The synthesis of the low affinity PBP5 in *E. hirae* was previously proposed to be negatively controlled by the *psr* gene located immediately upstream of the *pbp5* gene. A reexamination of the nucleotide sequence of the *E. hirae psr* leads to the conclusion that it is substantially longer than previously reported [88]. Similar *psr* genes of different clinical strains of *E. faecium* are located immediately upstream the *pbp5* gene [27].

Cloning and sequencing of a *psr* gene of the *E. faecalis* JH2-2 strain and its resistant derivative JH2-2R reveal that the *psr* gene is not located in the vicinity of a PBP-encoding gene. In addition, both genes are perfectly identical even in the putative promoter region therefore excluding the existence of a direct relationship between the observed overproduction of PBP4 and a modification of the *psr* gene or its promoter region in JH2-2R. This result raises the question of the exact function of the *psr* product.

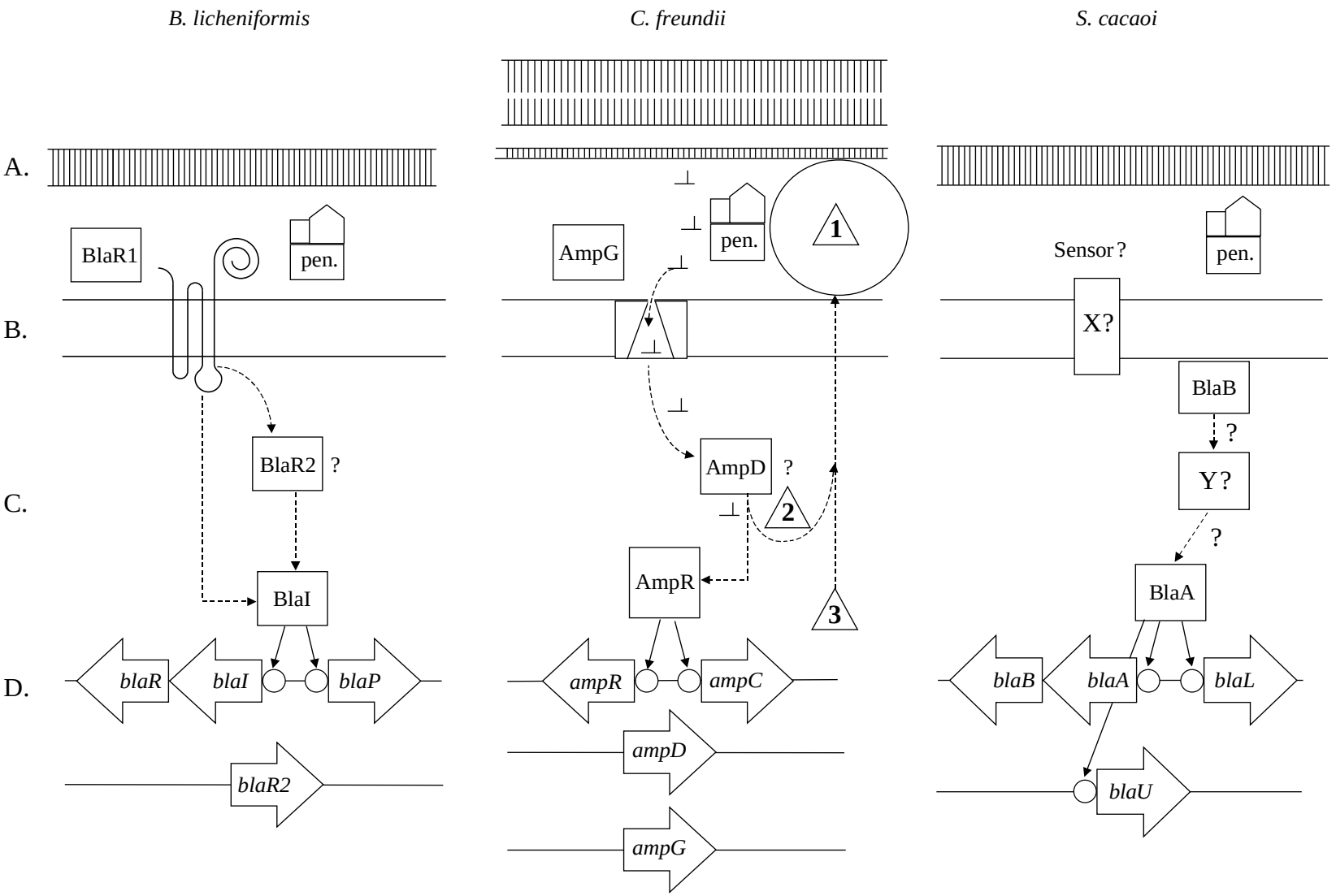
Crude and purified preparations of *E. hirae* Psr overproduced in *E. coli* are unable to bind DNA fragments containing the *pbp5* promoter region as shown by electrophoretic mobility shift assays. In addition, neither disruption nor deletion of the *psr* gene of *E. hirae* by homologous recombinations change the susceptibility of the mutants to penicillin or the amount of PBP5 synthesized in the membranes. Again these results seem to indicate that the *psr* product of *E. hirae* has no direct implication in the control of the PBP5 synthesis.

2.4. β-Lactamase induction as a model for signal transduction and gene regulation

To survive and continuously adapt their cellular machinery to the external environment, living cells have acquired membrane proteins which allow communication between the outside and the inside of the cell. These proteins perform a diversity of functions as different as metabolite uptake and cell signalling.

In several bacteria, the synthesis of one or several β-lactamase(s) is the main factor of β-lactam antibiotic resistance and is induced by the presence of the antibiotic. As this type of antibacterial agents does not significantly cross the cytoplasmic membrane, bacteria in which the β-lactamase is inducible have a mechanism which signals the presence of the antibiotic outside the cell. In the literature four different mechanisms are reported [87] and we have studied those of *Citrobacter freundii* [55], *Bacillus licheniformis* [52] and *Streptomyces cacaoi* [62] (see Fig. 5).

The aim of this research is to understand the mechanism of bacterial resistance and the coupling between signal transduction and gene regulation.


B. licheniformis
C. freundii
S. cacaoi
A.
B.
C.
D.
BlaR1
pen.
BlaR2
?
BlaI
blaR
blaI
blaP
blaR2
AmpG
pen.
1
AmpD
?
2
AmpR
3
ampR
ampC
ampD
ampG
Sensor?
pen.
X?
BlaB
?
Y?
?
BlaA
blaB
blaA
blaL
blaU

2.4.1. Induction of AmpC β-lactamase in *C. freundii*: the Gram-negative model

In *C. freundii*, the expression of the gene encoding the AmpC β-lactamase is controlled by the DNA-binding protein AmpR which acts both as a repressor and a transcriptional activator of *ampC*. Both in uninduced or induced conditions AmpR is always bound to its DNA operator [Bartowsky and Normark, 1991]. Two other genes, *ampD* and *ampG*, are also involved in the induction phenomenon [Lindquist *et al.*, 1989; 1993]. In our previous works, we have demonstrated that the peptidoglycan recycling in Gram-negative is linked to β-lactamase induction [Jacobs *et al.*, 1994].

In collaboration with S. Normark and J. Park we have elucidated the mechanism of induction. In the absence of the antibiotic, the cytoplasmic peptidoglycan precursor, the UDP-N-acetyl-muramic acid-pentapeptide (MurNAc-pentapeptide) is bound to AmpR which acts as a repressor.

In the presence of penicillin (cell wall synthesis is impaired), the major metabolite is probably the anhydro-MurNAc-pentapeptide and its cytoplasmic concentration becomes sufficient to restore the activating properties of AmpR by displacing the UDP-MurNAc peptide ligand. This is facilitated by a concommitant and presently unexplained decrease of the cytoplasmic concentration of UDP-MurNAc-pentapeptide [55].

Thus, the control of the *ampC* gene transcription rests upon a delicate balance between the concentrations of peptidoglycan precursors and degradation products. It can be suggested that “this system functions as a means

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On opposite page:

Fig. 5. Schematic representation of the regulatory pathways presently proposed for β-lactamase production in *B. licheniformis*, *C. freundii* and *S. cacaoi*. A: peptidoglycan layer; B: cytoplasmic membrane; C: cytoplasm; D: genome.

In *B. licheniformis*, the synthesis of β-lactamase, BlaP, is controlled by three factors: BlaR1, detector of β-lactams, at the external side of the cytoplasmic membrane, BlaI, transcription repressor of *blaP* gene, and BlaR2 whose the function is not known.

In *S. cacaoi*, two regulatory elements have been described: BlaA, a transcription activator, controls two β-lactamase genes, *blaL* and *blaU*, and BlaB, of unknown function, localized at the internal side of the cytoplasmic membrane.

In Gram-negative bacteria (*C. freundii*), at least four genes are involved in the regulation of the gene coding for the class C β-lactamase, AmpC: *ampR* acts as repressor in the absence of induction, as activator in the presence of induction. Induction depends on the product of *ampG* (that codes for a permease), *ampD* (that codes for an amidase implied in the recycling of the peptidoglycan degradation products that enter the cell via AmpG) and *ampE* (the product of which is of unknown function). Circle 1 represents the multi-enzymatic complex (hydrolases-synthetases) of peptidoglycan biosynthesis-remodeling. Arrows 2 and 3 show the recycling and the biosynthesis pathways, respectively. Hypothetical steps or intermediates are indicated by question marks.

of monitoring cell wall integrity and maintaining a proper balance between murein synthesis and degradation during bacterial growth" [55].

During growth, normal Gram-negative cells recycle up to 40 % of the peptidoglycan fragments formed by the autolytic sytem. This recycling does not occur in cell lacking either *ampG* or *ampD* and the β-lactamase induction is altered. The *ampG* mutants release peptidoglycan-derived material into the medium. In contrast, an *ampD* mutant accumulates anhydro-MurNAc-tripeptide into the cytoplasm [Jacobs *et al.*, 1994]. AmpG is the permease for anhydro-MurNAc-tripeptide and AmpD an amidase which cleaves the amide bond linking the sugar moiety to the tripeptide [3].

To elucidate the transport mechanism of the anhydro-MurNAc-tripeptide, the topology of the integral cytoplasmic membrane protein AmpG (53 kD) was analysed according to the method described by Broome-Smith [Broome-Smith *et al.*, 1990]. Out of the 14 putative transmembrane segments (PTSs) predicted by the hydropathy curve, 10 have been found as transmembrane α-helices [A. Chahboune and B. Joris, unpublished results].

In order to understand the mode of action of the AmpD enzyme (21 kD) at the atomic level, the determination of its 3D structure by NMR has been undertaken in collaboration with G. Otting (Karolinska Institutet, Sweden). AmpD was labelled with ^{15}N or/and ^{13}C isotopes and purified to 99 %. At the present time, 90 % of NMR signals have been assigned [C. Généreux and B. Joris, unpublished results]. Meanwhile, crystallization attempts have been made in order to determine the 3D structure by X-ray diffraction. Several crystal forms have been obtained.

2.4.2. Induction of the BlaP β-lactamase in *B. licheniformis*: the Gram-positive model

Two genes products, BlaI and BlaR1, are known to affect the expression of the inducible BlaP β-lactamase in *B. licheniformis*. BlaI is a DNA-binding protein and BlaR1 is a membrane protein which plays the role of a penicillin receptor. In the *B. licheniformis* chromosome, *blaI*, *blaR1* and *blaP* are organized as a divergeon in which *blaI* and *blaR1* form an operon. In the absence of β-lactam antibiotic, the inducer BlaI binds to three operators (OP1, OP2 and OP3) in the intercistronic region between *blaP* and *blaI*. Addition of the inducer converts BlaI into an inactive form that cannot bind to the operators. The nature of the signal launched through the cytoplasm by BlaR1 is still unknown.

The topology of BlaR1 has been experimentally determined. The N-terminal domain contains four transmembrane segments (TM1, 2, 3, 4) which could be associated to form an α-helix bundle and play the role of transducer [52]. The C-terminal penicillin sensor module (BlaR1 C-terminal

domain, residues 351 to 601) contains all the sequence motifs of penicilloyl serine transferases. The BlaR soluble C-terminal domain (BlaR-CTD) has been cloned and purified as a soluble protein of 26 kD. It was crystallized as very thin plates of $10 \times 300 \times 300\,\mu m^3$ of unknown unit-cell. The N-terminal domain of BlaR1 (BlaR1-NTD, residues 1–349) contains the transducer and the transmitter modules. It seems that the large cytoplasmic loop (residues 137 to 320), connecting the transmembrane segments TM3 and TM4, is the transducer module which is responsible for the launching of the cytoplasmic signal. In this loop, a Zinc binding motif ($H^{212}EXXH$) which might be the signature of a metallo-protease belonging to the family of thermolysin, was predicted. Site-directed mutagenesis of this motif results in the loss of β-lactamase induction confirming its importance for the functioning of the receptor [K. Benlafya and B. Joris, unpublished results].

BlaI was overexpressed in *E. coli* and purified. In solution BlaI forms a dimer which has been characterized by molecular sieve chromatography, cross-linking and NMR. The interaction between BlaI and its target DNA OP1 has been characterized by quantitative fluorescent band shift assay using an ALF Express DNA sequencer, a new method to analyse DNA-binding proteins [P. Filée and B. Joris, unpublished results].

The BlaI protein crystallizes as small prisms of $100 \times 100 \times 300\,\mu m^3$, in the monoclinic $P2_1$ space group, with a unit-cell of $a = 66\,Å$, $b = 49\,Å$, $c = 57\,Å$, $\beta = 123°$. Cocrystals of BlaI with a 21 bp DNA fragment have also been obtained.

The phasing procedure for determining the structures of both BlaR1-CTD and BlaI involves the production of selenomethionine-labelled proteins. The proteins contain 3 and 4 methionine residues respectively. Crystals of selenomethionine-labelled-BlaI have already been obtained.

2.4.3. β-Lactamase induction in *Streptomyces* [J. Magdalena, C. Gérard, Ph. D. theses]

β-Lactamase production is very common in the *Streptomyces* genus (75 % of the species) but this production is generally constitutive. Exceptionally the β-lactamase production is inducible by β-lactam compounds. *Streptomyces cacaoi* is one of these exceptions and has been studied in the laboratory for several years. The present knowledge of the regulatory system is summarized in Figure 5. *S. cacaoi* possesses two structural genes, *blaL* and *blaU*, encoding two different class A β-lactamases. In 1994, it was still believed that both genes were inducible, but controlled by different mechanisms. It has now been demonstrated that both *blaU* and *blaL* are regulated by a pair of regulatory genes, *blaA* and *blaB*, laying upstream of *blaL* [62].

The similarity of the upstream regulatory regions of *blaL* and *blaU* is rather high, and both of them contain the same BlaA-binding motif (a palindromic sequence called T-N_{11}-A). They however differ upstream of this motif. The *blaL* regulatory region is preceded by the *blaA* and *blaB* genes, whereas the *blaU* regulatory region is preceded by several direct repeats. If these repeats are deleted, *blaU* is no longer inducible, but how these repeats influence the regulation mechanism remains unclear [C. Gérard, Ph. D. thesis].

BlaA, a classical transcriptional activator, was not studied further. We focused on BlaB, a protein similar to the β-lactam-recognizing enzymes, with two of the usual conserved motifs (SXXK, KTG), a modified SDN, and most of the secondary structures present in this family of enzymes. BlaB has been tentatively localized on the internal face of the cytoplasmic membrane but its role in induction and basal expression of *blaL* remains unknown. It does not act as a β-lactamase, a PBP or a D-aminopeptidase and it is not a substrate for phosphorylation [18].

A site-directed-mutation study is under progress in order to elucidate the mode of binding of BlaB to the membrane and its role in the regulation process.

BlaB appears to contain two amphiphilic segments (a central and a C-terminal one) which could take part in binding to the membrane. Their deletions or modification (without disrupting their helical structures) abolished the regulatory activity of the protein, but did not modify its cellular localization. Mutations introduced at the levels of the conserved motifs SXXK (S → A) and KTG (K → A) appeared to have different effects. Mutation of K suppressed the induction and the system was permanently repressed. By contrast, mutation of S resulted in high-level constitutive β-lactamase production.

Further study of this protein would require the knowledge of its 3D structure. A lot of work has been done to obtain purified BlaB preparations but the results were not satisfactory since overproduced BlaB formed inclusion bodies.

2.5. Xylanase induction in *Streptomyces* [C. Servais, F. Giannotta, Ph. D. theses]

Study of the regulatory system of the XlnC xylanase in *Streptomyces* sp. strain EC3 has revealed the presence of three operator sequences, important for the transcriptional regulation of the *xlnC* gene [36, 42]. These operating sequences consist in 10-bp palindromic structures, 5′-CGAAA-N-TTTCG-3′ and are conserved in most of the xylanase promoter sequences in *Streptomyces*. They specifically bind a protein complex which is present only in xylanolytic strains of the genus *Streptomyces*. Attempts to identify the proteins involved in this complex allowed the isolation of two regulatory genes. *xlnR*, the first to be cloned and sequenced, codes for a DNA-binding "helix-turn-helix" protein, belonging to the GntR family. XlnR mainly acts as a repressor of the xylanase genes. The second, *xlnI*, a regulator similar to CAP in *E. coli* is probably indirectly involved in xylanase regulation.

3. Integrated models

The high second-order rate constants characterizing the acylation of *B. licheniformis* PBP1 by penicillins and cephalosporins were determined by the combined utilization of fluorescein labelled penicillins and of a new competition method. The method was also suitable for measuring the kinetic parameters of PBP1 in intact cells. The results confirmed that PBP1 is probably the main target of most β-lactam antibiotics [9].

However, in growing cells, but not in resting cells, the penicillin targets appeared to be somewhat protected from the action of the inactivators. Compounds mimicking the peptide moiety of the nascent peptidoglycan significantly interfered with the acylation of PBP1 by the antibiotics. The level of PBP1 saturation at the minimum inhibitory concentration (MIC) was not constant, suggesting that additional, still undiscovered factors might be necessary to account for the experimental observations [6].

With β-lactamase overproducing mutants of *Enterobacter cloacae*, the MIC values were practically independent of the sensitivity of the target PBP even for poor β-lactamase substrates. This apparent paradox was explained by analysing the equation which relates the antibiotic concentration in the periplasm to that in the external medium. In conditions which are frequently encountered in clinical isolates, the factor characterizing the PBP sensitivity became negligible. The conclusions can be extended to all antibiotics that are sensitive to enzymatic inactivation and active efflux mechanisms and that must overcome permeability barriers. This consideration is of the highest importance in the design of future antibacterial agents [107].

4. Folding mechanisms

4.1. Class A β-lactamases

These monomeric proteins of about 29 kD have been found to be useful models for studying the stability and folding properties of proteins [96].

Five representative class A enzymes produced by various mesophilic bacterial species were selected in order to probe the determinants of protein stability. Despite significant similarities in their amino acid sequences and in their overall 3D structures, these enzymes exhibit very different stabilities. Careful structural analysis indicated that higher stability appears to correlate with increased numbers of intramolecular hydrogen bonds and of salt bridges [8]. By contrast, the global hydrophobicity of the protein seems to play a relatively minor role. A strongly unfavourable balance between charged residues, and the presence of a *cis*-peptide bond might also account for the particularly low stability of two of the enzymes. In addition, the single Cys-77-Cys-123 disulfide bond of the TEM-1 enzyme was removed by introducing a Cys77 → Ser mutation, and the enzymatically active mutant was found to be 3.4 kcal/mol less stable that the wild-type protein [50].

The folding mechanism of two enzymes, namely TEM-1 and NmcA was investigated by equilibrium and kinetic experiments, with the help of fluorescence spectroscopy and circular dichroism (CD). The equilibrium unfolding of the TEM-1 enzyme induced by guanidinium hydrochloride (GndHCl) occurs in two discrete steps, indicating the existence of a thermodynamically stable intermediate state. This species is ~ 5.2 kcal/mol less stable than the native enzyme and ~ 5.7 kcal/mol more stable than the fully denatured protein. It is devoid of specific tertiary organization but exhibits a high content of secondary structure elements [7]. By contrast, with the NmcA β-lactamase both activity and optical measurements (i.e., intrinsic fluorescence emission and far UV CD) suggest that the enzyme is unfolded reversibly and cooperatively in a single transition.

The kinetics of refolding of the two β-lactamases have been investigated by following the recovery of both the optical properties and the catalytic activity. In manual mixing experiments, a transient intermediate, which displays a significant proportion of secondary and tertiary structure organization is observed with both enzymes. This major folding event (~ 10 s) is typically followed by two very slow phases (> 10 s). Using the double jump technique, the first of these two slow phases [time-constant τ ($= 1/k$) $\simeq 30$ s] was shown to be associated with the *cis-trans* isomerization of still unassigned peptide bond(s) containing proline residues. The second slowest phase is due to the unusual *cis* configuration of the E166-X167 peptide bond in the Ω-loop of all class A β-lactamases of

known structures. In the TEM-1 and NmcA enzymes, residues 167 are a proline and a leucine, respectively, and the rate-limiting step for refolding to the functional enzyme ($\tau \simeq 300$ s and $\simeq 1000$ s, respectively) has been proposed to reflect mainly the *trans* to *cis* isomerization of the E166-X167 peptide bond [25; A. Lejeune and J.M. Frère, unpublished results].

When rapid mixing methods (dead-time $\simeq$ 2–4 ms) are used, additional fast phases become visible. With the TEM-1 β-lactamase [84], the binding of 1-anilino-8-naphtalenesulfonic acid (ANS) within the dead-time of the stopped-flow apparatus points to an early hydrophobic collapse of the protein. This is accompanied by the regain of ~ 60 % of secondary structure as indicated by far-UV CD measurements. Interestingly, quenching of fluorescence by acrylamide suggested that the tryptophan residues, which are largely exposed to solvent in the native protein, become less accessible to acrylamide during the first 2–4 ms, thus suggesting the formation of transient non-native hydrophobic interactions [84]. With the NmcA enzyme, three fast phases (with time constants values of ~ 0.2 s, ~ 1 s and ~ 5 s) are visible. Our next aim will be to analyse these fast folding events in more details.

4.2. Single chain antibody fragments from camelids

Dromedaries and related species (Camelids) display antibodies composed of heavy chains only [Hamers-Casterman *et al.*, 1993]. Besides the conventional heterotetrameric antibodies common to all vertebrates, these animals present homodimeric heavy-chain antibodies. Although the relative proportions seem to vary, an average of about 50 % is common for dromedaries [Muyldermans and Lauwereys, 1999]. Following immunization, the DNA fragments coding for the variable N-terminal domains of these antibodies (VHH) can easily be cloned and expressed as phage libraries, from which specific binders can be selected. Finally, these minimal-sized recognition units can be produced as soluble monomers in *E. coli*.

Within the frame of a collaborative project with the group of Lode Wyns and Serge Muyldermans (V.U.B.), where the single chain antibody fragments are constructed, we have embarked on the characterization of the folding properties of these small all-β-sheet protein fragments ($\sim$ 130 amino acids). The equilibrium folding properties of three fragments, namely cAb-Lys3 (anti-hen lysozyme), cAb-Bla02 (anti-NmcA) and cAb-BCII10 (anti-BcII β-lactamase) were analysed. In all cases, by using both spectroscopic and affinity (with the help of a Biacore instrument) measurements, folding was shown to be fully reversible, and to proceed *via* a simple two-state mechanism. The characteristic thermodynamic parameters were determined, and rather high values were

obtained for the free energy of folding of cAb-BCII10 ($\sim 12\,\mathrm{kcal\,mol^{-1}}$) compared to the values obtained for the two other fragments ($\sim 8\,\mathrm{kcal\,mol^{-1}}$). This will be further analysed in the future.

Preliminary stopped-flow experiments performed with cAb-Bla02 seem to indicate the occurrence of at least three phases in the refolding kinetics of this fragment.

4.3. Co-folding experiments

With the various antigen-antibody pairs available, we plan to study the influence of one partner on the other, depending on their relative stabilities. Thus, the stability of the NmcA β-lactamase, in the presence of cAb-Bla2 has been found to be slightly but significantly increased. Now, the unfolding and refolding kinetics of the enzyme in the presence of the antibody fragment are being investigated. Similar studies will be performed with some other fragments and their corresponding antigens.

5. Stabilization of xylanases

Xylanases used to facilitate the bleaching of kraft pulp must exhibit a high thermostability and activity in a broad pH range. The *xyl1* gene encoding Xyl1, a family-11 xylanase from *Streptomyces* sp. S38 which was fully responsible for the biodelignification potential of the crude culture extract [1], was cloned and sequenced [129]. In a general approach to the understanding of protein adaptation to high temperature, molecular models of the closely related mesophilic *Streptomyces* sp. S38 Xyl1 and thermophilic *Thermomonospora fusca* TfxA xylanases were built and compared with the 3D structures of homologous enzymes (Fig. 6). Some of the structural features identified as potential contributors to the different thermostabilities were introduced in Xyl1 by site-directed mutagenesis in an attempt to improve its thermostability and thermophilicity. A new Y11-Y16 aromatic interaction, similar to that present in TfxA, was created in Xyl1 by a T11Y mutation and improved the thermophilicity and thermostability. Indeed, the optimum activity temperature (70 *vs* 60 °C) and the apparent T_m were increased by about 9 °C and the enzyme half-life at 57 °C increased six fold [146]. Mutations A82R/F168H/N169D/Δ170 potentially creating a R82-D169 salt bridge homologous to that present in TfxA improved the thermostability but not the thermophilicity. Mutations R82/D170 and S33P seemed to be slightly destabilizing and devoid of influence on the optimal activity temperature of Xyl1. Structural analysis revealed that residues

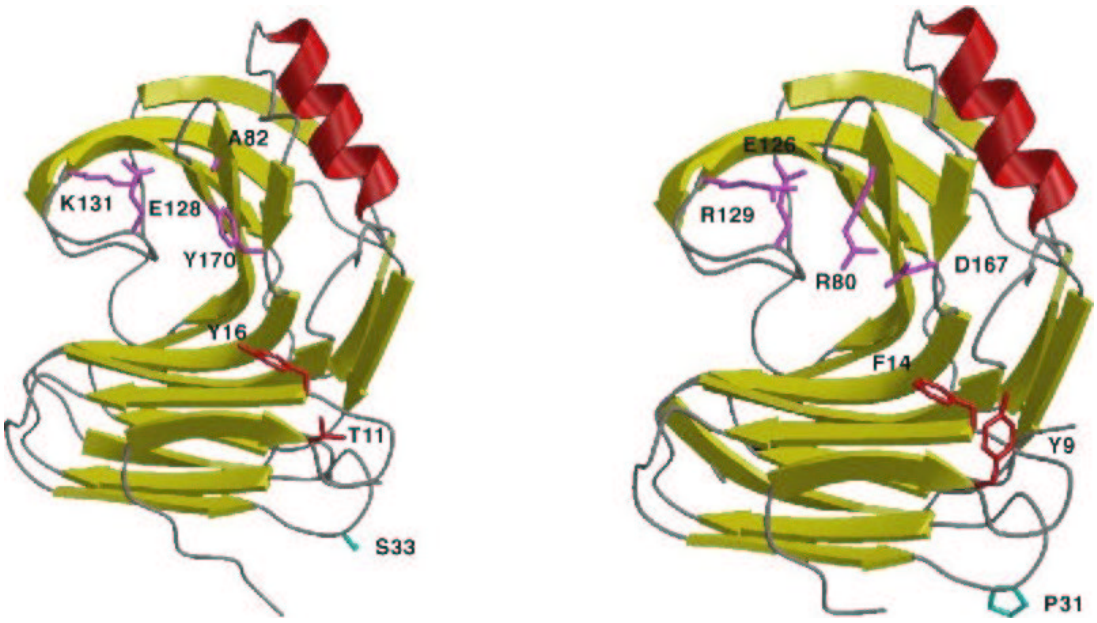

Fig. 6. Ribbon structures of Xyl1 and TfxA xylanases showing the residues of interest.

Y11 and Y16 were located on β-strands B1 and B2, respectively. This interaction should increase the stability of the N-terminal part of Xyl1. Moreover, Y11 and Y16 seem to form an aromatic continuum with five other residues forming putative subsites involved in the binding of xylan $(+3, +2, +1, -1, -2)$. Y11 and Y16 might represent two additional binding subsites $(-3, -4)$ and the T11Y mutation could thus improve substrate binding to the enzyme at higher temperatures and thus the thermophilicity of Xyl1.

6. Theoretical chemistry and molecular modelling

Molecular systems can be described by their potential energy surfaces. Depending on the number of degrees of freedom associated to the 3D representation, the dimension of the surface grows extremely fast and only a small part can be investigated in order to locate equilibrium structures like conformers, reactants, products and transition state structures.

This energy function is estimated for a given electronic state. When several of them lie in the same energetic range, they lose their simple physical meaning of potential energy. In such regions, the Hartree-Fock wave function becomes unstable.

Several applications have been analysed along these topics particularly in electronic transfer phenomena and in the study of pharmacological molecules involved in putative redox systems.

Catalytic reaction pathways have also been approached by quantum chemistry [97] mainly in serine peptidases such as α-chymotrypsin, elastase and β-lactamases.

6.1. Vicinity of several electronic states

The numerical solution of Schroedinger's equation is the wave function of the molecular system in an electronic state such as the singlet at the fundamental level of a closed-shell structure. For radicals in doublet state, α and β spin electrons are no more paired and separate solutions are calculated for both sets of electronic distributions.

6.1.1. Electronic transfer

Intervalence transitions can be experimentally observed in the near infrared for symmetrical molecules bearing two tetrathiafulvalene moieties grafted onto an aromatic ring like a benzene or a pyrazine. Surprisingly, the geometry optimization of the radical cation generates a non-symmetrical structure and thus asymmetric electronic and spin distributions. From these results, experimental data have been explained for so-called mixed valence organic compounds [20].

This first study deals with aromatic derivatives. Nevertheless, several other chemical classes of non aromatic molecules exhibit the same experimental behaviours. An in-depth analysis reveals the same loss of symmetry in the geometry and the electronic properties [143] confirming the previous results and underlining several features involved in the electronic transfer.

More recently, a reinvestigation of the first set of molecules allows to locate an equilibrium structure with a higher level of symmetry than the previous one. This structure could represent a transient geometry adopted by the system during the electronic transition to excited states. These numerical results have been correlated and discussed in the frame of the model developed in literature by Marcus and Husch [submitted].

6.1.2. Pharmacological molecules

Mivazerol is a benzylimidazole compound with an α-2 agonist profile. At physiological pH, this molecule can be protonated and the interactions between the imidazole ring and the carboxamide moiety can generate several tautomeric forms. Among all the equilibrium structures, quinoidic forms have been located and can be seen as intermediate conformers on the pathway followed by a proton going from one side of the molecule to the other. Moreover, in all the forms, the singlet electronic state is perturbed by a near lying triplet state giving rise to an instable Hartree-Fock (HF) wave function [49].

Several adrenergic system receptors are sensitive to molecules bearing an imidazoline ring and are named I receptors. These molecules belong to very heterogenous aromatic families and only share a common substituent. Moreover, biochemical investigations reveal that these receptors could be coupled to mono amine oxydase (MAO) redox systems. A semi-quantative relationship has been found between the biological response and HF wave function instability of about twenty molecules with high and low I receptor affinity [submitted]. These observations allowed the design of new compounds with dissociation constants in the nanomolar range.

6.1.3. Hartree-Fock (HF) wave function instability

From all these studies on very different molecular systems, it appears that the existence of an HF instability is a characteristic of most unsaturated molecules. An extensive study of one hundred molecules confirms this feature [145]. The triplet perturbation is related with spin properties of systems that possess electrons with nonnull orbital angular momentum, typically π electronic systems. The spin coupling pattern between the singlet and the triplet states is maybe influenced by this orbital angular momentum. So, one can expect the occurrence of triplet instabilities in systems that do not present unsaturated sites as such, but that acquire, in a certain region of nuclear conformations, a nonnull orbital angular momentum. This feature can be correlated with the very local instability found in models of enzymatic systems due to the aromatic residues.

6.2. Exploration of energy hypersurfaces

The location of equilibrium structures on an energy hypersurface remains a very complex task due to the topological profile which considerably varies with the nature of the explored molecular system. The valleys and the saddles of the energy mountains are characterized by positive and negative curvatures, respectively, along the reaction coordinate. Nevertheless some regions can exhibit a very shallow curvature and can be associated to bifurcation or branching regions between several paths [144].

6.2.1. Conformational map

In the case of a new I-selective imidazoline molecule, the 2D conformational analysis of this subsituent in interaction with an alcohol moiety allowed to locate about twenty equilibrium structures which can be connected by very complex paths. In this subspace, several energy maxima in the two-dimension maps exhibit a lower energy than other ones in one direction only [in press].

6.2.2. Acylation mechanism of the serine proteases

The previous studies concerning α-chymotrypsin models highlighted the energy deformation of the two partners involved in the formation of the Michaelis complex. In order to model the acylation reaction at the *ab initio* quantum level, only small models can be considered. In a stepwise manner, a series of such models have been built by progressive addition of molecules mimicking the involved amino acid residues: a methanol for a serine and an imidazole for the histidine of the charge relay system. For a series of models of increasing complexity, the equilibrium structures have been localized and the intrinsic reaction pathway which connects them has been determined. The most interesting feature is the correlation between the free energy differences calculated and observed for several mutants of the catalytic triad [48]. The same approach has been applied to coumarinic derivatives as mechanism-based inhibitors of α-chymotrypsin and human leukocyte elastase [148].

In class A β-lactamases, the two amino acids directly involved in such a reaction are Ser70 and Ser130. In the Michaelis complex between TEM1 and benzylpenicillin, optimized at the molecular mechanics level, the two Ser residues are already very well positioned to easily reach the transition state conformation. The role of the oxyanion hole environment is very important in the stabilization of this transient structure. The role of Lys73, close to the two Ser residues, is indirect and coupled with that of the oxyanion hole stabilizers [134].

6.3. Drug design and Molecular modelling

In collaboration with other laboratories, several applications have been analysed in the fields of drug design, modelling of chemical reactions and electronic spectroscopy.

6.3.1. Drug design

In the past, our collaboration with the *Pharmacochimie moléculaire* laboratory of Prof. J.J. Godfroid (Paris VII) allowed to define electronic properties of platelet-activating-factor (PAF) molecules.

A novel interpretation has been proposed concerning the electronic cloud generated by trimethoxybenzoyl groups grafted on a piperazine cycle in the design of new PAF antagonists [46, 47]. The structure-activity relationships have been underlined in dual PAF and acetylcholinesterase inhibitors derived from tetrahydrofuran [45].

The imidazoline ring attached to a piperazine does not induce an I-receptor affinity. By contrast, new derivatives influence glucose homeostasis in a rat model of type II diabetes [79, 136].

In the search of new RGD peptidomimetics, the conformational disposition of the pharmacophores plays an important role in the bio-derivatization of polymer substrates [103].

6.3.2. Organic synthesis models

The search of equilibrium structures on energy surfaces leads to the determination of the reaction heat. This approach has been applied to the asymmetric oxyamination of dienes by acylnitroso compounds synthetized in the laboratory of Prof. L. Ghosez (Louvain-la-Neuve) [104]. Similarly, access to pentaradialene has been investigated by a stepwise introduction of π-electron cross-conjugation [78].

6.3.3. Electron spectroscopy

The vibrational analysis determined by high level *ab initio* calculations can be correlated with the high resolution UV photoabsorption spectrum of CH_3F [in press] and photoelectron spectroscopy of vinylbromide [149].

7. Other projects

7.1. D-aminopeptidases

7.1.1. DAP

Despite a very low degree of sequence identity, striking structural similarities have been highlighted between DD-peptidases and β-lactamases (classes A, C and D enzymes) by comparing their known 3D structures. On the basis of this observation, all active serine enzymes which interact with penicillin form the superfamily of serine penicillin-recognizing enzymes. Members of this superfamily exhibit enzyme activities such as DD-carboxypeptidase, DD-transpeptidase, β-lactamase and D-aminopeptidase or behave as penicillin sensors. The D-aminopeptidases and DD-peptidases act on different ends of D-alanine containing peptides. For this reason, we have purified and crystallized the D-aminopeptidase from *Ochrobactrum anthropi* (DAP) [Asano *et al.*, 1992]. DAP (520 residues) shares 25 % sequence identity with the R61 DD-peptidase and class C β-lactamases and is inhibited by various β-lactam antibiotics. The crystal structure of DAP has been determined to 1.9 Å resolution in collaboration with C. Bompard-Gilles and J. Van Beeumen (Rijksuniversiteit-Gent, Belgium) [submitted]. In the crystal and in solution, DAP is a dimer. The monomer consists of three domains A, B and C. The structure of domain A (residues 1-331) which contains the conserved catalytic residues, has the classical fold of serine penicillin-recognizing enzymes. Both domains B and C are antiparallel eight-stranded β-barrels. This result emphasizes that this superfamily of enzymes provides a good example of molecular adaptation.

7.1.2. DmpA and DppA

7.1.2.1. DmpA

In contrast to the numerous DD-carboxypeptidases, only a very small number of aminohydrolases recognize the N-terminal end in a D-stereospecific way. Besides DAP, *O. anthropi* LMG799 produces very small amounts of another protein, DmpA, which also hydrolyses D-alanyl-paranitroanilide (D-Ala-pNa). This enzyme was characterized after the corresponding gene was cloned and sequenced [124]. The stereospecificity of DmpA depends on the nature of the substrate. This enzyme hydrolyses the simple derivatives of D-alanine (D-alanyl-amide, -methyl ester and –p nitroanilide) faster than their L-homologues. In contrast, with peptides, DmpA is a strict L-aminopeptidase the activity of which depends upon the nature of the first N-terminal residue. Devoid of activity towards dipeptides exhibiting an acidic N-terminal residue, DmpA is fully active towards dipeptides with a basic residue in the same position [125]. DmpA belongs to the structural family of the N-terminal nucleophile residue

(Ntn) hydrolases in which the amino group of the N-terminal nucleophile residue (Ser, Thr or Cys) plays the role of general base in the activation of an active serine, cysteine or threonine [119]. An exploration of the databases highlights the presence of a number of open reading frames (ORF) encoding related proteins in various bacterial genomes. Thus DmpA is very probably the prototype of an original family of Ntn amino hydrolases.

7.1.2.2. DppA

In order to find new D-aminopeptidases, strains of Bacilli were screened with the chromogenic compound D-Ala-pNa. The cloning and characterization of a new D-alanyl-amino-peptidase from *B. subtilis* 161 were achieved. This protein is encoded by the first ORF of the dipeptide transport operon, a gene called *dppA* the product (DppA) of which had not been biochemically characterized before. DppA has been overproduced in *E. coli*, purified and sent to the *Laboratorium voor Eiwitengineering* (Prof. J. Van Beeumen, Gent) where crystals of DppA and of its heavy derivatives have been obtained. DppA is a strict D-stereospecific aminopeptidase far more active on D-Ala-pNa ($k_{cat}/K_M = 100{,}000\ M^{-1}s^{-1}$) than on its best peptide substrates $(D\text{-}Ala)_2$ and D-Ala-Gly-Gly (turnover numbers of about $5\ min^{-1}$). Molecular sieve chromatography suggested that the active enzyme was an octamer composed of identical subunits. Inhibition was observed in the presence of Zn^{2+}-chelators and the initial activity could be recovered by addition of $10\ \mu M\ Zn^{2+}$. An exploration of the databases revealed 11 ORFs encoding putative proteins similar to DppA. Some amino acid residues, involved in Zn^{2+} binding in metallo-enzymes, are conserved in these DppA-related sequences. A DppA-like enzyme seems to exist in very different genera (Gram-positive, Gram-negative, *Streptomyces*, Archaeae), both sporulating (*Bacillus*, *Clostridium*, ...) and non-sporulating (*Deinococcus*, Archaeae). No protein homologous to DppA had been biochemically characterized before. In consequence, DppA is the prototype of a new family of D-aminopeptidases.

7.2. Construction of *Bacillus* production vectors

The most generally used organism for the production of recombinant proteins is *Escherichia coli*. This organism however presents some inconvenients: it contains endotoxins and the overproduced proteins are mainly localised in the cytoplasm or in the periplasm of the cell which can render purification of the protein of interest difficult. It also occurs that the protein of interest is not produced, is degraded or is trapped in inclusion bodies and hardly recoverable in its native form. To overcome these inconvenients, several vectors have been developed in alternative systems like *Pichia pastoris* or the baculovirus.

The genus *Bacillus* appeared to be another possible choice for the production of recombinant proteins. *Bacillus* is used in industry to produce a large range of hydrolytic enzymes and presents several advantages. It does not produce endotoxins and can secrete large quantities of proteins in the culture medium. Moreover, the genetics of *Bacillus* are well documented. In spite of that, vectors for the production of proteins in *Bacillus* are not commercially available. Therefore, *Bacillus* production vectors were constructed.

These vectors contain a cassette including the following elements:
- a promoter (regulated or constitutive),
- a ribosome-binding site,
- a secretion signal sequence,
- a multiple cloning site to introduce the gene of interest,
- a stop codon (preceded or not by a 6 His-tag),
- two different transcriptional terminators located upstream of the promoter and dowstream of the stop codon.

This cassette has been introduced in several vectors:
- an integrative vector, the cassette being integrated in the *amy*E locus of the *Bacillus subtilis* chromosome,
- a high-copy shuttle plasmid (*E. coli – Bacillus*),
- a low-copy shuttle plasmid (*E. coli – Bacillus*).

Three different proteins have been successfully produced with these different vectors using *B. subtilis* as the host.

In order to improve the production, a genetic algorithm program was written, that can optimize the culture medium composition. The best medium obtained with this program is now in use for studying the production in a 15-litre fermentor.

In industry, one of the most frequently used strains is *Bacillus licheniformis*. However, this bacterium cannot be transformed with classical methods. A system, based on the use of a conjugative transposon, has now been constructed that allows the introduction of genetic material in *B. licheniformis*.

7.3. Construction of *Streptomyces* secretion vectors

Streptomyces is another possible alternative host organisms for the secretory production of recombinant proteins.

We are working on the construction of a secretion shuttle vector, where gene expression is controlled by a regulatory protein from *Streptomyces*.

An *E. coli–Streptomyces* shuttle vector was constructed where the gene of interest was under the control of one of two natural regulated systems. The first one is based on the *Streptomyces cacaoi* β-lactamase system, regulated by β-lactam

antibiotics. The second one involves the hemicellulase regulated system of *Streptomyces* sp., induced by a degradation product of a wood component. Some homologous and heterologous productions are currently under progress.

7.4. Metal ion binding capacity of *Enterococcus hirae* cell wall derivatives

Nickel was used to compare the metal binding capacity of purified cell walls of three *Enterococcus hirae* strains, exhibiting different penicillin sensitivities. The cell walls of the penicillin-resistant strain R40 (MIC: 80 $\mu g\,ml^{-1}$) showed a higher affinity than that of the wild type (MIC: 1 $\mu g\,ml^{-1}$) and the sensitive (MIC: 0.05 $\mu g\,ml^{-1}$) strains.

From the application of several adsorption models to the nickel binding, it was concluded that (i) nickel adsorption results from a multilayer sorption mechanism, (ii) two sites with high and low affinities are involved in this process, (iii) negative cooperativity occurs between sites. The removal of phosphate groups (teichoic acids) increased nickel binding to carboxyl groups [76].

7.5. Genetic studies of a peptide antibiotic production by *Enterococcus faecalis* S48

A 7.8 kb region of the plasmid pMB2 from *Enterococcus faecalis* S48 carrying the information necessary for production of the peptide antibiotic AS-48 and responsible for its immunogenic properties was cloned and sequenced. It contains the *as-48A* structural gene plus five other open reading frames. Besides AS-48D, the four other gene products are thought to be membrane-bound and involved in AS-48 production and secretion. Immunity to AS-48 is due to AS-48D1. Transcription analysis suggests that the different genes are organized in two constitutive operons [114].

7.6. Sequencing of the *Bacillus* genome

In the framework of the EC-Biotechnology programme (EC contracts: BIO2-CT93-0272 and BIO2-CT94-2011), we have sequenced 71.6 kb of the *Bacillus* genome [80, 85].

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[148] L. Pochet, C. Doucet, G. Dive, J. Wouters, B. Masereel, M. Reboud-Ravaux and B. Pirotte. Coumarinic derivatives as mechanism-based inhibitors of α-chymotrypsin and human leukocyte elastase. *Bioorg. Med. Chem.*, 2000, **8**, 1489–1501.

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COLLABORATIONS

ARGENTINA

Universidad de Buenos Aires — Departamento de Microbiologia y Immunologia (G. Gutkind, M. Mollerach, A. Amoroso).

BELGIUM

FUSAGx — Bioindustries (P. Thonart, P. Jacques).

FUSAGx — Département de Biophysique Moléculaire Numérique, Gembloux (R. Brasseur, X. Gallet).

FUSAGx — Service de Chimie générale et organique (M. Marlier, J. Delcarte).

FUNDP — Unité de Recherches en Biologie Moléculaire, Namur (J. Vandenhaute, E. Depiereux, J. Wouters).

KUL — Faculteit Farmaceutische Wetenschappen, Leuven (H. De Bondt, C. De Ranter).

RUG — Department of Biochemistry, Physiology and Microbiology, Gent (M. Claeyssens).

RUG — Laboratorium voor Eiwitbiochemie en Eiwitengineering, Gent (J. Van Beeumen, B. Devreese).

SmithKline Biologicals — Usine des Antibiotiques (Ph. Dehottay, M. Piecq).

UCL — Biochimie Physique et des Biopolymères, Louvain-la-Neuve (J. Fastrez).

UCL — Service de Chimie Organique de Synthèse, Louvain-la-Neuve (L. Ghosez, J. Marchand-Brynaert).

UCL — Service de Chimie Quantique, Louvain-la-Neuve (G. Leroy, D. Peeters).

UCL — Unité de Génétique Moléculaire, Louvain-la-Neuve (J. Delcour).

UCL — Unité de Génie Biologique, Louvain-la-Neuve (P. Ledent).

UCL — Unité de Pathogénie Microbienne, ICP, Bruxelles (G. Cornélis).

ULB — Laboratoire de Chimie Physique des Macromolécules aux Interfaces, Bruxelles (J.M. Ruysschaert).

ULB (CERIA) — Service de Microbiologie, Bruxelles (M. Penninckx, V. Stalon, V. Villeret).

ULg — Centre Wallon de Biologie Industrielle, Liège (Ph. Thonart).

ULg — Cyclotron, Physique Nucléaire Expérimentale, Liège (G. Weber).

ULg — Département de Chimie Physique, Liège (E. de Pauw).

ULg — Laboratoire de Bioénergétique, Liège (F. Sluse, C. Duyckaerts).

ULg — Laboratoire de Biologie Moléculaire et de Génie Génétique, Liège (J. Martial).

ULg — Laboratoire de Référence SIDA, Liège (C. Forceille).

ULg — Laboratoire de Technologie Pharmaceutique, Liège (L. Delattre, G. Piel).

ULg — Service de Biochimie, Liège (C. Gerday).

ULg — Service de Chimie Industrielle, Liège (M. Crine).

ULg — Service de Chimie Organique, Liège (L. Christiaens).

ULg — Service de Chimie Pharmaceutique, Liège (J. Delarge, B. Pirotte).

ULg — Service de Chimie Macromoléculaire et de Chimie Physique, Liège (C. Houssier).

ULg —+ Service d'Écologie Microbienne et de Radioécologie, Liège (J. Remacle).

ULg — Service de Physique Expérimentale, Liège (G. Llabres).

ULg — Service de Pharmacognosie, Liège (L. Angenot, M. Frederich).

VUB — Laboratorium voor Anthropogenetica, Brussel (M. Kirsch-Volders).

CANADA

Shriners Hospital for Crippled Children — Genetics Unit, Montréal (A. Moreau).

Université du Québec, Institut Armand Frappier — Centre de Microbiologie appliquée, Ville de Laval (R. Morosoli).

Université Laval — Département de Microbiologie, Sainte-Foy (R.C. Levesque).

University of Alberta — Department of Microbiology and Biochemistry, Edmonton, Alberta (M. James, S. Jensen).

EGYPT

National Research Centre — Natural and Microbial Products Department, Cairo (H.A. El-Enshasy).

FRANCE

CNRS — Institut de Biologie Structurale, Laboratoire de Cristallographie Macromoléculaire, Grenoble (O. Dideberg, J.P. Ebel).

CNRS — Institut de Pharmacologie et de Biologie Structurale, Toulouse (J.P. Samama)

ESRF — Grenoble (G. Léonard, M. Roth).

Hoechst Marion Roussel — Romainville, France (J. Aszodi, M. Lampilas).

Hôpital Cochin — Paris (A. Philippon).

Institut Pasteur — Unité des Agents Antibactériens, Paris (P. Courvalin, M. Arthur).

Institut Pasteur — Unité de Génétique des Mycobactéries, Paris (B. Gicquel).

Institut Pasteur — Unité de Génie Microbiologique, Paris (B. Gicquel, J. Timm, J. Trias).

Servier-Adir — Recherche et Développement, Courbevoie/Paris (P. Renard).

Station Biologique de Roscoff — Université Paris VI et CNRS (B. Kloareg, T. Barbeyron, D. Flament).

Université Claude Bernard Lyon I — Laboratoire de Biochimie Microbienne, Villeurbanne (M. Guinand, M. Malissard, F. Chavagnat).

Université Paris VI — Laboratoire de Recherches Moléculaires sur les Antibiotiques, Paris (L. Gutmann).

Université Paris VII — Département de Pharmacochimie Moléculaire, Paris (J.J. Godfroid).

Université Paris-Sud — Département de Physique des Solides, Orsay (A. Moradpour).

Université Paris-Sud — Département de Biochimie Moléculaire et Cellulaire, Orsay (J. van Heijenoort, D. Mengin-Lecreulx, J. Nguyen).

Université Paris-Sud — LURE, Orsay (R. Fourme, A. Lewit-Bentley, B. Shepard).

GERMANY

EMBL — Outstation, Hamburg (V. Lamzin).

EMBL — Department of Molecular modelling, simulation and design, Heidelberg (R.C. Wade).

Max-Planck Institut für Entwicklungsbiologie — Abteilung Biochemie, Tübingen (J.V. Höltje).

RWTH — Institut für Biologie I, Aachen (K. Hoffmann).

Universität Bayreuth (F.X. Schmid).

Universität Kaiserslautern — Fachbereich Biologie, Abt. Mikrobiologie (R. Hakenbeck).

Universität des Saarlandes — Fachberichtung 12.4 Biochemie, Saarbrücken (M. Zeppezauer, H.W. Adolf).

INDIA

Bose Institute — Department of Chemistry, Calcutta (P. Chakrabarti, J. Basu, M. Kundu).

ITALY

Università dell'Aquila — Dipartimento di Scienze e Tecnologie Biomediche e di Biometria, L'Aquila (A. Oratore, G. Amicosante, M. Perilli, B. Segatore, N. Franceschini, A. Felici).

Università di Cagliari — Dipartimento di Microbiologia, Cagliari (O. Massidda).

Università di Siena — Dipartimento di Biologia Moleculare, Siena (G. Rossolini).

JAPAN

Meiji College of Pharmacy — Department of Biochemistry, Tokyo (H. Ogawara, H. Urabe).

Saitama University — Department of Biochemistry and Molecular Biology, Urawa (H. Hara).

MOROCCO

Université de Meknès — Département de Biologie (F. Rhazi-Filali, A. Zaid).

POLAND

Jagellonian University — Department of Crystallography, Krakow (B. Oleksyn).

SLOVENIA

Jozef Stefan Institute — Ljubljana (R. Pain).

SPAIN

Universidad Autonoma de Madrid — Centro de Biologia Molecular, Madrid (J.A. Ayala, J. Berenguer).

Universidad de Leon (P. Liras, J.F. Martin).

SWEDEN

Karolinska Institutet — Microbiology and Tumor Biology Centre, Stockholm (S. Normark).

National Research Defence Establishment — Department of Cell- and Microbiology, Umeå (M. Forsman).

SWITZERLAND

Université de Lausanne — Institut de Génétique et de Biologie microbienne (D. Karamata).

THE NETHERLANDS

University of Amsterdam, Department of Molecular Cell Biology, Section of Molecular Cytology (N. Nanninga, T. den Blaauwen).

UNITED KINGDOM

Rowett Research Institute — Bucksburn, Aberdeen (H.J. Flint).

SmithKline Beecham Pharmaceuticals — Harlow (J. Bateson, I. François, D. Payne).

University of Leicester — Biological NMR Centre, Leicester (G.C.K. Roberts, L.Y. Lian).

University of Newcastle-upon-Tyne — Department of Biochemistry and Genetics, Newcastle-upon-Tyne (R. Virden).

University of Oxford — Oxford Centre for Molecular Sciences, Oxford (C. Schofield, J.E. Baldwin).

University of York (J. Brannigan).

USA

Baylor College of Medicine — Houston, Texas (T. Palzkill).

California Institute of Technology — Pasadena, CA (J.H. Richards).

Cornell University — Department of Biochemistry, Molecular and Cell Biology, Ithaca, NY (D.B. Wilson, D. Irwin).

Harvard Medical School — Department of Microbiology and Molecular Genetics (J. Beckwith, N. Buddelmeijer).

The State University of New Jersey — Piscataway, NJ (J.O. Lampen).

Temple University — Medical School, Department of Microbiology, Philadelphia, Pennsylvania (L. Daneo-Moore).

University of California — Department of Biochemistry, Santa Cruz, California (A. Fink).

University of Connecticut — Biological Sciences Group, Storrs, Connecticut (J.R. Knox, J.A. Kelly, P.C. Moews).

DOCTORATE THESES

11/01/95 — Alain Dubus (Chemistry).
Mécanisme catalytique des β-lactamases de classe C.

13/01/95 — Willy Zorzi (Biochemistry).
Étude des gènes et caractérisation biochimique des PBPs de faible affinité conférant une résistance aux β-lactamines chez les espèces *Enterococcus faecium* et *Enterococcus faecalis*.

23/03/95 — Xavier Raquet (Biochemistry).
Les variants cliniques de la β-lactamase TEM-1 : étude de la stabilité et des propriétés catalytiques.

06/04/95 — Alain Brans (Biochemistry).
Étude de la machinerie de sécrétion des protéines chez *Streptomyces*.

28/04/95 — Juana Magdalena (Biochemistry).
Étude de la régulation de la β-lactamase BlaL de *Streptomyces cacaoi*.

13/06/95 — Philippe Ledent (Biochemistry).
Les β-lactamases de classe D : propriétés catalytiques et mécanisme d'inactivation partielle induit par les substrats.

21/06/95 — Karin Hardt (Biochemistry).
Topology of *Bacillus licheniformis* membrane receptor BlaR1.

13/10/95 — Marc Vanhove (Biochemistry).
Étude des phénomènes de dénaturation et de folding des protéines. Cas des β-lactamases de classe A.

13/10/95 — Kurt Hoffmann (Chemistry).
Étude du mécanisme catalytique des DD-peptidases de *Streptomyces* K15 et *Streptomyces* R61.

03/04/96 — Christian Damblon (Chemistry).
Contribution de la résonance magnétique nucléaire à l'étude des enzymes qui reconnaissent la pénicilline.

21/03/96 — Sophie Lepage (Biochemistry).
Modélisation de la croissance d'une culture de *Bacillus licheniformis* en présence d'antibiotiques.

24/06/96 — Guohua Zhao (Biochemistry).
The mechanism of DD-peptidases.

11/10/96 — Christine Jacobs (Biochemistry).
Signaling pathway for β-lactamase induction in enterobacteria.

29/11/96 — Olivier Dardenne (Sciences).
Chez les entérocoques (*E. hirae* et *E. faecium*) la résistance aux pénicillines est déterminée par le gène de la protéine de faible affinité liant la pénicilline et par le gène répresseur *psr*.

08/10/97 — Laurence Fanuel (Biochemistry).
À la recherche de D-aminopeptidases chez *Ochrobactrum anthropi* et *Bacillus subtilis*.

10/12/97 — Pierre Partoune (Sciences).
Production, purification et étude d'une forme soluble de la protéine membranaire PBP5 conférant une résistance aux β-lactamines chez *Enterococcus hirae*.

17/12/97 — Nezha Laraki (Biology).
β-Lactamase à zinc de *Pseudomonas aeruginosa* 101/1477 : propriétés et support génétique.

05/06/98 — Gilliane Guillaume (Biochemistry).
Étude des résidus de la boucle Ω dans le mécanisme catalytique des β-lactamases de classe A.

19/06/98 — Christine Gérard (Biochemistry).
La régulation de la β-lactamase BlaU chez *Streptomyces cacaoi*.

06/07/98 — Axelle Loriaux (Biochemistry).
Identification des éléments impliqués dans la machinerie de sécrétion des protéines chez *Streptomyces coelicolor* A3(2).

21/08/98 — Fabrizio Giannotta (Sciences).
Éléments *cis* et *trans* dans la régulation de la xylanase C de *Streptomyces* sp. EC3.

29/09/98 — Maria Hernandez-Valladares (Biochemistry).
The *Aeromonas hydrophila* AE036 metallo-β-lactamase.

12/10/98 — Raquel Paul-Soto (Biochemistry).
The role of Zn ions in the *Bacillus cereus* and *Bacteroides fragilis* β-lactamases.

25/02/99 — Jacques Georis (Biochemistry).
Étude de l'adaptation sélective aux hautes températures d'une xylanase mésophile.

09/06/99 — Éric Sauvage (Sciences).
Étude structurale et modèle théorique de l'activité enzymatique des β-lactamases de classe A.

FINAL YEAR LICENCE PROJECTS

1994-95	Biochemistry:	Myriam Bruls Pascale Frare Annabelle Lejeune
	Chemistry:	Fabrice Bouillenne
1995-96	Biochemistry:	Annick Pierrard
	Botany:	Marie-Alice Meeuwis Frédéric Sapunarić
	Chemistry:	Florence Gillman
	Zoology:	Arnold Weber
1996-97	Biochemistry:	Pascal Brouillard Olivier Corvilain Dominique de Seny Etienne Goossens Séverine Hallut Serge Leimanis
	Botany:	Frédéric De Lemos-Esteves Françoise De Longueville Frank Smitz Sébastien Rigali
	Zoology	David van Lochem
1997-98	Biochemistry:	Michael Delmarcelle Jean-Denis Docquier Philippe Noël Catherine Raskin Patrick Stefanić
	Zoology:	André Piette

1998-99	Biochemistry:	Carine Bebrone Maria-Luigi Colombo Jean-Baptiste Mayet Nadia Ruth Anne Sanglier
	Zoology:	Christina Franssen
	DES in Protein Engineering:	Cédric Beauvois

VISITORS

Amoroso, Ana (Universidad de Buenos Aires, Argentina)	8/1/98 to 22/12/98 20/1/99 to 20/5/99
Arnout, F. (Puratos N.V., Groot-Bijgaarden, Belgium)	19/1/98
Ayala, Juan A. (Universidad Autonoma de Madrid, Spain)	5 to 11/10/98 10 to 16/5/99
Barbeyron, Tristan (Station Biologique de Roscoff, France)	3 weeks in March 96
Basu, Joyoti (Bose Institute, Calcutta, India)	one week each year
Dauvrin, Van Este, Janssen (Frimond S.A., Andenne, Belgium)	19/1/98
Fairley-Marrec, Monique (HMR, Romainville, France)	May 96 to Feb. 99
Flament, Didier (Station Biologique de Roscoff, France)	3 weeks in March 96
Geneste, Florence (Laboratoire de Physique du Solide, Paris XI)	20/11/95 to 3/12/95
Gutmann, Laurent (Université de Paris VI, France)	13/1/95
Hai Fen Ye (Laboratoire de Pharmacochimie Moléculaire, Paris VII)	8/12/97 to 17/12/97
Hassan, Ibrahim (University of Bristol, U.K.)	13/2 to 7/3/97
Ishii, Yoshikazu (Toho University, Tokyo, Japan)	Jan. to Dec. 1999
Kelly, Judith (University of Connecticut, Storrs, CT, USA)	11 to 15/10/95 3 to 7/8/97
Kessler, R. (Bristol Myers, Wallingford, CT, USA)	8/3/95
Knox, Jim R. (University of Connecticut, Storrs, CT, USA)	3 to 7/8/97
Magnus, Walter (University of Oxford, U.K.)	10 to 14/7/95
Magyar, Csaka (Institute of Enzymology, BRC, Budapest, Hongrie)	3 weeks in Sept. 99
Moyano, Sandra (Universidad de Los Andes, Bogota, Colombia)	1/6/98 to 15/4/99
Nanninga, Nanne (University of Amsterdam, The Netherlands)	25/10 to 14/11/98
Pain, Roger (Jozef Stefan Institute, Ljubljana, Slovenia)	20 to 23/1/95 19 to 24/5/95 3 to 7/3/97
Pérez-Llarena, F. Jose (Universidad de Léon, Spain)	1/2/95 to 29/4/95 1/2/99 to 29/2/00
Rhazi-Filali, Fousia (Université de Meknès, Morocco)	1/1/95 to 31/3/95
Rodriguez, Grisel (Universidad de Montevideo, Uruguay)	1/8/98 to 31/12/99
Rossolini, Gianmaria (Università di Siena, Italy)	24/6 to 20/7/96

Samama, Jean-Pierre (CNRS, Toulouse, France)	9/6/99
Turk, Boris (Jozef Stefan Institute, Ljubljana, Slovenia)	9/3 to 22/3/97
van Heijenoort, Jean (Université de Paris-Sud, France)	26/1/2000
Vilar, Mateja (Jozef Stefan Institute, Ljubljana, Slovenia)	8/3 to 30/5/98 19/4 to 1/6/99
Wéry, Jean-Pierre (Eli Lilly, Indianapolis, IN, USA)	25/11/98
Wouters, Johan (FUNDP, Namur, Belgium)	11 to 15/10/99
Zanin, Valérie (Université de Compiègne, France)	15/5/95 to 30/5/95

CONFERENCES, CONGRESSES*

Bacteriology 1995, Pasteur Institute Lectures, Paris, France, February 9, 1995 (**J.M. Frère**).

7th European Congress on Biotechnology, Nice, France, February 13–19, 1995 (J. Georis, B. Granier).

Euregio Biotechnology Symposium, Maastricht, The Netherlands, March 1995 (J. Georis).

19th International Congress of Chemotherapy, Montreal, Quebec, Canada, 1995 (**J.M. Ghuysen**, member of the International Scientific Committee).

6th International β-Lactamase Workshop, Holy Island, U.K., April 9–13, 1995 (**P. Charlier**, J.M. Frère, J. Brasseur, C. Damblon, E. Fonzé, M. Galleni, G. Guillaume, P. Ledent, R. Paul-Soto, B. Quinting, X. Raquet, E. Sauvage, M. Vanhove).

Jozef Stefan Institute, University of Ljubljana, Slovenia, April 18–19, 1995 (**J.M. Frère**).

Workshop on "Structure, Function and Controls in Microbial Division", Instituto Juan March de Estudios e Investigaciones, Madrid, Spain, May 22–24, 1995 (**J.M. Ghuysen**, **M. Nguyen-Distèche**).

14th International Conference of the Molecular Graphics and Modelling Society "Molecular Design down under", Cairns, Australia, August 27 – September 1, 1995 (J. Brasseur, X. Raquet).

"Peptidomimetics: Design, Synthesis, Structure", Spa, Belgium, September 4–8, 1995 (**J.M. Frère**).

Synphar 4th International Conference, "Antibiotics: Current Problems and Future Trends". Radium Hot Springs, Alberta, Canada, September 10–13, 1995 (**J.M. Frère**).

International Symposium "The Envelope in Bacterial Physiology and Antibiotic Action", Garda, Italy, September 10–14, 1995 (**J.M. Ghuysen**, member of the Organizing Committee; P. Charlier, **J. Coyette**, O. Dardenne, C. Duez,

* Speakers are indicated in bold letters.

E. Fonzé, C. Fraipont, C. Goffin, B. Joris, M. Nguyen-Distèche, P. Partoune, D. Raze).

8th Workshop of the French-German Society for Development in Research and Technology (DFGWT/AFAST) "*Streptomyces*, *Corynae* and *Bacillus* as Cell Factories", Frankfurt am Main, Germany, November 19–21, 1995 (J. Dusart, **A. Brans**, A. Loriaux, **J. Magdalena**, C. Gérard, F. Giannotta).

Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm, Sweden, March 12, 1996 (**J.M. Frère**).

CERC-3 Meeting "Advanced Computational Studies of Increasingly Complex Chemical Systems", Vienna, Austria, March 17–21, 1996 (**G. Dive**).

Semestrial meeting of the *Comité National Belge de Cristallographie*, Liège, Belgium, March 20, 1996 (**P. Charlier**, E. Fonzé, E. Sauvage).

4th Meeting of the National Committee of Microbiology "Biosafety: a Scientific Challenge in Medicine and Biotechnology, Brussels, Belgium, March 22, 1996 (J. Dusart, member of the Organizing Committee).

Conference on "Biology of Streptomycetes", Osnabrück, Germany, April 10–15, 1996 (J. Dusart, C. Gérard, **J. Magdalena**, F. Giannotta).

15th International Conference of the Molecular Graphics and Modelling Society "Molecular Interactions", York, U.K., April 16–19, 1996 (J. Brasseur, G. Guillaume).

3rd Cycle Course – Paris V, VI and VII Universities "Relations structure-activité et modélisation moléculaire : *partim* Chimie théorique", 20 hours, Paris, France, May 1996 (**G. Dive**).

10es Journées Franco-belges de Pharmacochimie « De la Conception à la Réalisation en Pharmacochimie », Luxembourg, G.D. Luxembourg, May 30–31, 1996 (G. Dive).

International Meeting "Recent Advances in Macromolecular Crystallization", Obernai-Bischoffsheim, France, June 2–4, 1996 (P. Charlier).

"Structure and Biology of Receptors: Key Role in Design and New Drugs", Brussels, Belgium, June 3–4, 1996 (**J.M. Frère**).

Gordon Research Conference "Bacterial Cell Surfaces", New England College, Henniker, N.H., USA, June 30 – July 7, 1996 (**J. Coyette**).

44th Harden Conference "The Biochemical Basis of Microbial Morphogenesis", Circester, U.K., September 8–12, 1996 (M. Nguyen-Distèche).

"Quantum Chemistry in Belgium II", Leuven, Belgium, September 26, 1996 (**D. Dehareng**, G. Dive).

École Doctorale Biologie-Santé, Université de Nancy, France, November 21, 1996 (**J.M. Frère**).

Gaussian Software Workshop, Liège, Belgium, December 1996 (**G. Dive**).

7th Sanibel Symposium Quantum Theory Project, St Augustine, Florida, USA, March 1–7, 1997 (**D. Dehareng**, G. Dive).

Réunion de la Société Belge de Biochimie et Biologie Moléculaire, Liège, Belgium, April 26, 1997 (J. Coyette, C. Duez, N. Hoyez, P. Partoune, F. Sapunarić).

Table Ronde Roussel Uclaf n° 86 "Peptidoglycan Assembly and the Bacterial Cell Cycle", Versailles, France, May 28–30, 1997 (**J.M. Ghuysen**, chairman of the Scientific Committee; **M. Nguyen-Distèche**, J. Coyette, C. Fraipont, C. Duez, C. Goffin, B. Joris).

10th International Symposium on the Biology of Actinomycetes, Beijing, China, May 27–30, 1997 (**J. Dusart**).

Institut de Biologie Structurale, Grenoble, France, May 30, 1997 (**J.M. Frère**).

Euroconference "New Targets for New Antibiotics", Institut Pasteur, Paris, France, June 4–6, 1997 (J. Coyette, M. Nguyen-Distèche, A. Brans, J.M. Wilkin).

Comité SOLEIL-Synchrotron, Lille, France, June 11–12, 1997 (**P. Charlier**, E. Fonzé, E. Sauvage).

Jagellonian University, Department of Crystallography, Krakow, Poland, June 11, 1997 (**J. Brasseur**).

9th International Conference on Bacilli, Lausanne, Switzerland, July 15–19, 1997 (**B. Joris**, A. Brans, K. Benlafya, P. Filée, S. Hanique).

International Symposium dedicated to Prof. J.M. Ghuysen "From Peptidoglycan Biosynthesis to Antibiotic Resistance. Present and Future", Liège, Belgium, August 4–6, 1997 (**P. Charlier**, **J. Coyette**, **G. Dive**, **J.M. Frère**, **A. Matagne**, **M. Nguyen-Distèche**, J. Brasseur, J. Dusart, members of the Organizing Committee).

First European β-Lactamase Workshop, Anthisnes, Belgium, September 13–14, 1997 (J. Brasseur, P. Charlier, J.M. Frère, E. Fonzé, M. Galleni, E. Sauvage).

"The Bacterial Cell Cycle", Chorin (Berlin), Germany, September 13–17, 1997 (M. Nguyen-Distèche).

Quantum Chemistry in Belgium III, Brussels, Belgium, October 9, 1997 (**D. Dehareng**, G. Dive).

First Symposium of the Belgian Society for Microbiology "Evolution and Gene Transfer in Microorganisms", Leuven, Belgium, October 17, 1997 (S. Bossrez, J. Coyette).

First International Conference Paris-Shanghaï "From Traditional Medicine to Molecular Modelling", Shanghaï, China, November 4–7, 1997 (**G. Dive**, D. Dehareng).

7th International β-Lactamase Workshop, Holy Island, U.K., April 5–9, 1998 (J.M. Frère, J. Brasseur, M. Galleni).

"Computational Chemistry and the Living World", Chambéry, France, April 20–24, 1998 (G. Dive, D. Dehareng).

Académie Royale des Sciences, Brussels, Belgium, April 25, 1998 (**J.M. Frère**).

ASM Conference on Streptococcal Genetics "Genetics of the Streptococci, Enterococci and Lactococci", Vichy, France, April 26–29, 1998 (A. Amoroso, **J. Coyette**, S. Hallut, N. Hoyez, S. Leimanis, F. Sapunarić).

« Informatique Appliquée à la Chimie », Louvain-la-Neuve, Belgium, May 1998 (**G. Dive**, invited teacher).

Université Catholique de Louvain, Pharmacologie Cellulaire et Moléculaire, Brussels, Belgium, June 2, 1998 (**J.M. Frère**).

FNRS Contact Group « Étude des Fonctions d'Onde Atomiques et Moléculaires », Louvain-la-Neuve, Belgium, June 5, 1998 (**G. Dive**, D. Dehareng, D. Peeters).

Meeting of the Zn^{++} β-Lactamase Network, L'Aquila, Italy, July 3–5, 1998 (J.M. Frère, M. Galleni, P. Mercuri).

13th Canadian Symposium on Theoretical Chemistry, The University of British Columbia, Vancouver, British Columbia, Canada, August 2–7, 1998 (**G. Dive**, D. Dehareng).

International Society of Quantum Biology and Pharmacology Meeting "Molecular Structure and Dynamics in Biology", Elba, Italy, September 8–11, 1998 (**G. Dive**, D. Dehareng).

141st Meeting of the Society for General Microbiology "Portrait of an Organism: the Genetic Analysis of *Streptomyces coelicolor* A3(2) biology", Norwich, U.K., September 9–10, 1998 (J. Dusart).

Symposium to mark the contribution by David Hopwood to *Streptomyces* genetics, Norwich, U.K., September 10–12, 1998 (J. Dusart).

Biochemical Society Meeting no. 667, Leicester, U.K., September 21–23, 1998 (**J.M. Frère**).

Université Libre de Bruxelles, Institut de Recherche Interdisciplinaire, Brussels, Belgium, September 24, 1998 (**J.M. Frère**).

Workshop on "Targets for New Antimicrobials Directed to Inhibit Bacterial Proliferation", Verona, Italy, September 30 – October 1, 1998 (**M. Nguyen-Distèche**).

Europa Bio 98, Brussels, Belgium, October 27–30, 1998 (**J.M. Frère**).

2nd Symposium of the Belgian Society for Microbiology "Apoptosis and Microorganisms", Leuven, Belgium, November 12, 1998 (J. Dusart).

Colloque de Chimie Organique, Louvain-la-Neuve, February 25, 1999 (**G. Dive**).

"Microbial Adaptation and Diversity", Brussels, Belgium, March 24, 1999 (**J.M. Frère**).

Biovision – Forum Mondial des Sciences de la Vie, Lyon, France, March 26–29, 1999 (**J.M. Frère**).

EC Sectorial Meeting "Controlling the Proliferation of the Microbial Cell Factory", Verona, Italy, April 19–21, 1999 (C. Fraipont, M. Nguyen-Distèche).

3^{e} Cycle Interuniversitaire en Chimie Physique Moléculaire du FNRS, May 1999 (**G. Dive**, teacher and co-organizer).

University of Connecticut, Department of Biochemistry, Storrs, USA, May 28, 1999 (**J.M. Frère**).

99th General Meeting of the American Society for Microbiology, Chicago, USA, May 30 – June 3, 1999 (**J.M. Frère**).

Lilly Research Laboratories, Indianapolis, USA, June 2, 1999 (**J.M. Frère**).

Procter & Gamble Pharmaceuticals, Cincinnati, USA, June 3, 1999 (**J.M. Frère**).

"β-Lactamase Mediated Resistance: Molecular Aspects and Clinical Implications", L'Aquila, Italy, June 17–20, 1999 (**J. Brasseur**, J.D. Docquier, **J. Dusart**, M. Galleni, **J.M. Frère**, G. Guillaume, **F. Mahy**, A. Matagne).

Chemotherapy for the 21st Century, Birmingham, U.K., July 4–7, 1999 (**J.M. Frère**).

9th European Congress on Biotechnology, Brussels, Belgium, July 11–15, 1999 (**J.M. Frère**).

3rd International Conference on Molecular Structural Biology, Vienna, Austria, September 8–12, 1999 (J. Brasseur).

3rd European Symposium of the Protein Society, Garmisch-Partenkirchen, Germany, September 19–22, 1999 (A. Matagne, S. Hanique, A. Lejeune).

3rd Meeting of the Slovenian Biochemical Society, Portoroz, Slovenia, September 25–29, 1999 (**J.M. Frère**).

"EC Biotechnology Consortium", Edinburgh, U.K., September 27–28, 1999 (**M. Nguyen-Distèche**).

Euregio Biotechnology Symposium, Liège, Belgium (J. Georis).

Lilly Laboratories, Mont Saint-Guibert, Belgium, October 8, 1999 (**J.M. Frère**).

11th International Symposium on the Biology of Actinomycetes, Heraklion, Greece, October 24–29, 1999 (A. Brans, J. Dusart, J. Georis, F. Giannotta, C. Raskin, S. Rigali).

First Belgian Crystallography Symposium, Academy House, Brussels, Belgium, October 28, 1999 (E. Fonzé, E. Sauvage, F. Kerff).

"Proteins: from Structure towards their Design", Brussels, Belgium, November 18, 1999 (**J.M. Frère**).

Quantum Chemistry in Belgium IV, Antwerpen, Belgium, November 23, 1999 (**M.N. Ramquet**, G. Dive, D. Dehareng).

3rd Symposium of the Belgian Society for Microbiology, "Microbial Identification: an Integral Approach", Leuven, Belgium, December 17, 1999 (J. Dusart).

STAYS ABROAD

Different stays at the LURE Laboratory, Université de Paris-Sud, Orsay, France, Dec 3–5, 1996; June 17–18, 1997; July 6–8, October 21–23, 1998; July 7–10, Dec. 1–4, 1999 (Crystallography team).

25th Crystallographic Course: Direct Methods for Solving Macromolecular Structures, NATO Advanced Study Institute, Erice, Italy, May 22 – June 2, 1997 (E. Fonzé, E. Sauvage).

EMBL Laboratory bei DESY, Hamburg, Germany, May 27–29, 1997 (P. Charlier, E. Fonzé).

Department of Crystallography, Jagellonian University, Krakow, Poland, June 7–16, 1997 (J. Brasseur).

Biochimie Moléculaire et Cellulaire, Université Paris-Sud, Orsay, France, February 17 – June 20, 1997 (M. Terrak).

Department of Molecular Modelling, Simulation and Design, European Molecular Biology Laboratory, Heidelberg, Germany, March 1 – April 30, 1998 (J. Brasseur).

Centro de Biologia Molecular, Universidad Autonoma de Madrid, Spain [June 1 – July 31, 1995 (J. Georis); March 21–26, 1998 (M. Nguyen-Distèche); December 9–19, 1998 (M. Terrak)].

HERCULES 99 Course (Higher European Research Course for Users of Large Experimental Systems), session B "Neutron and Synchrotron Radiation for Biomolecular Structure and Dynamics", Grenoble, France, February 22 – April 1, 1999 (F. Kerff).

Different stays at the ESRF Laboratory (European Synchrotron Radiation Facilities), Grenoble, France, April 12–13, June 5–6, August 29–30, November 21–22, 1999 (Crystallography team).

LIST OF THE ORGANISMS AND SOCIETIES HAVING SUPPORTED THE CIP

— Les Pôles d'Attraction Interuniversitaires
— Le Secrétariat Permanent à la Politique Scientifique
— Les Actions de Recherche Concertée
— La Région Wallonne
— Le Commissariat Général aux Relations Internationales
— Le Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture
— Le Fonds National de la Recherche Scientifique
— Le Fonds pour le Recherche Fondamentale Collective
— Le Fonds pour la Recherche Scientifique Médicale
— L'Université de Liège
— La Loterie Nationale
— La Communauté Économique Européenne
— L'Organisation du Traité de l'Atlantique Nord
— Hoechst Marion Roussel (France)
— Servier-Adir (France)
— SmithKline Beecham (U.K.)
— Glaxo, Bruxelles
— SmithKline Biologicals, Rixensart
— UCB Bioproducts, Braine l'Alleud
— UCB Pharma, Braine l'Alleud

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* December 1999.

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