University of Liège

CENTRE FOR PROTEIN ENGINEERING

ACTIVITY REPORT 2000–2004

University of Liège

CENTRE FOR PROTEIN ENGINEERING

ACTIVITY REPORT 2000–2004

Table of contents

INTRODUCTION

Over the 2000–2004 period, the analysis of the mechanisms of bacterial resistance to β-lactam antibiotics has remained the major research field of the Centre for Protein Engineering (CIP).

Bacteria utilize four distinct mechanisms to avoid the lethal effects of antibiotics:

- inactivation of the antibiotics by enzymatic mechanisms;
- modification of the antibiotic targets to decrease their affinity for these compounds;
- modification of the permeability barrier between the target and the outer medium (in Gram-negative bacteria and mycobacteria);
- active efflux systems which "pump out" the antibiotic molecules against the concentration gradient.

With β-lactam antibiotics, the first two mechanisms are prevalent. β-lactamases inactivate penicillins and related compounds by hydrolysing the endocyclic β-lactam amide bond. The targets of penicillins are DD-carboxypeptidases and transpeptidases (also called Penicillins-binding proteins or PBPs) responsible for the last biosynthesis step of peptidoglycan, a polymer whose integrity is essential to the survival of bacteria. The emergence of PBPs with vastly decreased affinities for β-lactams now represents a major public health problem. The study of these two types of enzymes constitutes the major topic of the CIP's activities. Interestingly, PBPs and active-site serine β-lactamases are structurally related. The latter enzymes have received sustained attention and several structures have been solved, sometimes in complex with inactivators or poor substrates. A lot of work has been devoted to the Zn^{++} metallo-β-lactamases whose emergence is becoming an important clinical problem, due to the ability of these enzymes to hydrolyse carbapenems, compounds which are usually not hydrolysed efficiently by the active-site serine enzymes and often utilised as last-resort antibiotics in the clinical setting. Within the frame of two successive European networks, major advances have been recorded in this field, including the determination of several structures and a better understanding of the roles of various residues in the binding of the zinc ions and the catalytic process. The study of penicillin-binding proteins (PBPs) has also produced a score of important results, including the

determination of the structure of the penicillin-resistant PBP5 of *Enterococcus faecium*, a good model for the analysis of the factors which determine the sensitivity of PBPs to β-lactam antibiotics.

Recently, the structure of the *Actinomadura* R39 low-molecular mass DD-transpeptidase also highlighted the presence of two additional domains, only encountered in a small number of PBPs and which might be involved in regulatory phenomena. In a major breakthrough, the bifunctional PBP1b of *E. coli* has been obtained as a soluble and homogeneous protein in the presence of detergents. This enzyme exhibits two functional domains, a DD-transpeptidase, penicillin-binding domain and a transglycosylase domain. The latter is a potential new target for original antibiotics which might inhibit the transglycosylase activity. No such compound of clinical utility is presently available. These studies are presently extended to other transglycosylases and glycosyl hydrolases involved in cell wall metabolism.

The cell division machinery is another potential target for new antibiotics. The study of the interaction between PBP3, involved in septum formation in *E. coli* and other proteins identified as playing a role in this process has been initiated.

Other complex mechanisms are also the goals of intense research. The induction of β-lactamase biosynthesis in Enterobacteria and in *Bacillus licheniformis* and *Staphylococcus aureus* occurs according to very different mechanisms. In Enterobacteria, peptidoglycan degradation products reenter the cell and can alter the properties of the AmpR protein. Under normal conditions, AmpR binds a precursor of peptidoglycan biosynthesis and acts as a repressor of the transcription of the β-lactamase gene. In the presence of penicillin, larger amounts of degradation products are formed and they transform AmpR into a transcriptional activator. In *Bacillus* and *Staphylococcus*, penicillin binds to the external domain of an integral membrane protein whose four transmembrane segments transmit the message inside the cell by activating the peptidase activity of a cytoplasmic domain. Whether this activity affects the Bla1 repressor directly or indirectly remains a subject of controversy, but the final result is clearly that the repressor becomes unable to bind the operator sequence, thus allowing the transcription of the β-lactamase gene. A third mechanism occurs in *Streptomyces* but although several participating proteins have been identified, their exact roles remain mysterious, with the exception of BlaA, a repressor of the LysR family.

D-aminopeptidases represent a group of enzymes which share with PBPs the unusual property of acting on substrates containing D-amino acid residues. Various D-aminopeptidases have been studied, DAP, DmpA and DppA and

their structures solved within the frames of two successive Interuniversity Attraction Poles (IUAP). The structure of the catalytic domain of DAP is similar to that of the DD-carboxypeptidase of *Streptomyces* R61 but it does not interact covalently with β-lactams. Comparison of the structures allowed to design mutations of DAP which yielded a protein exhibiting low but significant DD-carboxypeptidase and penicillin-binding activities. DmpA is a member of the N-terminal nucleophile hydrolase families. It acts as a D-alanyl esterase but with peptide substrates, only hydrolyses the peptide bond in α of Glycyl and L-alanyl N-terminal residues. The reasons for this modification of stereospecificity remain mysterious.

DppA is a decamer whose preferred substrate appears to be D-alanyl-D-alanine. The active-sites are in an internal pocket accessible through a rather narrow tunnel. In consequence it behaves as a self-compartimentalizing peptidase, but its physiological role remains poorly understood. Note that DmpA and DppA are not related to PBPs.

Protein folding is another important topic developed within the IUAP network. The proteins which serve as experimental models are several lysozymes, various active-site serine or metallo-β-lactamases and the V_HH fragments of camel antibodies (characterized by their absence of light chains) directed against some of these enzymes. Variants of human lysozyme are known that easily form amyloid fibres, which can accumulate and result in rare but lethal diseases. A V_HH fragment of anti-lysozyme antibody was found to very significantly inhibit the formation of these amyloid fibres, a finding which might have implications for the therapy of diseases due to the accumulation of this type of fibres.

The xylanase of *Streptomyces* sp S38 was used as a model for trying to increase the thermostability and modify the pH-activity profile of an enzyme by performing mutations chosen on the basis of sequence comparisons with thermophilic, acidophilic or alkalophilic enzymes. Although several of these mutations yielded the expected results, it is not clear yet why others failed to reach the expected goal.

A variety of hybrid proteins have been constructed, using the TEM β-lactamase as a template. These hybrid proteins might be used in numerous applications such as the production of vaccines or biosensors.

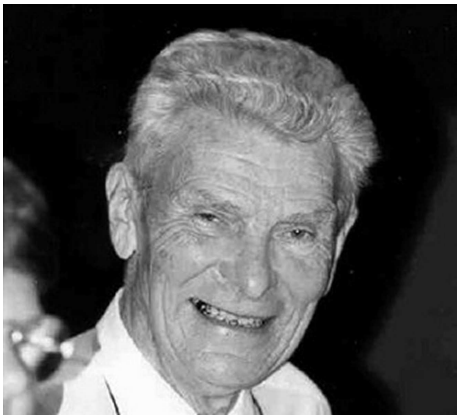
A metagenomic approach has been initiated to identify genes encoding proteins with known functions in non-cultivable organisms from soils. Indeed, the majority of bacteria cannot be presently cultivated, and metagenomics allows to get access to this new genetic diversity.

Another topic is the biodiversity of cyanobacteria, studied on the basis of molecular taxonomic markers (e.g., parts of the rRNA operon). In Antarctic

microbial mats, this study has revealed new taxa that seem to be endemic. In the frame of an EC project coordinated by CIP, a DNA chip was created and tested, to follow the dynamics of planctonic cyanobacteria in freshwater lakes where they can form toxic blooms in summer and in autumn. This resulted in an internationally patented DNA chip.

Finally, several other applied projects which all rest on the experience of the CIP in the analysis of protein structure chemistry and enzymatic properties are underway.

IN MEMORIAM JEAN-MARIE GHUYSEN



Bacteriology has recently lost one of its great contributors. Jean-Marie Ghuysen left us suddenly last August 31st as the result of septicaemia.

Born in Blégny-Trembleur, a large village next to Liège, on January 26th 1925, Jean-Marie Ghuysen was raised in the local pharmacy owned by his father. As a boy, he went to the primary school in Blégny and then to the Saint-Hadelin secondary school in Visé, a small town north of Liège.

Before he was eighteen, the young Jean-Marie had virtually no contact with scientific matters except through his father's activities. The only influence he could remember was when his father, the mayor of the village, decided around 1933–34 to install a water supply in the village to replace the central well. Together they wandered around the countryside collecting samples from springs, and analysing them in a small laboratory next to the pharmacy. As a youth, Jean-Marie lived in a literary world (old Greek, Latin, philosophy, novels), but he also dreamt of becoming a sailor. His adventurous nature led him, when he left high school, to head for Antwerp with the hope of boarding a ship. This plan was foiled when he was arrested by the police (in war time) and brought back home.

He had then no other choice but to follow his father's steps and to become a pharmacist. He discovered the Sciences at the University of Liège and became fascinated by chemistry and physics. His passion for chemistry was so great that he decided to study both pharmacy and chemistry. He obtained his degree in pharmacy in 1947 and finished the chemistry curriculum in 1948, presenting a final term essay on the "Isolation and purification of RNA".

During the occupation, Jean-Marie was not regularly registered as a university student in order to avoid being sent to Germany as a "voluntary worker".

Instead, he joined the resistance movement where he helped as a messenger and a bomb maker. His degrees were authenticated after the war was over.

He obtained a research fellowship, from 1948 to 1951, to pursue his work on RNA with Prof. V. Desreux in the department of chemistry. He published four papers which formed the core of a PhD thesis on "The study of the heterogeneity of RNA" that he presented in October 1951 and for which he received the Stas-Spring award, the first of many prizes.

He was approached by the Labaz Laboratories, a company which was planning to create a biochemistry and microbiology facility. They offered him the post of director of the laboratory and while the laboratory was being completed, he decided to improve his microbiological knowledge. With the support of the IRSIA and the Labaz Laboratories, Jean-Marie worked with Prof. M. Welsch, director of the general and medical microbiology unit of the medical faculty of the University of Liège. A young chemical engineer was hired at the same time as his deputy. The team studied bacteriolytic enzymes that were known to be part of the *Actinomycetine* secreted by some Streptomycetes. Quite rapidly they identified and separated several proteolytic activities as well as different bacteriolytic enzymes. Two enzymatic fractions FI and FII were subjected to detailed study and shown to have different peptidase activities on bacterial cell walls. Results collected over a six year period were published in twelve papers and in a thesis for the Agregation Diploma in Pharmaceutical Sciences which was awarded in 1957. In the meantime, Jean-Marie had received, in 1955, the Louis Empain prize.

When the unit at the Labaz Laboratories was ready, Jean-Marie initiated research on brain glutamate decarboxylase. However, he resigned from the company after a few months and returned to university research in January 1958. In negotiations with the University, it was agreed that he would not be involved in practical courses for the students but be free to manage his research as he pleased. With the help of M. Welsch and the rector of the University, Prof. M. Dubuisson, he was rapidly promoted and became associate professor in April 1966.

From 1958 to 1969 his research focussed on determination of the chemical structure of bacterial cell walls, using the various bacteriolytic enzymes he had purified and characterized previously. This was a period in which he collaborated extensively with a number of workers in related fields: his collaborations with Milton Salton (structure), Jack Strominger (biosynthesis) and Gerald Shockman (lytic enzymes) were particularly fruitful and established the main thrust of his research for the next two decades. The investigations he carried out were an essential contribution to the understanding of the chemical structure of the bacterial cell wall. In 1966, at a roundtable organized during a

Symposium of the American Chemical Society in Detroit, Jean-Marie proposed, with colleagues including G.D. Shockman, the word "peptidoglycan" to define the macromolecular structure which forms the skeleton of the cell wall and is the target of autolysins and other lytic enzymes. These results were gathered in an important review entitled 'Use of Bacteriolytic Enzymes in Determination of Wall Structure and their Role in Cell Metabolism' which was published at the end of 1968 in Bacteriological Reviews. The work was developed by K. Schleifer and O. Kandler who in 1972 used the different peptidoglycan types as taxonomic criteria for bacterial classification.

He was appointed full Professor in Liège in 1969 and formed a nucleus of local research workers which included Melina Leyh-Bouille, Jacques Coyette, Martine Distèche, Jean Dusart and Jean-Marie Frère. The stimulation provided by Jean-Marie and his laboratory attracted a plethora of international scientists (microbiologists, biochemists, chemists). It is not surprising that such an invigorating atmosphere increased the enthusiasm of all participants and paved the way for future successes. At the same time, the general orientation of the research also shifted from the elucidation of the structure of the bacterial peptidoglycan (a problem Jean-Marie considered as solved in 1971) to that of the mode of action of penicillin, which was known to interfere with peptidoglycan biosynthesis. The enzymes produced by various strains of actinomycetes which Jean-Marie had used as tools for the study of peptidoglycan structure became model proteins for the analysis of penicillin-target interactions. Model substrates and assay systems were developed to enable the complex peptidoglycan transpeptidation reaction to be studied using purified enzymes so that the kinetics of the interactions could be investigated. The studies were later extended to Penicillin-Binding Proteins from *E. coli* and enterococci. In 1976, the team contributed a major advance in the field by demonstrating that penicillin acylated a serine residue in one of the *Streptomyces* penicillin-sensitive D-alanyl-D-alanine carboxy transpeptidases, a reaction which was later found to account for the inactivation of all PBPs by β-lactam antibiotics. Surprisingly, this seminal paper is seldom cited, it seems that the result is now considered as self-evident! Not surprisingly, the "sensitivity" of a PBP to a particular penicillin was found to be dependent mainly on the rate of this acylation reaction.

Further progress required the utilisation of new methods and collaborations were initiated with crystallographers in Connecticut (Jim Knox and Judith Kelly) and Liège (Otto Dideberg). The first complete structure of a D-alanyl-D-alanine carboxypeptidase was solved in Liège in 1978. It was a Zn^{++} metallo enzyme which was in consequence not sensitive to penicillin. It was the first protein 3D structure to be solved in Belgium. At this time, Jean-Marie also felt the need for theoretical approaches and Georges Dive and Josette

Lamotte joined the team to start a group specialising in molecular modelling and quantum chemistry.

The group had now become multidisciplinary, covering such diverse areas as microbiology, enzymology, protein crystallography and theoretical chemistry. New staff members, namely Paulette Charlier, Bernard Joris and Colette Duez obtained permanent positions. The need for large quantities of proteins became an important factor and, since the focus was still mainly on *Streptomyces*, Jean Dusart visited David Hopwood's laboratory in Norwich to acquire expertise in the cloning of streptomycete genes and in expression systems for the production of large amounts of streptomycete proteins. Several genes encoding D-alanyl-D-alanylpeptidases and β-lactamases were cloned to satisfy the requirements of the protein chemists and crystallographers. The structural and functional characteristics of several enzymes were thus determined and attempts were made to establish correlations between both types of properties, an old dream of Jean-Marie's. In 1990, when Jean-Marie reached the age of mandatory retirement, the Centre for Protein Engineering was created to avoid dispersion of the various experts that formed the team and of the equipment. The Rector of the University organized the appointment of Jean-Marie as the first director of the Centre until, at 70, he had to retire though he remained active as a scientific advisor. Meanwhile, new collaborations had been initiated, mainly with Josef Van Beeumen, a protein chemist at the Universiy of Ghent and with Léon Ghosez, an organic chemist at the University of Louvain. Jean-Marie had an encyclopaedic knowledge of microbiology and biochemistry. He was generous with his time and keen to discuss their data or more general scientific problems with his younger collaborators: this often led to the birth of new ideas, many of which turned out to be seminal. In his most recent publications (out of a total of more than 350), he discussed the molecular basis of the lack of efficiency of penicillins against mycobacteria (the "Mycobacterial Paradox") and the evolutionary relationship between penicillin-binding enzymes.

Over the years, he was the recipient of a large number of prestigious awards: the Prix Joseph Maisin of the National Research Foundation of Belgium (FNRS), the Prix de l'Innovation Technologique of the Walloon Region (shared with four of his co-workers), the Gairdner Foundation International Award in Medical Science, the UNESCO Carlos J. Finlay Award in Microbiology, the Albert Einstein World Award of Science and the Bristol-Myers-Squibb Award in Microbiology. He was chairman of numerous symposia at international conferences and a much sought after lecturer at several Belgian and foreign universities and at a large number of meetings. He received "Honoris Causa" doctorates from the Universities of Nancy, Debrecen and Montreal.

The last years were saddened by his wife's poor health. To the end, in August 2003, he devoted a lot of time, attention and love to her.

Jean-Marie leaves behind him a thriving Centre, composed of more than 70 scientists and technicians now headed by Jean-Marie Frère (not unexpectedly called "Jean-Marie the second"). He has made a lasting mark on Belgian and international science and his numerous contributions (more than 350 publications) will long be remembered not only by scientists all over the world, but also by his three children and eight grandchildren, one of whom is … a pharmacist!

J. Coyette
J.M. Frère
P.E. Reynolds

SCIENTIFIC REPORT

1. Protein-ligand interactions (substrates or inhibitors)

1.1. β-lactamases

1.1.1. CENTA as a chromogenic substrate for the study of β-lactamases

Nitrocefin and, to a lesser extent, PADAC have been used as chromogenic substrates of β-lactamases. Such substrates, whose hydrolysis can be directly monitored in the wavelength range of visible light are of particular interest for the kinetic characterization of β-lactamases. Nitrocefin also allows the rapid identification of active fractions during β-lactamase purification. Both compounds, however, are rather difficult to synthesize. A third chromogenic cephalosporin, known as CENTA, can represent an interesting alternative. It can easily be prepared from the commercially available cephalothin.

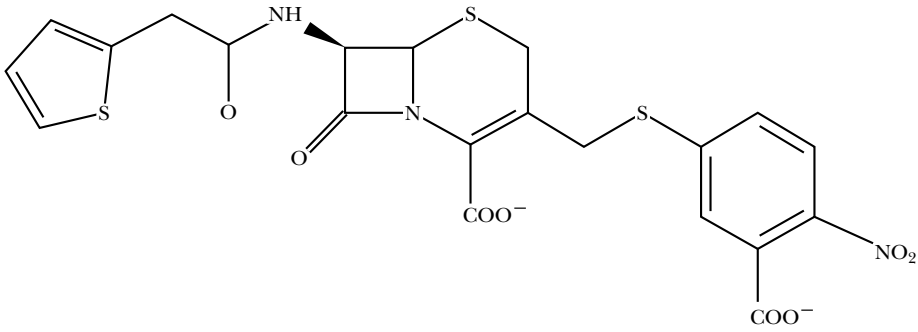


Fig. 1. Structure of CENTA

In this contribution [34], we determined the kinetic parameters characteristic of the interactions between CENTA and a set of representative β-lactamases and Penicillin Binding Proteins (PBPs). CENTA was a relatively good substrate of all β-lactamases tested, with the sole exception of the CphA enzyme, which is very specific for carbapenems and similarly exhibits a very poor activity versus nitrocefin. It was a poor substrate and inactivator of the PBP5 from *E. coli* ($k_{\mathrm{cat}}/K_m = 22\ \mathrm{M}^{-1}\mathrm{s}^{-1}$). In contrast, it behaved as a good inactivator for the

Streptomyces R61 and *Actinomadura* R39 enzymes, with second-order inactivation rate constants in the same order of magnitude as those observed with cephalothin (2000 ± 200 $M^{-1}s^{-1}$ and 90,000 ± 10,000 $M^{-1}s^{-1}$, respectively).

Finally, the chemical properties of CENTA are also favorable. In contrast to nitrocefin whose stock solution must be prepared in dimethylsulfoxide or dimethylformamide, CENTA is highly soluble in aqueous buffers. Its stability was analysed in the 4–12 pH range at 25°C. Up to pH 9, no significant spontaneous hydrolysis of CENTA could be detected after 60 min incubation. At higher pH values, the compound turned out to be less stable. Thus, at pH 12 the hydrolysis rate constant value of CENTA was found to be around 10^{-3} s^{-1}, very close to the value measured with nitrocefin (2.6×10^{-3} s^{-1}) under the same conditions. Incubation of 100 μM CENTA in rabbit serum did not result in significant hydrolysis after 1 h, whereas the half-life of nitrocefin was 13 min under the same conditions.

These experiments demonstrate that CENTA can advantageously be used in a variety of β-lactamase assay, e.g., for the detection of these enzymes in bacterial crude extracts or in chromatographic fractions during enzyme purification, and in high throughput screening test for the selection of new β-lactamase inactivators. Unfortunately, the absorption spectrum of the C-3 leaving group of CENTA is such that it hampers the direct detection of β-lactamase producing colonies on agar plates and on paper strip tests, or the localization of β-lactamases on IEF gels.

1.1.2. Class A β-lactamases

1.1.2.1. The *Bacillus licheniformis* BS3 β-lactamase

Crystalline complexes and analysis at the atomic level of the binding modes of several inhibitors have been done. We have identified the molecular details of the interactions of the β-iodopenicillanic (BIP) inactivator and citrate (CIT) or isocitrate (ISOCIT) anions, identified as a potential novel class of inhibitors for class A β-lactamases.

a) Complex with the 6β-iodopenicillanic acid (BIP)

The crystallographic structure of the BS3-BIP complex has been solved using the coordinates of the BS3 enzyme (PDB code 1I2S) [56] as initial phasing model. The BS3-BIP structure was refined at 1.8 Å resolution with R and R_{free} factors of 21.7% and 26.8% respectively. The electron density in the adduct crystal structure unambiguously reveals a covalent acyl-enzyme corresponding to the irreversibly inactivated enzyme, with the acyl moiety of β-iodopenicillanate rearranged into the dihydrothiazine chromophore. In this rearranged form of BIP, the iodine atom is relaxed. This iodine ion, identified in the active site

by an important electron density sphere, makes interactions with the catalytic residues Ser130 and Thr235. The carbonyl oxygen of the β-lactam ring replaces Wat303 (observed in the unliganded structure) in the "oxyanion hole", at distances of 2.7 Å and 2.8 Å from the main-chain nitrogens of Ser70 and Ser237, respectively. The carboxylate oxygen atoms form hydrogen-bond to the side-chain nitrogens of Asn132 and Asn105, at distances of 2.8 Å and 3.1 Å. The interaction between the BIP carboxylate and the catalytic residue Asn132 seems to induce electronic perturbations in the active site, together with a slight shift of the Ω-loop (residues Glu166 to Glu171). This movement of the essential residues Glu166 and Asn170 enhances steric incompatibility with the presence of the so-called hydrolytic water molecule, known as Wat302 in the free enzyme. Further investigations on the binding mode of BIP, which is very different to the one observed with classical β-lactam substrates, should be performed in order to better understand the action mechanism of this inactivator and related molecules.

b) Complexes with the citrate (CIT) and isocitrate (ISOCIT) anions

The crystallographic structure of the BS3-CIT (PDB code 1I2S) complex has been solved using the coordinates of the *Bacillus licheniformis* 749/C enzyme (PDB code 4BLM) and the BS3-ISOCIT (PDB code 1W7F, S. Petrella *et al.*) complex has been solved using the coordinates of the BS3-CIT complex as initial phasing model. The BS3-CIT and BS3-ISOCIT structures were both refined at 1.7 Å. The citrate ion interacts with the hydroxyl oxygen atoms of both Ser70 and Ser130 and with the side chains of Thr235 and Arg244, which border the active site and are generally considered to be involved in the positioning of substrates. Those multiple interactions can explain the kinetic results observed when experiments are performed in sodium acetate or citrate buffers. With cephalothin as substrate, K_m values are of 42 μM and higher than 1 mM, with unchanged k_{cat} values, for sodium acetate and citrate buffers, respectively. This kinetic result suggests that citrate ion behave as a competitive inhibitor. Both citrate and isocitrate ions have been further tested as inbithors with different β-lactamases belonging to the four classes (A, B, C and D). The K_i values show clearly that those molecules are competitive inhibitors mostly for the class A β-lactamases, with values ranging from 53 μM to 730 μM for the citrate ion and 60 μM to 1500 μM for the isocitrate ion, for the NMCA and TEM enzymes, respectively. The isocitrate ion adopts a different conformation and has a slightly different position in the active site, subsequently losing some interactions. For example, the loss of the interaction with the Arg244 side chain may explain the difference in the K_i values. Based on these observations, new com-

pounds will be synthesized in order to enhance their efficiency and specificity.

1.1.2.2. Carbapenem-hydrolyzing β-lactamases belonging to class A

We studied the NMCA carbapenemase, an enzyme produced by *Enterobacter cloacae*.

The structure of the enzyme was solved by X-ray crystallography. In order to determine the residues involved in the carbapenem-hydrolysing activity, the three-dimensional structures of NMCA and TEM-1 were compared. On this basis, we decided to perform by site directed mutagenesis the substitutions of the following residues of NMCA: H105S, N132Q and N132A, R141A, R220L, A240V and A240E and A244V. Furthermore, a major difference between the NMCA and non-carbapenemase class A β-lactamases is the presence of a disulfide bond between C69 and C238. Hence, both residues were replaced by an alanine.

Removal of the disulfide bridge in the NMCA active site yielded a highly unstable enzyme, which could not be produced and purified to homogeneity. In the case of TEM-1, the substitution of the asparagine132 decreased strongly the activity of the β-lactamase. The same results were obtained for NMCA. The catalytic efficiency of the N132A and N132Q mutants was significantly impaired (for example, k_{cat}/K_m decreased more than 10^3 fold for ampicillin). These data underlines that N132 is an important residue involved in the catalytic process of class A β-lactamases, but it is not typical of the carbapenmase activity.

Mutations at positions 141 and 240 did not affect the NMCA activity profile, whereas substitution of residues 105 and 237 affected the activity profile of NMCA. Both mutants were unable to hydrolyse second and third generation cephalosporins, and cephamycins. They both retained their catalytic activity against carbapenems but their specificity was modified. Their best substrate was meropenem ($k_{\text{cat}}/K_m \sim 10^6\,\text{M}^{-1}\,\text{s}^{-1}$) compared to imipenem ($k_{\text{cat}}/K_m \sim 10^5\text{M}^{-1}\text{s}^{-1}$). The inverse substrate preference was observed for the WT NMCA.

1.1.2.3. TOHO-1, a CTX-M class A β-lactamase

Class A β-lactamases are still today classified as penicillinases on the basis of their substrate profile. However, an increasing number of class A β-lactamases are able to hydrolyse efficiently antibiotics belonging to different β-lactam families (e.g., third generation cephalosporins and monobactams). These enzymes are known as extended-spectrum β-lactamases (ESBL). TOHO-1 is an ESBL and it is included in the CTX-M family on the basis of amino acid sequence identities. The kinetic analysis of the enzyme indicated that it was active against both penicillins ans cephalosporins. Cephaloridine and

cefotaxime were among its best substrates ($k_{cat}/K_m \sim 2.7\ 10^6\ M^{-1}\ s^{-1}$), while ceftazidime remained poorly hydrolyzed. This phenomenon can be explained by the fact that the presence of a bulky lateral chain (a carboxy-propioxyimino group) in position 7βof ceftazidime affects the TOHO-1 activity. Interestingly, new β-lactam compounds such as cefdinir, cefcapene, cefepime and S-1090 behaved as good substrates of Toho-1. Conversely, carbapenem compounds behaved as potent inactivators. Their interaction with Toho-1 is characterized by a high acylation efficiency ($k_2/K \sim 10^5\ M^{-1}\ s^{-1}$).

Finally, tazobactam and sulbactam, two mechanism based inactivators were poorly recognized by the enzyme ($k_2/K \sim 500\ M^{-1}\ s^{-1}$) [91].

1.1.3. Class C β-lactamases

1.1.3.1. Plasmid encoded AmpC β-lactamases

Class C β-lactamases (AmpC) were described as chromosomal enzymes. However, over the last fifteen years, more than 20 plasmid-encoded class C enzymes have been identified. AmpC β-lactamases are classified into 6 sub-groups based on amino acid sequence similarities. Each sub-group contains plasmid-borne class C enzymes and their closest chromosomal relatives. Clinical isolates harbouring plasmid encoded AmpC exhibit a high resistance toward β-lactam antibiotics such as cephamycins and monobactams [64]. Nevertheless, it was not clear how the production of plasmid-derived enzymes modified the resistance patterns of the hosts. For some authors, the acquisition of bacterial resistance was due to new catalytic properties of these class C â-lactamases. By contrast, other authors indicated that plasmid-derived AmpCs differe from the chromosomal ones by the localization of their genetic material only [90].

The present work was focused on an extensive characterization of four plasmid-borne class C β-lactamases [i.e., ACT-1 and MIR-1 (subgroup 2), CMY-2 (subgroup 1) and CMY-1 (subgroup 6)], in order to determine whether their kinetic properties were significantly different from those of the chromosome-encoded enzymes, as suggested by some microbiological studies.

The steady state kinetic parameters of these four plasmid-encoded AmpCs were determined and compared to the values obtained with their closest chromosomal class C β-lactamases. The turnover of plasmid-encoded enzymes was typically smaller for penicillin than for cephalosporins, and no major differences are seen between the two types of enzymes. In contrast to what was suggested on the basis of MIC values, CMY-1, ACT-1 and MIR-1 have lower catalytic efficiencies toward ampicillin (2 to 6 times smaller) than the other class C enzymes.

The fact that strains producing plasmid-encoded class C β-lactamases are characterized by very high MICs values with the different β-lactam families cannot be explained on the basis of increased activities against these compounds. To determine if β-lactamase overproduction could not be responsible for these resistance profiles, we compared the MICs values for *E. coli* DH5α producing either the plasmid-encoded ACT-1 and MIR-1 enzymes, or the chromosomal *E. coli* AmpC encoded by a plasmid (pAD7). MICs values obtained for the *E. coli* AmpC were similar to those obtained with the MIR-1 and ACT-1 enzymes.

In order to complete our studies, we also determined the structures of CMY-2 and ACT-1. The structure of ACT-1 was determined by Dr. Ibuka As (Dept. of Food and Nutritional Sciences, University of Shizuoka, Japan). The final model (pdb code: 1ZC2) of CMY-2, refined to 2.1 Å resolution contains two polypeptidic chains, A and B, of respectively, 357 (4−361) and 355 (6−361) residues, 445 water molecules and two citrate molecules. 90.2% of the residues are included in the most favourable region of the Ramachandran plot and model quality was assessed using WHAT_CHECK. CMY-2 β-lactamase presents an overall fold very similar to that of the chromosomal AmpC β-lactamase from *Citrobacter freundii*. Indeed, superimposition of both proteins indicated a 0.5 Å positional root-mean-square deviation (r.m.s.d.) for 1432 atoms in the two chains (all common non-hydrogen atoms including backbones and side chains). All secondary structures are conserved and as far as active side residues are concerned (principally Leu-62, Gly-63, Ser-64, Val-65, Ser-66, Lys-67, Thr-68, Tyr-150, Asn-152, Ala-220, Tyr-221, Lys-315, Thr-316, Gly-317 and Ser-318), no major variation was observed. Furthermore, the separate refinement results of monomers A and B resulted in very similar results (superimposition of both monomers was less than 0.3 Å r.m.s.d. for 1424 atoms). A Fo-Fc difference electron density map contoured at 4 σ revealed an additional density that was interpreted as a citrate molecule. This molecule stems from the crystallization solution where it is used as the buffering agent. In the structure, one citrate molecule is localized in the active site of each monomer and the interactions made by this molecule are the same in each monomer. The majority of these interactions, predominately polar, take place with residues essential for activity, in particular Ser-64, Tyr-150, Lys-315 and Ser-318. Furthermore, many water molecules are present in close vicinity to the buffer molecule and form a complex hydrogen-bonding network, interacting directly with other water molecules and also with the active site residues and the citrate molecule. Interestingly, previous kinetic studies performed with ACT-1 β-lactamase revealed that this enzyme was competitively inhibited by citrate with a relatively weak inhibition constant ($K_i = 1.1$ mM).

1.1.3.2. Chromosomal class C β-lactamase

The structures of the class C β-lactamase from *Enterobacter cloacae* 908R, alone and in complex with the transition state analogue 4-iodo-acetamido-phenyl boronic acid (IAPB, PDB code 1S6R) [89] and the BRL 42715, a C6-(N1-methyl-1,2,3-triazolylmethylene) penem inhibitor (PDB code 1Y54, Michaux *et al.*), have been determined by X-ray crystallography at 2.1, 2.3 and 2.07 Å resolution, respectively. The structure of 908R class C β-lactamase was solved by molecular replacement using the *Enterobacter cloacae* P99 enzyme (PDB code 1BLS) as search model. The overall structure of *E. cloacae* 908R is similar to those previously reported for other class C β-lactamases, including *E. coli* AmpC, *E. cloacae* P99 and *Citobacter freundii.*

a) Complex with 4-iodo-acetamido-phenyl boronic acid (IAPB)

Clear electron density connects the Oγ atom of the catalytic serine Ser64 to the tetrahedral boron atom of the phenyl boronic acid inhibitor, suggesting that a covalent bond is formed between IAPB and the enzyme. One of the oxygen atoms (O1) of the boron adduct sits in the "oxyanion hole", forming direct hydrogen bonds to the main-chain nitrogen atom of Ser64, and the backbone N and O atoms of Ser318. This covalent bond between IAPB and the protein is consistent with the proposed mechanism of inhibition, and is similar to the one observed in other structures of AmpC in complex with aryl boronic acid.

b) Complex with the BRL 42715, C6-(N1-methyl-1,2,3-triazolylmethylene)penem inhibitor

BRL 42715, C6-(N1-methyl-1,2,3-triazolylmethylene)penem is an active-site-directed inhibitor of a broad range of bacterial β-lactamases, including the class C enzymes. This compound is 10 to 100 times more active than other clinically used β-lactamase inhibitors, like clavulanic acid, sulbactam and tazobactam.

The structure of the complex is in good agreement with spectral properties of the product, that are identical to those of the dihydrothiazepine obtained after sodium hydroxide hydrolysis and with mass spectrometry results. It confirms a mechanism that implies opening of the five-membered thiazole ring system upon hydrolysis at the C5-S bond and rearrangement via a Michael addition to form a seven-membered dihydrothiazepine ring system. The binding mode and geometry of the covalent adduct of BRL 42715 in the 908R β-lactamase are quite different from that of a penem analogue in complex with the extended-spectrum GC1 β-lactamase. In particular, the absolute configuration of the cyclic β-aminoacrylate-enzyme complex with BRL 42715 is *S* while the other enantiomer prevails for the penem analogue upon complexation with the GC1 β-lactamase.

1.1.3.3. New efficient inactivators of class C β-lactamases

Two new eniantiomeric carbapenems compounds (Fig. 2) were found to be very efficient inactivators of the model class C β-lactamase from *Enterobacter cloacae* 908R [37]. Complete inactivation occurs with a second-order rate constant, k_2/K', of 2×10^{-4} to 4×10^{-4} M^{-1} s^{-1}, and reactivation is very slow, with a half-life > 1 h. Accordingly, Lek 157 significantly decreases the MIC ("Minimal Inhibitory Concentration") of ampicillin for *E. cloacae*, a constitutive class C β-lactamase overproducer. Preliminary experiments indicated that various other class C enzymes were also inactivated. This is a very unusual, specific and interesting property, as most other mechanism-based inactivators of active-site serine β-lactamases (e.g., clavulanic acid and tazobactam) generally exhibit rather poor activity against class C enzymes.

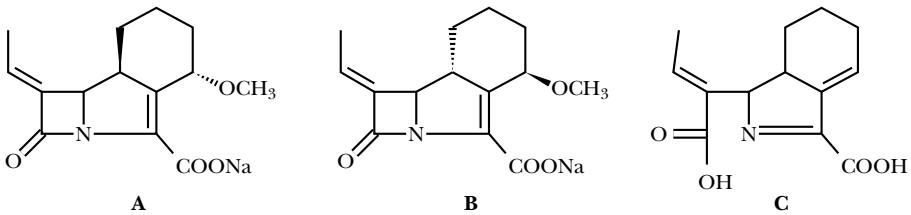


Fig. 2. Structures of Lek 156 (A), Lek 157 (B), and Lek1A (C), which is obtained from base- or enzyme catalyzed hydrolysis of the first two compounds.

1.1.4. Class D β-lactamases

1.1.4.1. β-Lactamase OXA2 from *Salmonella typhimurium*

The structure of the OXA2 class D β-lactamase was solved by molecular replacement using the OXA10 class D β-lactamase from *Pseudomonas aeruginosa* (PDB code 1K4F) as search model. The OXA2 structure was refined at 1.5 Å resolution with R and R_{free} factors of 19.8% and 21.1% respectively (PDB code 1K38, Kerff *et al.*).

OXA-2 is in the form of a dimer in the crystal. Each monomer is defined by 2 domains as in classes A and C β-lactamases and in penicillin-binding proteins (PBPs). The α/β domain is made up of a 6-stranded β-sheet and 4 α-helices. The second all-α domain includes 6 helices and two characteristic loops: the Ω-loop (by analogy with the class A β-lactamases) and a moving loop connecting α3 and α4. The active serine is at the N-terminal of the α3 helix and the catalytic site lies at the interface of the two domains (Fig. 3a). When compared to other penicilloyl serine transferases, the overall structure of OXA2 can be

easily superimposed, except for the two loops that are extremely different. However, OXA-2 and more generally class D β-lactamases are closer to the D-D-transpeptidase domain of PBP2x from *Streptococcus pneumoniae* (r.m.s.d. = 1.28 Å for 121 Cα) and to the low-affinity PBP5 from *Enteroccocus faecium* (r.m.s.d. = 1.21 Å for 152 Cα).

In OXA-2, as in the majority of the class D β-lactamases, the three structural elements characteristic of the penicilloyl serine transferase active site are preserved. The first element (Ser67-Thr68-Phe69-Lys70) containing the active serine, is conspicuous by the presence of a carbamylated Lys70 (Fig. 3b). The second motif (Ser115-Xaa-Val117) strictly preserved in the class D enzymes, forms the left edge of the active site. As in the majority of cases, the third motif is composed of Lys205-Thr206-Gly207. It belongs to the strand β5 and forms the flat rim of the active site, together with Arg244 of the helix α10. This arginine is equivalent to Arg244 of the TEM-1 class A β-lactamase.

In class D β-lactamases, Lys70 is located inside a hydrophobic cavity formed by the residues Val117, Tyr/Phe120, Trp154 and Leu/Ile155, which would support the deprotonated state of this lysine and consequently its carbonation. The carbamylated lysine is then stabilised by hydrogen bonds with residue Ser67, residue Trp154 and with a water molecule close to His/Asn73 (and in some cases, with Ser115). Three of the residues implied in this environment are strictly conserved (Ser67, Ser115 and Val117) and are the equivalents of the three residues implied in a hydrogen bond with the ε-amino group of the corresponding lysine in the classes A and C β-lactamases. The replacement of Asn of the second preserved motif (Ser/Tyr-Xaa-Asn) by a Val thus seems to play a dominating role for the carbonation. The structure characterised by a carbamylated lysine is the most active form. The presence of a carbamylated lysine seems to be essential for a correct conformation of the active site in the absence of substrate.

1.1.4.2. β-Lactamase OXA-10 from *Pseudomonas aeruginosa*

The structure of the OXA10 class D β-lactamase was solved by the MAD (Multiwavelenght Anomalous Dispersion) technique with a selenomethionyl derivative of the protein. The OXA10-SeMet and the OXA10-WT structures were refined at 2.0 and 1.5 Å resolution with R (R_{free}) factors of 20.1% (22.6%) and 18.9% (22.8%), respectively (PDB code 1K4E and 1K4F, Kerff *et al.*).

a) Complex with 4-iodo-acétamido phenylboric acid (IAPB)

The complex was obtained by soaking crystal of OXA-10 in a IAPB solution of 1 mM for 4 months. The structure was refined to a resolution of 2 Å and was deposited in the Protein Data Bank under the code 1K6S. The derivatives

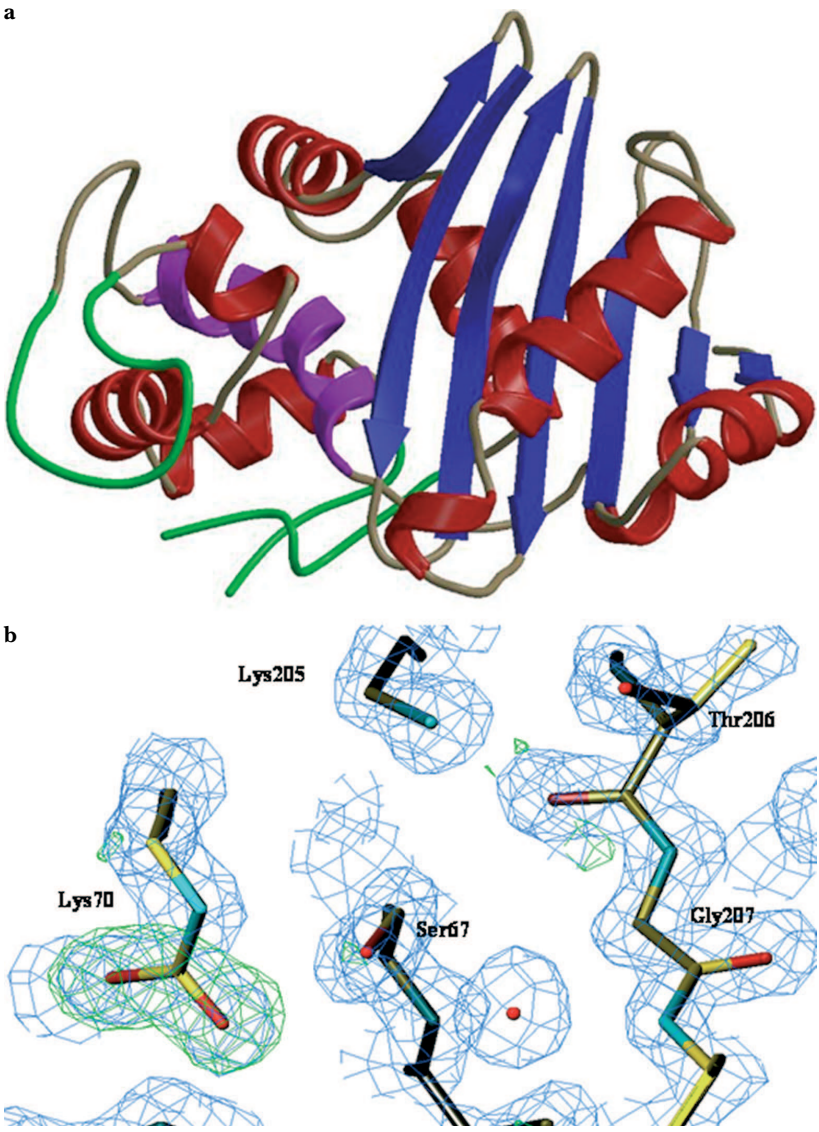


Fig. 3. **a**) Ribbon representation of the OXA-2 monomer. The α/β domain includes a β-sheet made of 7 antiparallel strands (in blue), the N and C terminal helices α1 and α10 and two small helices α2 and α9 (in red). The second all a domain includes the helices α3 to α8 (in red) and two characteristic loops (in green), the Ω-loop and a moving loop connecting α3 and α4 and covering the α5 helix (in red). The active serine is at the N terminal end of the α3 helix (in purple) and the catalytic site lies at the interface of the two domains. **b**) Electron density (in blue) around the active site residues of motif 1 (Ser67 and Lys70) and motif three (Lys205-Thr206-Gly207), showing (in green) the additional density attributed to a carbonate on the Ne atom of Lys70 side-chain.

of the boric acid are generally reversible inhibitors and it is considered that the structure of these complexes is similar to the structure of the transition state leading to the desacylation. In the OXA10-IAPB adduct, the boron atom is covalently bound to the hydroxyl oxygen of the active serine Ser67 and has a tetrahedral conformation. One of the oxygen atom bound to boron occupies the "oxyanion hole" at hydrogen-bound distances from both main chain nitrogens of Ser67 and Phe208. The second oxygen atom interacts with the serine 115 of the second conserved motif. If this structure is analogous to that of the transition state preceding the desacylation, the position of this oxygen atom would reveal the direction of the nucleophilic attack by a water molecule responsible for this step in the kinetic process. In that case, the hydrolytic water molecule is activated by the serine 115 residue. The OXA10-IAPB adduct structure also shows that the iodo-acetamide group of the inhibitor reacts with the sulfur atom of the Met99 residue *via* a carboxymethylation reaction. This reaction implies the departure of iodine and of the Cε carbon of the methionine.

b) Complex with moxalactam (MOXA)

The complex was obtained by soaking crystal of OXA-10 in a solution of moxalactam of 50 mM during 7 h. The structure was refined to a resolution of 2.3 Å and was deposited in the Protein Data Bank under the code of 1K6R. With an oxygen atom substituent to the sulphur of the dihydrothiazine ring of the cephalosporins, the moxalactam belongs to the family of the oxacephems. The chain attached to this ring includes a tetrazolium ring. In addition to this very bulky side chain in 7β position, this molecule is characterized by the presence of a methoxy group in position 7α. The electron density in the complex OXA10-MOXA crystal unambigously reveals a covalent acyl-enzyme adduct in all molecules, but in a quite surprising way, the substrate adopts completely different positions in the two monomers. In monomer A, the moxalactam exhibits a binding mode similar to the one observed in various complexes formed by this type of substrate with other penicilloyl transferase enzymes. The carbonyl oxygen of the open β-lactam ring occupies the oxyanion hole formed by nitrogen atoms of the main chain of Ser67 and of Phe208, and the carboxylate oxygens form hydrogen bonds to the guanidium group of Arg250. In monomer B, the moxalactam adopts a conformation totally different, which has never been observed so far. In this case, the carboxylate group of the oxacephem ring does not interact with Arg250, rather, it is bounded to the Nε atom of Trp154, whereas Arg250 interacts with the carboxylate group of the 7β side chain. The carbonyl group of the open cycle β-lactam ring forms a hydrogen bond with Lys205 of the third active site motif and a water

molecule replaces it in the "oxyanion hole". The structural difference between monomer A and B results from different crystallographic environments. The 'moving loop' of B is less constrained, probably allowing the substrate rotation after acylation.

1.1.4.3. β-Lactamase OXA29 from *Pseudomonas aeruginosa*

Primary structure revealed that OXA-29 is quite divergent from other class D β-lactamases, being more closely related (43–33% amino acid identity) to the OXA enzymes of groups III and IV (OXA-12, OXA-18, OXA-9, OXA-22 and OXA-1) than to other class D proteins. It is active exclusively as a dimer [44]. OXA-29 crystallizes as hexagonal needles in space group $P6_{1/5}22$, with a hudge unit-cell, $a = b = 82.7$ Å, $c = 407$ Å, $\alpha = \beta = 90°$, $\gamma = 120°$ containing 36 molecules. A complete data set has been collected on the ID14-1 beamline of ESRF to a resolution of 2.8 Å. Intensities are quite weak $[I/s(I) = 3]$ and data are of medium quality ($R_{\text{sym}} = 0.13$). Molecular replacement has been tried with the structure of OXA-1, without success. Diffusion of heavy atoms with anomalous scattering property will be tried in order to use the MAD phasing technique for solving the structure.

1.1.4.4. The *ybxI* gene product of *Bacillus subtilis* 168

The *ybxI* gene of *Bacillus subtilis* 168 encodes a pre-protein of 267 amino acid residues, including a putative signal peptide of 23 residues. The YbxI primary structure exhibits high similarity scores with two members of the superfamily of the serine penicillin-recognizing enzymes: the class D β-lactamases and the hydrophilic carboxy-terminal domains of the BlaR and MecR penicillin receptors. To determine the function and the activity of this putative penicillin-recognizing enzyme, we have subcloned the *ybxI* gene in the pET-26b expression vector. Transformation of *Escherichia coli* BL21(DE3) by the recombinant plasmid pCIP51 resulted in the export of the mature YbxI in the periplasm as a water-soluble protein [104]. The recombinant protein was purified to 95% homogeneity. YbxI interacts with several β-lactam antibiotics and can hydrolyze some of them. YbxI is not inactivated by clavulanic acid. The YbxI function and its enzymatic activity in *B. subtilis* remain unknown. The acyl-enzyme obtained after incubation of YbxI with a fluorescent derivative of ampicillin can be detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, confirming that YbxI can be acylated by β-lactam antibiotics. YbxI does not hydrolyze some of the standard substrates of D-alanyl-D-alanine peptidases, the targets of penicillin. YbxI belongs to the penicillin-recognizing enzyme family but has an activity intermediate between those of a penicillin-binding protein and a β-lactamase.

1.1.5. Class B β-lactamases

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

The BcII metallo-β-lactamase from *Bacillus cereus* 569/H/9 belongs to the subclass B1 [27] as other enzymes as VIM-1 [17]. It possesses a binuclear zinc centre. In an attempt to assign the involvement of the different zinc ligands in the catalytic properties of BcII, seven different β-lactamase mutants, with amino acid substitutions in or near the active site were obtained. The hydrolytic activities and metal ion contents of the different enzymes were analysed, and the results indicated that the majority of the substituted side chains have a role in the enzymic function of BcII [60, 74]. His116, His118 and His296 are the three residues constituting the so-called "first zinc-binding site". Substitution of His116 with serine did not drastically affect the catalytic efficiency or the zinc-binding capability of BcII. H116S binds one or two zinc ions in the presence of zinc concentrations of 0.4 or 100 μM, respectively. The dependence of the k_{cat}/K_m values on zinc-ion concentrations for H116S and the wild-type enzyme was found to be similar. With the H118S and H196S mutants, alteration of the kinetic parameters was more pronounced. With benzylpenicillin and nitrocefin as substrates, a 10^3 decrease of the k_{cat} value combined with an increase in the K_m value (by a factor of 10), resulted in a lowering of the catalytic efficiency (k_{cat}/K_m) of the mutants by 2 orders of magnitude. Addition of zinc ions had no significant effect on the activity of these two mutants. At pH 7.5, however, H118S and H196S were able to bind one and two zinc ions in the presence of metal concentrations of 0.4 and 100 μM, respectively. The study of the pH-dependence of the [Zn]/[E] ratio for the H196S and wild-type enzymes indicated that, below pH 8, the zinc content of H196S was always lower than that of the wild type, even at 100 μM zinc. As confirmed by X-ray crystallography, at pH 5 and at a zinc concentration of 100 μM, only the mono-zinc BcII wild-type β-lactamase structure can be obtained. These data also show that the affinity constant of the two zinc ions for the binding site is pH-dependent. The affinity for zinc was not significantly altered by the mutations, and each mutant could still bind two zinc ions. Substitution of Asp120 with an asparagine also drastically affects the activity, but not the zinc-binding capability, of the enzyme. The [Zn]/[E] ratio for the D120N mutant is similar to that determined for the wild-type enzyme. Nevertheless, even with a high zinc concentration, the catalytic efficiency of the enzyme is more than 100-fold lower than with the wild-type enzyme, with all the tested antibiotics. Our data identify Asp120 as an essential residue for the catalytic activity, but not for zinc binding. In the di-zinc BcII, Asp120 might participate in the orientation of the water/hydroxide during the nucleophilic attack of the β-lactam ring, and also in the orientation of the second water molecule involved

in the conversion of the apparent intermediate formed with this enzyme into free enzyme and product. The substitution of the cysteine residue with alanine was studied previously [R. Paul-Soto, R. Bauer, J.M. Frère, M. Galleni, W. Meyer-Klauke, H. Nolting, G.M. Rossolini, D. de Seny, M. Hernandez-Valladares, M. Zeppezauer and H.W. Adolph (1999). Mono- and binuclear Zn^{2+}-beta-lactamase. Role of the conserved cysteine in the catalytic mechanism, *J. Biol. Chem.* **274**, 13242–13249]. The mono- and the di-Zn forms of the C221A mutant exhibited different kinetic properties with respect to benzylpenicillin and nitrocefin, suggesting a role for the cysteine residue in efficient hydrolysis by the mono-Zn species, but not by the bi-nuclear enzyme [13]. Indeed, an increased value of the catalytic efficiency, mainly due to a higher k_{cat} value, was correlated with the zinc ion content of the protein. Finally, the H263S substitution led to a weakly active enzyme.

As observed for the hydrolysis of benzylpenicillin by the H118S and H196S mutants, the K_m value strongly increased, whereas the k_{cat} decreased by more than one order of magnitude. The catalytic efficiency of H263S towards cefotaxime is dependent on the zinc ion concentration. Surprisingly, there is no apparent correlation between an increasing [Zn]/[E] ratio and the modification of the catalytic efficiency of the mutant. We found that only one equivalent of zinc ion was bound to the mutant in the presence of 100 μM zinc. Nevertheless, EXAFS experiments performed at a zinc ion concentration of 1 mM indicated that the mutant binds two zinc ions [43].

The role of the mobile loop comprising residues 60–66 in subclass B1 metallo-β-lactamases has been studied by site-directed mutagenesis, determination of kinetic parameters for six substrates and two inhibitors, pre-steady-state characterization of the interaction with chromogenic nitrocefin, and molecular modelling [81]. The W64A mutation was performed in IMP-1 and BcII (after replacement of the BcII 60–66 peptide by that of IMP-1) and always resulted in increased K_i and K_m and decreased k_{cat}/K_m values, an effect reinforced by complete deletion of the loop. k_{cat} values were, by contrast, much more diversely affected, indicating that the loop does not systematically favor the best relative positioning of substrate and enzyme catalytic groups. The hydrophobic nature of the ligand is also crucial to strong interactions with the loop, since imipenem was almost insensitive to loop modifications. Our study demonstrates that a loop which is relatively far from the active site in the free enzyme significantly affects ligand binding and turnover numbers. It also highlights some residues which probably play a prominent role in the loop. Based on the kinetic data obtained in the IMP-1 background, W64 seems to account for approximately 50% of the loop effect, since all the trends observed for K_m and k_{cat}/K_m with W64A become enhanced around 2-fold upon loop deletion. Our

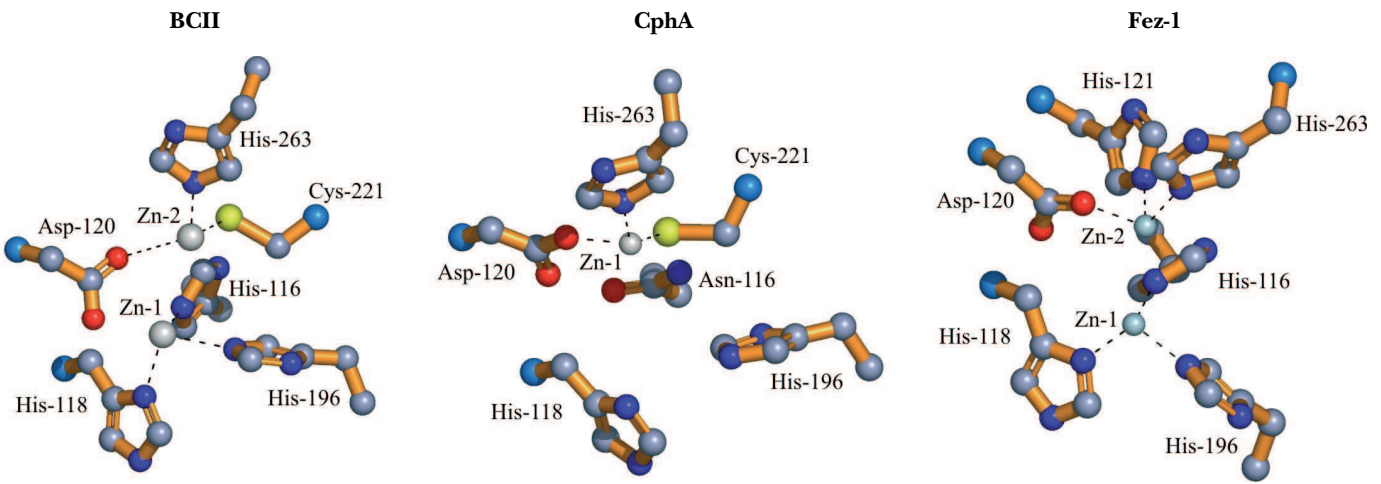


Fig. 4. Representation of the zinc binding sites of sub-class B2 (CphA), B1 (BCII) and B3 (Fez-1) β-lactamases. Amino acids that coordinate zinc ions are depicted.

data are in agreement with previous interpretations of the unusual mobility of W64, suggesting that it mainly contributes to create a plastic active site capable of binding a large variety of molecules with high affinity, probably through hydrophobic interactions. Interestingly, substrates with a weak hydrophobic character, such as imipenem, are not excluded from the substrate profile of subclass B1 enzymes, showing that this loop effect is not a necessary mechanism for efficient hydrolysis, at least in the presence of other anchoring points (for instance, S121 in the IMP-1-imipenem interaction). According to molecular modeling, residues 61 and 67, located at the base of the loop, seem to play an important role beyond their hinge function. Finally, we showed that the loop-induced increased affinity is imperfectely correlated with high turnover numbers, showing the limits of the "plastic strategy" developed by subclass B1 enzymes. The global effect measured in terms of k_{cat}/K_m clearly identifies the loop as a useful tool for metallo-β-lactamases, but it is certainly not the only one and can even be insignificant in the interaction between some enzymes and imipenem.

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

The CphA metallo-β-lactamase produced by *Aeromonas hydrophila* belongs to subclass B2 [10, 27]. It is characterized by a unique narrow specificity profile. CphA efficiently hydrolyses carbapenems only and shows very poor activity against penicillins and cephalosporins, a behaviour which contrast to that of metallo-β-lactamases of subclasses B1 and B3, which usually exhibit a broad activity spectrum against nearly all β-lactam compounds, with the exception of monobactams. CphA exhibits two zinc-binding sites. Maximum activity is obtained upon binding of one zinc ion, whereas binding of the second zinc ion results in a drastic decrease in the hydrolytic activity. The presence of a Zn^{2+} ion in a second low affinity-binding site non-competitively inhibits the enzyme, with a K_i value of 46 μM at pH 6.5. Recently, the structure of the mono-zinc CphA enzyme has been solved by X-ray cristallography. Similarly to the known structures of metallo-β-lactamases of subclasses B1 (e.g., BcII) and B3 (e.g., L1 and FEZ-1), the X-ray structure of CphA highlights a αββα sandwich fold with two central β-sheets and α-helices on the external faces. The active site is located at the bottom of the β-sheet core (Fig. 4). In agreement with previous spectroscopic results, these structural data show that the sole Zn^{2+} ion resides in the D120-C221-H263 site of the *A. hydrophila* metallo-β-lactamase. In the di-zinc form of subclass B1, the Zn ions occupy both the H116-H118-H196 and the D120-C221-H263 sites. The histidine residue in position 116 in most metallo-β-lactamases is replaced by an asparagine residue in CphA. We analyzed the role of Asn116 and Cys221, two residues of the active site. These residues

were replaced by site-directed mutagenesis and the different mutants were characterized. The C221S and C221A mutants were seriously impaired in their ability to bind the first, catalytic zinc ion and were nearly completely inactive, indicating a major role for Cys221 in the binding of the catalytic metal ion. By contrast, the binding of the second zinc ion was only slightly affected, at least for the C221S mutant. Mutation of Asn116 did not lead to a drastic decrease in the hydrolytic activity, indicating that this residue does not play a key role in the catalytic mechanism. However, the substitution of Asn116 by a Cys or His residue resulted in an approximately five-fold increase in the affinity for the second, inhibitory zinc ion.

We could also progressively, by site-directed mutagenesis, replace the metal binding site motif of subclass B2 metallo-β-lactamase (N116 × H118 × D120. H196..G219N220C221.H263) by those of subclass B1 (H116 × H118 × D120... H196.G219G220C221..H263) or subclass B3 (H116 × H118 × D120H121.. H196...H263), respectively. The N116H-N220G double mutant was not as active as the wild-type enzyme against imipenem, but several penicillins and cephalosporins were now found to be significantly hydrolysed. Thus, recreating the characteristic motif of the B1 sub-class clearly broadens the substrate profile. Its catalytic efficiency toward carbapenem and other β-lactam compounds is similar. In the case of the WT CphA, the k_{cat}/K_m against imipenem is 5 to 6 orders of magnitude higher compared to the activity against benzylpenicillin. With N116H-N220G and as already observed for N116H, the di-zinc form is more active against benzylpenicillin, cephaloridine, cefotaxime and nitrocefin than the mono-zinc form, contrary to what happens with imipenem. This suggests that imipenem on the one hand, and these latter compounds on the other, are hydrolysed via slightly different mechanisms. Accordingly, the K_{D2} values measured on the basis of the activation or inhibition curves were similar within the limits of experimental errors: 86 and 110 μM for N220G, and 3–9 μM for N116H-N220G.

Although obtaining a mutant similar to sub-class B3 enzymes proved more difficult, the mutants containing the R121H substitution exhibited an increased affinity for the second Zn (a B3 characteristic), and the triple mutant N116H-R121H-N220G, although less active than the N116H-N220G, also exhibited a broadened substrate profile. Moreover, the triple mutant was the only mutant for which the binding of the second zinc increased the activity *vs* imipenem.

1.1.5.3. Subclass B3 Zn β-lactamase of *Fluoribacter gormanii* (FEZ-1)

In subclass B3, the crystal structures of the metallo-β-lactamases L1 from *S. maltophilia* and FEZ-1 from *F. gormanii* have been solved [80, 117]. The active

site with the binuclear Zn center is located at the bottom of the β-sheet core. Zn1 is tetrahedrally coordinated by three histidines, His116, His118, His196 and a water molecule. In the subclass B3 enzymes, Zn2 is coordinated by His121, His263, Asp120 and two water molecules to form a trigonal bipyramid. FEZ-1 is a monomeric enzyme [30]. The sequence of the mature protein is easily aligned with that of the L1 enzyme with 33% of isology. In L1, it has been postulated that the carbonyl oxygen of the β-lactam substrate interacts with an oxyanion hole formed by Zn1 and the side-chain of Tyr228. This tyrosine is conserved and could play the same role in FEZ-1. In L1, the β-substituent on C6 or C7 of the β-lactam substrate generally fits in a hydrophobic pocket. In this pocket, the hydrophobic residues Phe156 and Ile162 of L1 are replaced by a Tyr and a Ser residue respectively in FEZ-1 [14]. These substitutions, together with Asn225, should influence the substrate specificity, with a facilitated interaction between FEZ-1 and β-lactams bearing a less hydrophobic β side-chain. We have studied the role of residues His121, Tyr156, Asn225, and Tyr228 in the catalytic activity of FEZ-1 [118].

We showed that His121 is essential for the production of a di-zinc form of FEZ-1 at low zinc concentration. The mono-zinc enzyme is active and stable. The addition of zinc ion allowed the production of a di-zinc enzyme as active as the wild type FEZ-1. All our data indicate that the main function of His121 is to interact with zinc ions. Our studies confirmed that Tyr156 does not play an important role in the subclass B3 β-lactamases. Substitutions of Ser221 and Asn225 modify the activity spectrum of the enzyme. In both cases, the catalytic efficiency of the two mutants against carbapenems decreases. These two residues are involved in the correct positioning of the carbapenem in the catalytic pocket. Finally, we could demonstrate that Tyr228 is important in the processing of bulky cephalosporins. The Tyr228Ala mutant is inactivated by the hydrolysis product of cephalosporin. A direct interaction between Cys200 and the cephem ring yields the formation of a covalent and inactive complex [118].

1.1.5.4. Clonal diversity and metallo-β-lactamase production in clinical isolates of *Stenotrophomonas maltophilia*

Stenotrophomonas maltophilia is a nosocomial pathogen with an intrinsic broad-spectrum resistance to β-lactam compounds and other antimicrobial agents. It produces two chromosomal β-lactamases: a clavulanic acid-resistant class A (L2) and a tetrameric carbapenemase (L1 or BlaS). We screened fourty *S. maltophilia* multidrug resistant isolates discovered between 1995 and 1998 in the Varese hospital (Italy) for the presence of L1. The isolates were investigated by phenotypic profiling (enzymatic activity and antibiotic resistant pattern) and molecular methods such as PCR and pulse-field gel electrophoresis (PFGE) to

reveal intraspecies diversity. For the tested *S. maltophilia* strains, we showed that the β-lactamase production could be induced by the presence of imipenem 50 μg/ml in the culture media. Addition of 1 mM dipicolinc acid completly inhibited the hydrolysis of imipenem but decreased that of nitrocefin in a strain dependent manner. Full activity of crude extract toward imipenem could be restored by addition of 1 mM $ZnCl_2$. Finally, the gene coding for the carbapenem hydrolysing β-lactamase from *S. maltophilia* ULA511 and 39/95, a clinical strain, were isolated and sequenced. These two strains have a different profile of multidrug resistance. The two metallo-β-lactamases were found to be isogenous. The differences of sensitivity of the two strains were associated to the level of production of the metallo-β-lactamase [65].

1.1.5.5. Search for potential Zn β-lactamase inhibitors

Most metallo-β-lactamases known to date efficiently hydrolyze most classes of β-lactams, including the broad-spectrum carbapenems, and are not inhibited by classic β-lactamase inhibitors. The first synthetic inhibitors of these enzymes were the α-amidotrifluoromethyl alcohols and ketones.

Several mercaptoacetic acid thiol ester derivatives also inhibit metallo-β-lactamases. For the *B. cereus* enzyme, a mechanism-based formation of mercaptoacetic acid was suggested, with the subsequent formation of a disulfide bond with the active-site cysteine residue. Compounds such as thiols, amino acid-derived hydroxamates, various carbapenem derivatives, and biphenyltetrazoles have been reported to be good inhibitors. Although cefoxitin, moxalactam and ceftriaxone are substrates for most known metallo-β-lactamases, they irreversibly inactivate the CphA enzyme [39]. Cefoxitin and moxalactam are both poor substrates of *A. hydrophila* metallo-β-lactamase (k_{cat}/K_m (cefoxitin) = 33 Ms^{-1}; k_{cat}/K_m (moxalactam) = 5.6 Ms^{-1}). Rates of inactivation by the hydrolyzed antibiotics are significantly higher, indicating that hydrolysis of the β-lactams is the rate-limiting step. For moxalactam, we have shown that the inactivation mechanism involves the formation of a mixed disulfide between the sole cysteine in the active site (C221) and the 3' leaving group of the hydrolyzed moxalactam. In the case of cefoxitin, formation of a thioester can be excluded. Two possible mechanisms remain. First, as shown by the reaction of hydrolyzed cefoxitin and cysteine, the free thiol of CphA reacts with the exo-methylene group, which appears after elimination of the 3' leaving group. The second alternative, based on MS data and dithiothreitol treatment of the inactivated complex, is formation of a disulfide bond between the cysteine residue of the enzyme and the dihydrothiazine sulfur.

Candidate inhibitors include mercaptocarboxylic acids [11], and we report studies of such a simple compound, thiomandelic acid [42, 83]. A series of

35 analogues were synthesized and examined as metallo-β-lactamase inhibitors. The K_i values (*Bacillus cereus* enzyme) are 0.09 μM for *R*-thiomandelic acid and 1.28 μM for the *S*-isomer. Structure-activity relationships show that the thiol is essential for activity and the carboxylate increases potency; the affinity is greatest when these groups are close together. Thioesters of thiomandelic acid are substrates for the enzyme, liberating thiomandelic acid, suggesting a starting point for the design of "pro-drugs." Importantly, thiomandelic acid is a broad-spectrum inhibitor of metallo-β-lactamases, with a submicromolar K_i value for all nine enzymes tested, except the *Aeromonas hydrophila* enzyme; such a wide spectrum of activity is unprecedented. The binding of thiomandelic acid to the *B. cereus* enzyme was studied by NMR. The results are consistent with the idea that the inhibitor thiol binds to both zinc ions, while its carboxylate binds to Arg121. Amide chemical shift perturbations for residues 30–40 (the β_3–β_4 loop) suggest that this small inhibitor induces a movement of the mobile loop. The modification of the loop position closes the active site by creating hydrophobic interactions between the aromatic group of thiomandelic and the hydrophobic residues present on the loop.

1.2. Penicillin-binding proteins (PBPs)

1.2.1. The low molecular mass PBPs/DD-peptidases

These LMM proteins (Mr $\leqslant$ 50,000) usually exhibit a detectable hydrolytic activity on peptides of general structure R–D–Ala–D–Ala. Their sequences can be aligned on those of the C-terminal modules of their HMM-counterparts or, in the case of the *Streptomyces* R61 enzyme on those of class C β-lactamases. Their structures are clearly similar to those of the known C-terminal modules of class B PBPs but also to those of class A β-lactamases, in the case of *Streptomyces* K15 enzyme and *E. coli* PBP5, with which the sequence similarities are barely above background [137]. Conserved motifs can be identified on the basis of the sequences and 3D structures. Intriguingly, site-directed mutagenesis experiments performed on four different enzymes (*Streptomyces* K15, *Streptomyces* R61 and *Actinomadura* R39 DD-peptidases and *E. coli* PBP5) show that modification of similarly situated and functionally related residues does not always result in similar alterations of activity, a result which underlines possible divergences in details of the catalytic mechanisms. This is in agreement with indications obtained from quantum chemistry studies which highlight the possible coexistence of multiple catalytic pathways separated by low energy barriers.

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

The catalytic mechanism of the *Streptomyces* K15 DD-peptidase (Mr: 28,000) has been extensively studied [85]. Crystallographic work has supported the biochemical and enzymatic kinetic analysis [84]. Several modified proteins obtained by site-directed mutagenesis have been solved to very high (up to 1.4 Å) resolution. The atomic coordinates and structure-factor amplitudes of mutants K38H, S96A, C98A, C98N, S216A, R248L have been deposited in the Protein Data Bank with the identification codes 1J9M, 1ES2, 1ES3, 1ES4, 1ES5, 1ESI, respectively. The alteration of kinetic parameters of the transpeptidation reaction and of the hydrolysis of various substrates and β-lactam compounds has been tentatively interpreted. The Lys38His mutation in motif 1 has the most drastic effect, yielding an almost totally inactive enzyme while the structure of the active site is essentially the same as the wild-type enzyme. The suppression of the hydroxyl group resulting from the Ser96Ala mutation in motif 2 is accompanied by the concomitant alteration of acylation and desacylation steps with donor substrates of the transpeptidation reaction. The reaction with β-lactam antibiotics is also severely impaired with the exception of cefoxitin which is still able to acylate the Ser96Ala enzyme. The mutations Cys98Ala/Asn have been designed by analogy with the class A β-lactamases and the high molecular mass penicillin-binding proteins, where the third residue of motif 2 is mostly an asparagine. Both mutations decrease the k_{cat}/K_m value for the peptide substrates but increase it significantly for the thiolester substrates. The substitution of a cysteine by an asparagine in position 98 has a remarkable positive effect on the interaction with β-lactam antibiotics, especially with cefoxitin. It is proposed that Lys38 acts as the catalytic base that abstracts a proton from the active serine Ser35 during nucleophilic attack and that Ser96 is a key intermediate in the proton transfer from the Oγ of Ser35 to the substrate leaving group nitrogen. Cys98 is proposed to play the same role as Asn in motif 2 of other penicilloyl serine transferases in properly positioning the substrate for the catalytic process.

1.2.1.2. Class C LMM PBP/DD peptidases

All class C PBPs, beside the DD-carboxypeptidase-transpeptidase/PBP domain, possess an additional domain different from what is observed in the other classes of PBPs.

The DD-carboxypeptidase-transpeptidase of *Actinomadura* R39 was solved by single anomalous dispersion at the cobalt resonance wavelength at a 2.85 Å resolution for the Co^{2+} derivative (PDB code 1W8Q) and 1.8 Å for the native protein (PDB code 1W79) [E. Sauvage *et al.*, 2005 accepted in *JBC*].

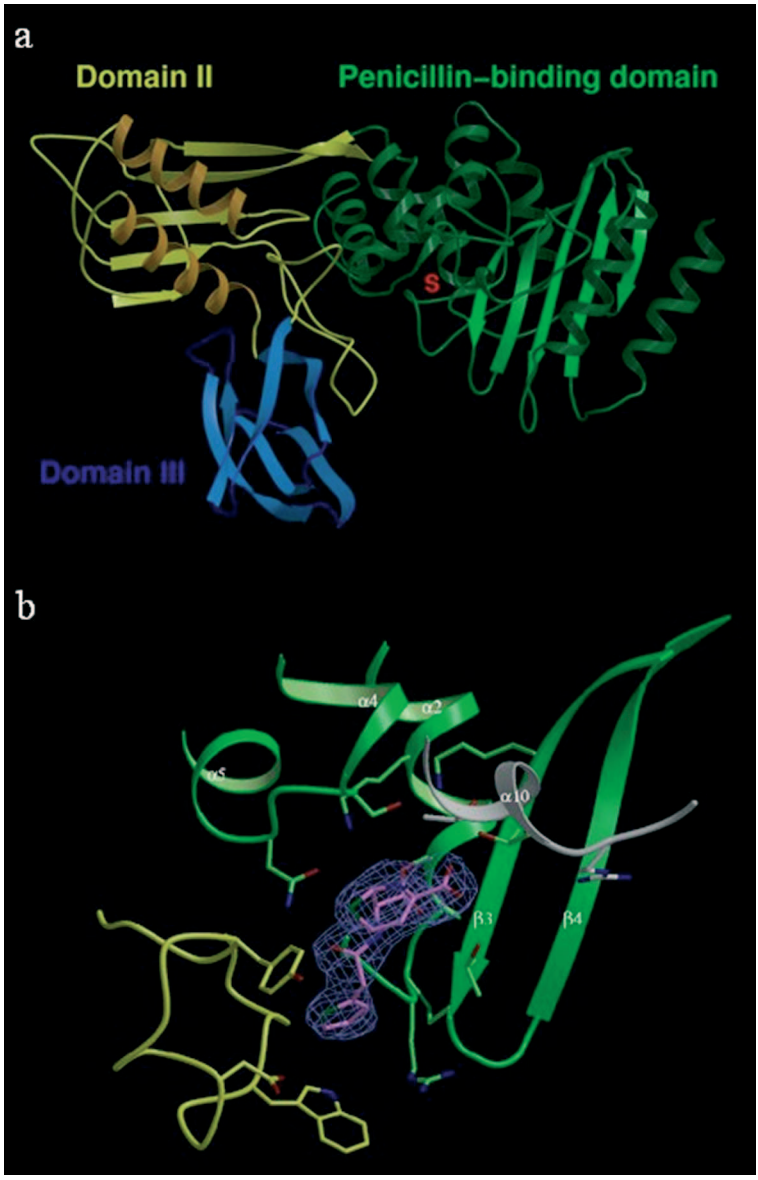


Fig. 5. **a**) Ribbon representation of the the DD-carboxypeptidase-transpeptidase of *Actinomadura* R39. The structure is composed of three domains, a penicillin-binding domain (in green) and two domains (in yellow and in blue) of unknown function, inserted in the penicillin-binding domain, between the SxxK and SxN motifs. The active site is represented by S (in red) for the active serine. **b**) Active site of the acyl-enzyme complex of R39 with nitrocefin, with the electron density (in blue) around nitrocefin (in purple).

The crystallographic R39 structure is a tetramer, each monomer containing 466 amino acids. The structure has been refined to an R factor of 21.4% ($R_{\text{free}} = 24.0\%$) and contains 13,394 protein atoms and 1,781 water molecules. It is composed of three domains, a penicillin-binding domain similar to the penicillin-binding domain of the *E. coli* PBP5 and two domains of unknown function (Fig. 5a). In most multimodular PBPs, additional domains are generally located at the C- or N-terminal end of the penicillin-binding domain. In R39, the other two domains are inserted in the penicillin-binding domain, between the SxxK and SxN motifs, in the way of "Matryoshka dolls". Domain II (residues 66–165 and 237–284) is a five stranded β-sheet with two helices packed on one side. Using a three dimensional DALI search, domain II was found to topologically resemble domains of *E. coli* d-Tyr-tRNATyr deacylase and Barley 1,3-1,4-b-glucanase, with rmsd values of 3.1 Å (82 aligned Cα) and 3.5 Å (74 aligned Cα), respectively. It is also topologically similar to the N-t domain of MinC and the 1A region of FtsA, two proteins interacting with FtsZ and implicated in the regulation of the septum formation in cell division. Domain III (residues 166–236) consists in two three-stranded β-sheets facing each other in a kind of incomplete β-barrel. A DALI search did not give any probing result of a structurally similar protein.

One complex with nitrocefin was solved at a 2.4 Å resolution (PDB code 1W8Y). R39 is highly sensitive to β-lactams ($k_2/K = 2600\ \text{mM}^{-1}\,\text{s}^{-1}$ for nitrocefin) and deacylation occurs at a very low rate ($k_3 = 1.5 \times 10^{-6}\,\text{s}^{-1}$), a favourable situation for the accumulation of an acyl-enzyme. The structure of the acyl-enzyme complex of R39 with nitrocefin at 2.4 Å reveals the absence of an active site conformational change upon binding by β-lactams (Fig. 5b).

The electron density of the R39 active site unambiguously reveals a covalent acyl-enzyme with a dihydrothiazolidine ring and the well defined carboxylate. The density map does not account for the R2 substituent of nitrocefin. Departure of the C3' substituent after electronic rearrangement upon opening of the β-lactam ring has been observed in acyl-enzyme complexes of R61 and PBP2x with cephalosporins but not in the acyl-nitrocefin-PBP2a structure.

The *B. subtilis* PBP4a is highly isologous to the *Actinomadura* R39 DD-peptidase and clearly exhibits DD-carboxypeptidase and thiolesterase activities *in vitro*. PBP4a however is only moderately sensitive to the β-lactam compounds, in opposition with the R39 enzyme. The PBP4a was overproduced for crystallization trials and an expression system was developed to enrich the protein in selenomethionine.

The *B. subtilis* PBP4a has then been solved at a 1.8 Å resolution by molecular replacement, using the R39 structure as model (PDB code 1W5D).

1.2.2. The high molecular mass PBPs

1.2.2.1. Class A PBPs

The PBP1b of *Escherichia coli*

The *E. coli* PBP1b catalyses the assembly of lipid-transported *N*-acetylglucosaminyl-β-1,4-*N*-acetylmuramoyl-L-Ala-D-Glu-mesoA2pm-D-Ala-D-Ala disaccharide pentapeptide units into polymeric peptidoglycan. These units are phosphodiester linked, at C1 of muramic acid, to a C55 undecaprenyl carrier. To gain insight into the catalytic mechanism of the glycosyl-transferase (GT), the specificity profile of the enzyme for the lipid II substrate, was carried out by using substrate analogues. The UDP-disaccharide pentapeptide compound in which the lipid moiety of lipid II was replaced by uridine functions rather as a glycosyl donor than a glycosyl acceptor. The GT activity required the presence of the two sugars for the recognition by the enzyme and a lipid part on the C1 of the MurNAc for the acceptor function. The peptide moiety of the substrate appears to be important for the GT activity.

The PBP1b of *Enterococcus faecalis*

Enterococcus faecalis, *Enterococcus faecium* and *Enterococcus hirae* possess each three class A PBPs designated by M. Arthur as PBP1, PBPF and PBPZ. Each of these PBPs is related to one class A PBP of *Streptococcus pneumoniae* and *Bacillus subtilis*. The enterococcal PBP1 (encoded by the *ponA* gene) is similar to PBP1a of *S. pneumoniae* and PBP1 of *B. subtilis*. PBPF is close to PBP2a and PBP2c respectively and PBPZ is related to PBP1b and PBP4 respectively.

A soluble derivative of the class A PBP1 of *E. faecalis* JH2-2 (encoded by the *ponA* gene) was overproduced in *E. coli* and purified. It exhibited a glycosyltransferase activity on the *E. coli* ^{14}C-labeled lipid II precursor. It could also hydrolyse thiolesters substrates with efficiencies similar to those of other class A PBPs. It also binds β-lactams, but with k_2/K (the parameter accounting for the acylation step efficiency) values characteristic of penicillin-resistant PBPs [111].

Deletion of the *ponA* gene of *E. hirae* (SR1 mutant) does not change much the susceptibility to β-lactams in comparison with the parental ATCC 9790 strain. However on solid medium, SR1 seems to be two times and five times more susceptible to vancomycin and streptomycin respectively. In addition, SR1 is more inhibited than ATCC 9790 when ampicillin, nisin (a peptidic antibiotic) or Triton X-100 (a detergent) are added at mid-exponential growth phase. When reaching stationary phase, the mutant cells are also more prone to lyse in the presence of lysozyme. Taken together these results point to a defect of the mutant cell wall that renders the cytoplasmic membrane more accessible.

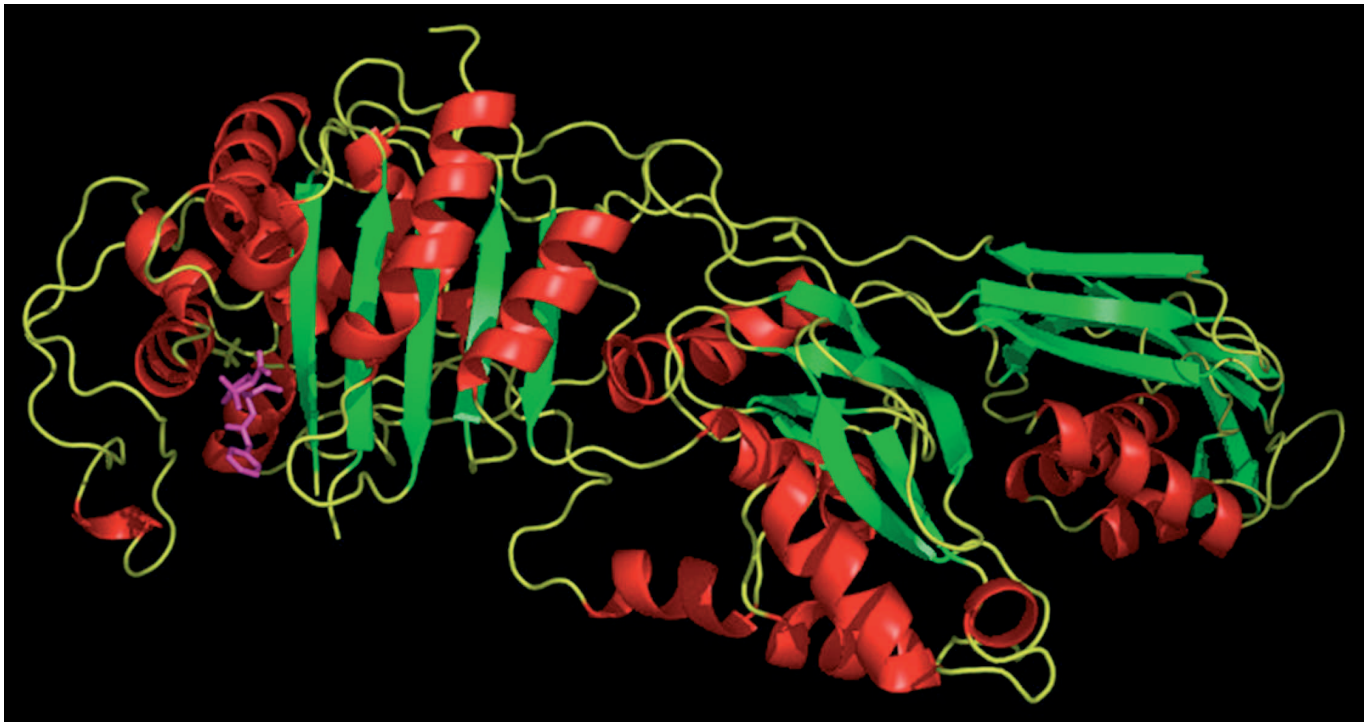


Fig. 6. Ribbon representation of the low-affinity PBP5 of *Enterococcus faecium* The structure is composed of three domains, a penicillin-binding domain (on the left) and two domains (on the right) of unknown function, at the N-terminal end of the protein. A benzylpenicillin molecule (in magenta) is covalently bound to the active serine inside the catalytic site.

1.2.2.2. Class B PBPs

The low-affinity PBP5 of *Enterococcus faecium*

The synthesis of low-affinity PBPs is responsible for the emergence of highly penicillin-resistant *Enterococcus* and *Staphylococcus* strains. They all belong to class B. A soluble form (Mr: 72,000) of the *E. faecium* low-affinity PBP5 (PBP5fm) has been produced, purified and crystallized. The structure of the complex of a selenomethionyl derivative of PBP5fm with benzylpenicillin has been solved at a resolution of 2.1 Å, using the MAD method [69]. The results underline a strong structural similarity between the penicillin-binding module and that of other penicillin-recognizing proteins (Fig. 6). Careful analysis of these structures indicated that two peptides, that seem to be highly conserved in different low-affinity PBPs and appear to control the access to the active site, could be responsible for the low propensity of PBP5 to bind β-lactams. Indeed, a characteristic of the active site, which distinguishes the PBP5fm from other PBPs of known structure, is the topology of loop 451–465 defining the left edge of the cavity. The Arg464 residue, while being involved with the Asp481 residue in a salt bridge, confers a greater rigidity to the active site. This structural characteristic coupled with the presence of the Val465 residue that is pointing inside the active site and thus can block accessibility of it, could account for the low affinity of PBP5fm for β-lactams. This loop is common to low-affinity PBPs, such as PBP2a of *Staphylococcus aureus* and PBP3 of *B. subtilis*. Moreover the existence of an insertion after residue 466 in the most resistant strains underlines even more the determining role of this loop in the recognition of the substrates.

Sequencing of the *pbp4* and *psr-like* genes of *Enterococcus faecalis*

The penicillin-resistant JH2-2r mutant was isolated from *E. faecalis* JH2-2 by successive passages on plates containing increasing concentrations of benzylpenicillin. A comparison of the PBP profiles in the two strains revealed a more intensely labelled PBP4 in JH2-2r. The sequences of the JH2-2 and JH2-2r *pbp4* genes, including their promoter regions, were determined and compared. They appeared identical. A *psr* gene analogous to that proposed to act as a regulator of the PBP5 synthesis in *E. hirae* and *E. faecium* was not found in the vicinity of *pbp4* in *E. faecalis* JH2-2 and JH2-2r. However, a *psr-like* gene distant from *pbp4* was identified. The cloning and sequencing of that *psr-like* gene from both strains indicated that they were also identical. The PBP4 overproduction in *E. faecalis* JH2-2r therefore results from the modification of a factor that remains to be identified [25].

1.3. D-aminopeptidases

Different strains of bacteria were screened for their ability to hydrolyse D-alanyl-para-nitroanilide. Activity was detected in several *Bacilli* and *Ochrobactrum anthropi* strains.

1.3.1. The self-compartmentalizing D-aminopeptidase DppA, a binuclear zinc-dependent enzyme from *Bacillus subtilis* 168

A D-alanylamidase was purified from *B. subtilis* and partially characterized. The determination of the N-terminal sequence identified the enzyme as the product of the *dppa* gene. *Dppa* gene, amplified by polymerase chain reaction from *B. subtilis* genomic DNA, was subcloned in an expression plasmid specific for *Escherichia coli*. The overproduced enzyme called DppA was purified to homogeneity. DppA is a multimeric zinc-dependent enzyme strictly D-stereospecific. Among the tested substrates, the highest activities were found with D-alanyl-D-alanine [18, 134].

The crystal structure obtained in Prof. Van Beeumen's laboratory reveals an elaborate quaternary organization similar to that found in the self-compartmentalizing proteases such as proteasomes: the DppA enzyme is composed of ten identical 30 kDa subunits arranged in a barrel-shape molecule. Two channels give access to a large central cavity. The active sites proceed in a zigzag course along the inner wall of the cavity shielded from the solvent. Two zinc ions are bound in each active site [35, 134].

An exploration of the databases showed that the genome of other organisms (Gram-positive bacteria, Gram-negative bacteria, *streptomyces*, archaea) contained ORFs encoding putative proteins similar to the *B. subtilis* DppA.

The physiological role of DppA, a cytoplasmic D-aminopeptidase that is produced at the end of the exponential phase of growth and able to hydrolyse D-alanyl-D-alanine, is probably an adaptation to nutrient deficiency.

1.3.2. The N-terminal nucleophile hydrolase DmpA, a L-aminopeptidase D-alanyl-amidase/-esterase from *Ochrobactrum anthropi* LMG 7991

From an expression library constructed in *E. coli* with *O. anthropi* genomic DNA, a gene coding for a D-alanylamidase was found. The corresponding protein called DmpA was overproduced in *E. coli* and characterized. The soluble active protein consisted of two distinct polypeptides corresponding to residues 2-249 (α-subunit) and 250-375 (β-subunit). The Gly249/Ser250 cleavage, producing a N-terminal nucleophile residue, is required for activity. With the alanyl-para-nitroanilide, the rate of hydrolysis of the D isomer is faster than that of the L isomer. In contrast, with oligopeptides like alanyl-glycyl-glycine, it is the reverse (Fanuel *et al.*, 1999).

The crystal structure obtained in Prof. Van Beeumen's laboratory confirmed that DmpA represents the first example of a novel family of aminopeptidases which shows structural similarity to members of the N-terminal nucleophile hydrolase superfamily. Active DmpA is organized as a homotetramer; the substrate binding site of a monomer is located at the interface with its two neighbour subunits [2, 141]. The study of DmpA is actually pursued.

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

2.1. Cell division machinery

2.1.1. Cell division in *E. coli*

In *Escherichia coli*, cell division is mediated by the concerted action of about twelve proteins that assemble at the division site to presumably form a complex called the divisome (Fig. 7). Among these essential division proteins, the multimodular class B PBP3 that is specifically involved in septal peptidoglycan synthesis consists of a short intracellular M1-R23 peptide fused to a F24-L39 membrane anchor that is linked via a G40-S70 peptide to a R71-I236 non-catalytic module itself linked to a D237-V577 catalytic penicillin-binding module. It has been proposed that the activity of the transpeptidase module of PBP3 is regulated by the interaction of its N-terminal non-catalytic module with other cell division proteins.

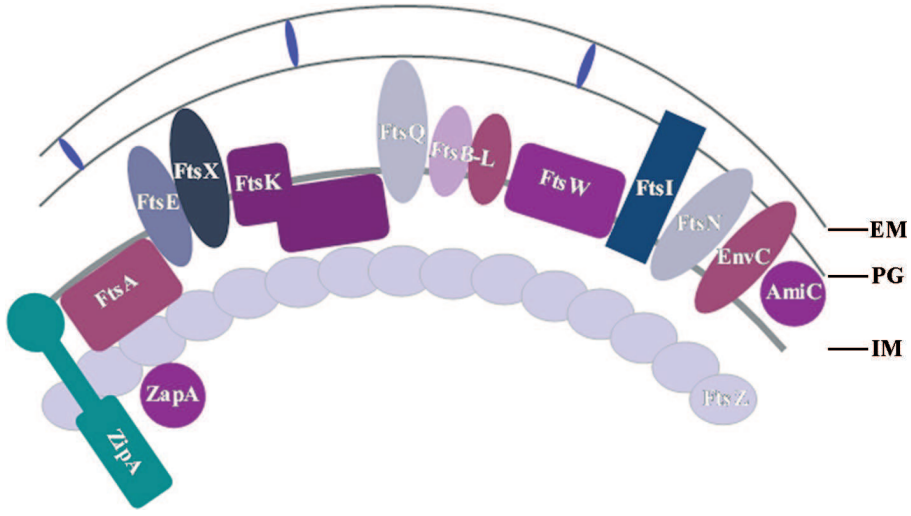


Fig. 7. Schematic representation of the divisome in *Escherichia coli*

Site-directed mutagenesis experiments allowed the identification in the non-catalytic module of PBP3 three peptide segments that have protein-protein interaction potentials and specific functions. The G40-S70 sequence and the membrane anchor-containing module appear to play an important role in the proper insertion of the protein within the divisome at the division site. The H160-G172 segment, located at the intermodule junction, seems to be involved in intramolecular interactions and plays an important role in the conformation of the protein. The E206-V217 segment that is exposed at the surface of the non-catalytic module of PBP3 plays an important role in cell septation presumably by interacting with other components of the divisome. FtsQ, FtsL, FtsW are plausible partners [23].

On the basis of localization analyses of PBP3 mutants fused to the Green Fluorescent Protein (GFP) by fluorescence microscopy (Fig. 8), it appears that the first 56 amino acid residues of PBP3 containing the membrane anchor and the G40-E56 peptide contain the structural determinants required to target the protein to the cell division site and that none of the putative protein interaction peptides present in the non-catalytic module are essential for the positioning of the protein to the division site [123]. By using an *E. coli* two-hybrid system, we showed that the first 56 amino acid residues of PBP3 interacted with FtsW (B. Wolf, C. Fraipont and M. Nguyen-Distèche, unpublished data).

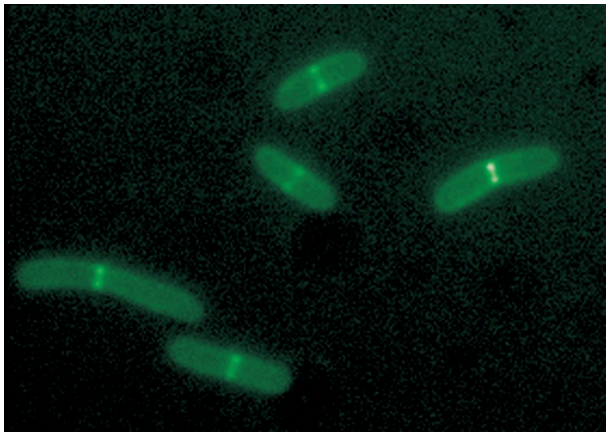


Fig. 8. Localisation of penicillin-binding protein 3 fused to the Green Fluorescent Protein (GFP) in *Escherichia coli*, observed by fluorescence microscopy.

FtsW that has 10 transmembrane segments (TMSs), is a late recruit to the division site and is required for subsequent recruitment of PBP3. Site-directed mutagenesis experiments combined with fluorescence microscopy allow to

identify several domains of the protein with distinct functions. The localization of PBP3 to the septum was found to be dependant on the periplasmic loop located between TMSs 9 and 10. The E240-A249 amphiphilic peptide in the periplasmic loop between TMSs 7 and 8 appears to be a key element of the functioning of FtsW in the septal peptidoglycan assembly machineries. The intracellular loop (containing the R166-F178 amphiphilic peptide) between TMSs 4 and 5, and Gly311 in TMS 8 are important components of the amino acid sequence-folding information [125].

Cell division proteins FtsZ, (FtsA, ZipA, ZapA), FtsE/X, FtsK, FtsQ, FtsL/B, FtsW, PBP3, FtsN, and AmiC, localize at mid cell in *E. coli* in an interdependent order as listed. To investigate whether this reflects a time dependent maturation of the divisome, the average cell age at which FtsZ, FtsQ, FtsW, PBP3, and FtsN arrive at their destination was determined by immuno- and GFP-fluorescence microscopy of steady state grown cells at a variety of growth rates. Consistently, a time delay of 14 to 21 min, depending on the growth rate, between Z-ring formation and the mid cell recruitment of proteins downstream of FtsK was found. We suggest a two-step model for bacterial division in which the Z-ring is involved in the switch from cylindrical to polar peptidoglycan synthesis, whereas the much later localizing cell division proteins are responsible for the modification of the envelope shape into that of two new poles (Aarsman *et al.*, 2005).

2.1.2. Cell division in *Enterococcus hirae*

The N-terminal module of PBP5fm is made of a long and narrow β-sheet that bears two small globular domains that could participate in protein-protein interactions. It is known that PBP5fm takes over the function of the other PBPs during inhibition with sub-MIC β-lactam concentrations. It can also replace PBP3 that is needed for cell division and probably part of a multiprotein complex.

Substitution of one or two amino acid residues by directed-mutagenesis in one of these globular domains, in the L200-K206 region predicted to be implicated in protein-protein interactions, almost completely abolishes the PBP5fm ability to replace PBP3. This was tested in a PBP5-deficient mutant of *E. hirae* ATCC 9790 which PBP3 synthesis was placed under the control of an inducible promotor. In the absence of the inducer, the cells stopped dividing unless PBP5fm was produced (S. Leimanis *et al.*, submitted).

2.2. Low-affinity PBP5 regulation in *E. hirae*

Expression of the *pbp5* gene (encoding the low-affinity PBP5) in *E. hirae* was proposed to be under the control of *psr*, a 882-bp gene starting 1kb upstream *pbp5*. It was also suggested that in addition to contributing in β-lactam resistance, the *psr* gene had a larger regulatory function on other properties such as, for example, cell growth and cell autolysis. Reexamination of the role of *psr* by specific gene disruption or deletion led to the conclusion that it does neither influence PBP5 synthesis and consequently β-lactam resistance nor cell autolysis or cell growth [101].

The *psr* and *pbp5* genes are preceded by the *ftsW* gene. All three form an operon that is overexpressed in the penicillin-resistant *E. hirae* R40 mutant. This seems to result from two genetic modifications: a joint mutation in the promotor site controlling the operon and a 87-bp deletion overlapping the 5'end of *psr*. The mutation occurred in a highly conserved palindromic sequence that acts as an operator site of different genes in Gram-positive bacteria and includes the −35 and −10 motifs. The deletion appears to suppress a secondary structure upstream of *pbp5* that might influence the stability of the mRNA (C. Franssen, PhD thesis).

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

A large number of proteins present in the bacterial cytoplasmic membrane serve as receptors that receive chemical messages and other signals from outside the cells. These receptors, which are sometimes enzymes, respond to these external stimuli by launching intracellular signals that control metabolism, cell growth and, in our case, the expression of a gene responsible for β-lactam resistance. In this latter case, a β-lactam resistant PBP or a β-lactamase is induced by the presence of a β-lactam antibiotic outside the cell. Four different induction mechanisms relaying the presence of the extracellular β-lactam antibiotic to the cytoplasmic protein that controls the gene of resistance, have been identified in the microbial world: two in Gram-negative bacteria [*Citrobacter freundii* (Jacobs *et al.*, *Cell. Mol. Life Sci.* 1997, **88**: 823–32) and *Aeromonas hydrophila* (Avison *et al.*, *J. Antimicrob. Agents Chemother.* 2004, **53**, 197–202)] and two in two Gram-positive bacteria [*Bacillus licheniformis*/*Staphylococcus aureus* (Zhang *et al.*, *Science* 2001, **29**, 1962–5.) and *Streptomyces cacaoi* (Lenzini *et al.*, *Mol. Genet. Genomics* 1992, **235**, 41–8)] (Fig. 9).

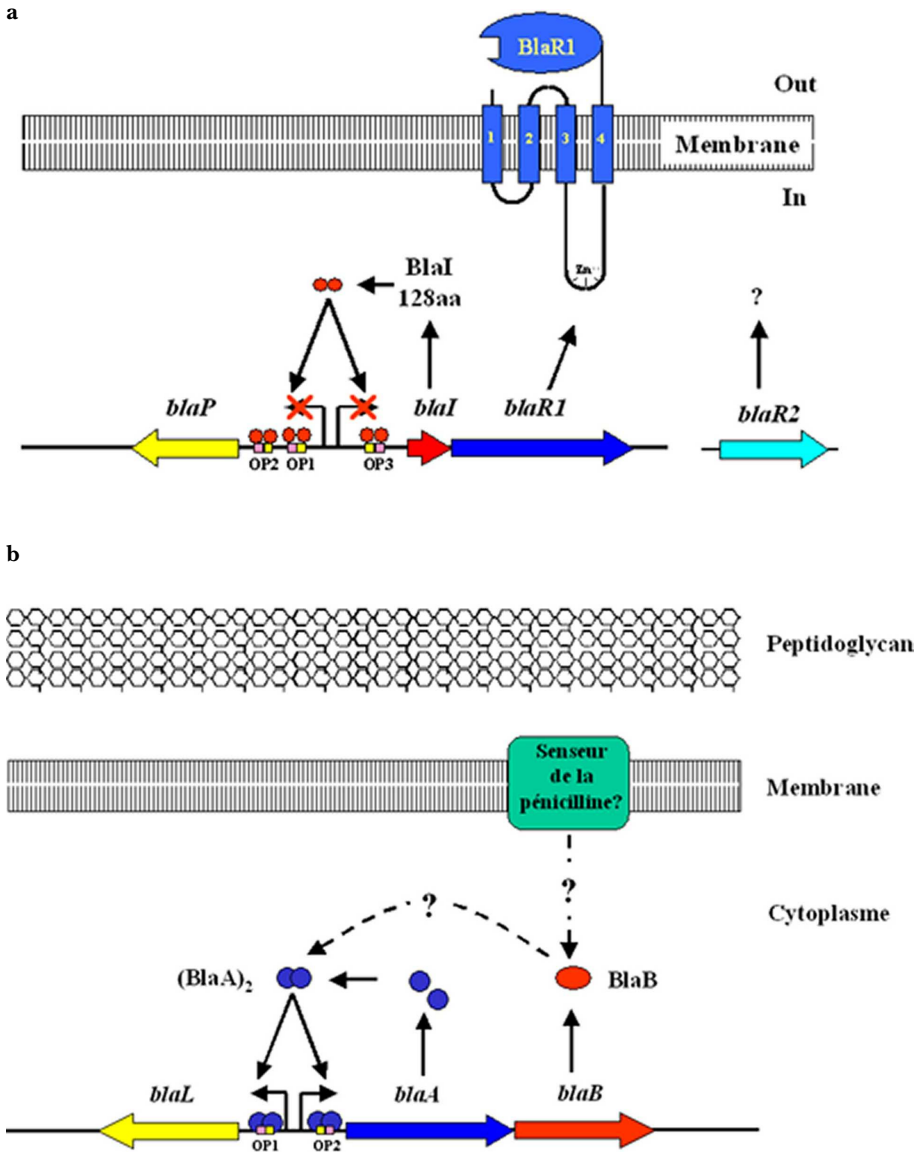


Fig. 9. β-lactamases induction mechanism in *B. licheniformis* (**a**) and *S. cacaoi* (**b**). (**a**) The products of the *blaI* and blaR1 genes are involved in the induction of the BlaP β-lactamase: BlaI is a DNA binding protein and acts as a repressor, BlaR1 is a membrane protein and acts as the penicillin receptor. The *blaR*2 gene has not been identified yet but its product could be involved in the clivage of the cytoplasmic loop L3 of the BlaR1 receptor. (**b**) The products of the *blaL*, *blaA* et *blaB* genes are, respectively, the BlaL β-lactamase, the BlaA activator-repressor and the cytoplasmic BlaB protein still of unknown function.

2.3.1. Induction of the BlaP β-lactamase in *Bacillus licheniformis*

The induction of the *Staphylococcus aureus* BlaZ and *Bacillus licheniformis* 749/I BlaP β-lactamases by β-lactam antibiotics occurs according to similar processes. In both bacteria, the products of the *blaI* and *blaR*l genes share a high degree of sequence homology and act as repressors and penicillin-sensory transducers respectively. It has been shown in *S. aureus* that the BlaI repressor, which controls the expression of BlaZ negatively, is degraded after the addition of the inducer. We have followed the fate of BlaI during β-lactamase induction in *B. licheniformis* 749/I and in a recombinant *Bacillus subtilis* 168 strain harbouring the pDML995 plasmid, which carries the *B. licheniformis blaP*, *blaI* and *blaR*l genes [57]. In contrast to the situation in *B. licheniformis* 749/I, β-lactamase induction in *B. subtilis* 168/pDML995 was not correlated with the proteolysis of BlaI. To exclude molecular variations undetectable by SDS-PAGE, two-dimensional gel electrophoresis was performed with cellular extracts from uninduced or induced *B. subtilis* 168/pDML995 cells. No variation in the Blal isoelectric point was observed in induced cells, whereas the DNA-binding property was lost. Cross-linking experiments with dithio-bis(succimidylpropionate) confirmed that, in uninduced recombinant *B. subtilis* cells, BlaI was present as a homodimer and that this situation was not altered in induced conditions. This latter result is incompatible with a mechanism of inactivation of BlaI by proteolysis and suggests that the inactivation of BlaI results from a non-covalent modification by a co-activator and that the subsequent proteolysis of BlaI might be a secondary phenomenon. In addition to the presence of this co-activator, our results show that the presence of a penicillin stress is also required for full induction of β-lactamase biosynthesis.

Three palindromic operator regions are recognized by BlaI: two in the *blaP* promoter (OP1 and OP2) and one (OP3) in the promoter of the *blaI-blaR*1 operon. For a better understanding of the BlaI regulation, the dissociation constant of the purified BlaI dimer has been estimated at 25 μM by equilibrium ultracentrifugation [82]. Quantitative Western blot analysis indicates that the intracellular concentrations of BlaI in *B. licheniformis* 749/I and *B. subtilis* transformed by a multicopy plasmid harboring the β-lactamase locus (*blaP-blaI-blaR*1) were lower than (1.9 μM) or in the same range as (75 μM) the dissociation constant, respectively. This suggests that BlaI is partially dimeric in the cytoplasm of these strains and interacts *in vivo* with its operators as a preformed dimer. This hypothesis is supported by band shift assays on an operator containing a randomized half-operator sequence. The global dissociation constants of the operator-BlaI dimer complexes were measured by band shift assays and estimated as

$$K_{d_{OP1}} = 1.7 \pm 0.5\,10^{-15}\,\mathrm{M}^2,$$
$$K_{d_{OP2}} = 3.3 \pm 0.9\,10^{-15}\,\mathrm{M}^2,$$
and
$$K_{d_{OP3}} = 10.5 \pm 2.5\,10^{-15}\,\mathrm{M}^2.$$

The noncovalent complexes between the BlaI protein dimer (wild-type and GM2 mutant) and its double-stranded DNA operator have been studied by nanospray mass spectrometry and tandem mass spectrometry (MS/MS) [70]. Reproducibility problems in the nanospray single-stage mass spectra are emphasized. The relative intensities depend greatly on the shape of the capillary tip and on the capillary-cone distance. This results in difficulties in assessing the relative stabilities of the complexes simply from MS spectra of protein-DNA mixtures. Competition experiments using MS/MS are a better approach to determine relative binding affinities. A competition between histidine-tagged BlaIWT (BlaIWTHis) and the GM2 mutant revealed that the two proteins have similar affinities for the DNA operator, and that they co-dimerize to form heterocomplexes. The low sample consumption of nanospray allows MS/MS spectra to be recorded at different collision energies for different charge states with 1 μL of sample. The MS/MS experiments on the dimers reveal that the GM2 ??? dimer is more kinetically stable in the gas phase than the wild-type dimer. The MS/MS experiments on the complexes shows that the two proteins require the same collision energy to dissociate from the complex. This indicates that the rate-limiting step in the monomer loss from the protein-DNA complex arises from the breaking of the protein-DNA interface rather than the protein-protein interface. The dissociation of the protein-DNA complex proceeds by the loss of a highly charged monomer (carrying about two-thirds of the total charge and one-third of the total mass). MS/MS experiments on a heterocomplex also show that the two proteins BlaIWTHis and BlaIGM2 have slightly different charge distributions in the fragments. This emphasizes the need for better understanding the dissociation mechanisms of biomolecular complexes.

The BlaI repressor is composed of two structural domains. The 82-residue NTD (N-terminal domain) is a DNA-binding domain, and the CTD (C-terminal domain) containing the next 46 residues is a dimerization domain. We have analysed the equilibrium unfolding of BlaI in the presence of GdmCl (guanidinium chloride) using different techniques: intrinsic and ANS (8-anilinonaphthalene-l-sulphonic acid) fluorescence, far- and near-UV CD spectroscopy, cross-linking, analytical ultracentrifugation, size exclusion chromatography and NMR spectroscopy [121]. In addition, the intact NTD and CTD were purified after proteolysis of BlaI by papain, and their unfolding by GdmCl was also studied. GdmCl-induced equilibrium unfolding was shown to be fully reversible for BlaI and for the two isolated fragments. The results

demonstrate that the NTD and CTD of BlaI fold/unfold independently in a four-step process, with no significant co-operative interactions between them. During the first step, the unfolding of the BlaI CTD occurs, followed in the second step by the formation of an 'ANS-bound' intermediate state. Cross-linking and analytical ultracentrifugation experiments suggest that the dissociation of the dimer into two partially unfolded monomers takes place in the third step. Finally, the unfolding of the BlaI NTD occurs at a GdmCl concentration of $\sim$ 4.0 M. In summary, it is shown that the BlaI CTD is structured, more flexible and less stable than the NTD upon GdmCl denaturation. These results contribute to the characterization of the BlaI dimerization domain (i.e., CTD) involved in the induction process.

We reported the first solution structure of the 82 amino acid residue DNA-binding domain of *B. licheniformis* BlaI which is very similar in primary sequence to the medically significant *Staphyloccocal* BlaI and MecI proteins [97]. This structure is composed of a compact core of three α-helices and a three-stranded β-sheet typical of the winged helix protein (WHP) family. The protein/DNA complex was studied by NMR chemical shift comparison between the free and complexed forms of BlaI. Residues involved in DNA interaction were identified and a WHP canonical model of interaction with the operators is proposed. In this model, specific contacts occur between the base-pairs of the TACA motif and conserved amino acid residues of the repressor helix H3. These results help toward understanding the repression and induction mechanism of the genes coding for β-lactamase and the resistant *S. aureus* PBP2'.

To study the properties of the BlaR penicillin-receptor involved in the induction of the *B. licheniformis* β-lactamase (Fig. 10a), the water-soluble carboxy terminal domain of the protein (BlaR-CTD) was overproduced in the periplasm of *Escherichia coli* JM105 and purified to protein homogeneity. Its interactions with various β-lactam antibiotics were studied [88]. The second-order acylation rate constants k_2/K' ranged from 0.0017 to more than 1 $\mu M^{-1}\,s^{-1}$ and the deacylation rate constants were lower than $4\,10^{-5}\,s^{-1}$. These values imply a rapid to very rapid formation of a stable acylated adduct. BlaR-CTD is thus one of the most sensitive penicillin-binding proteins presently described. In the light of these results, the kinetics of β-lactamase induction in *B. licheniformis* were re-examined. When starting with a rather high cell density, a good β-lactamase substrate such as benzylpenicillin is too sensitive to β-lactamase-mediated hydrolysis to allow full induction. By contrast, a poor β-lactamase substrate (7-aminocephalosporanic acid) can fully derepress β-lactamase expression under conditions where interference of the antibiotic with cell growth is observed. These results suggest that acylation of the penicillin receptor is a necessary, but not sufficient, condition for full induction.

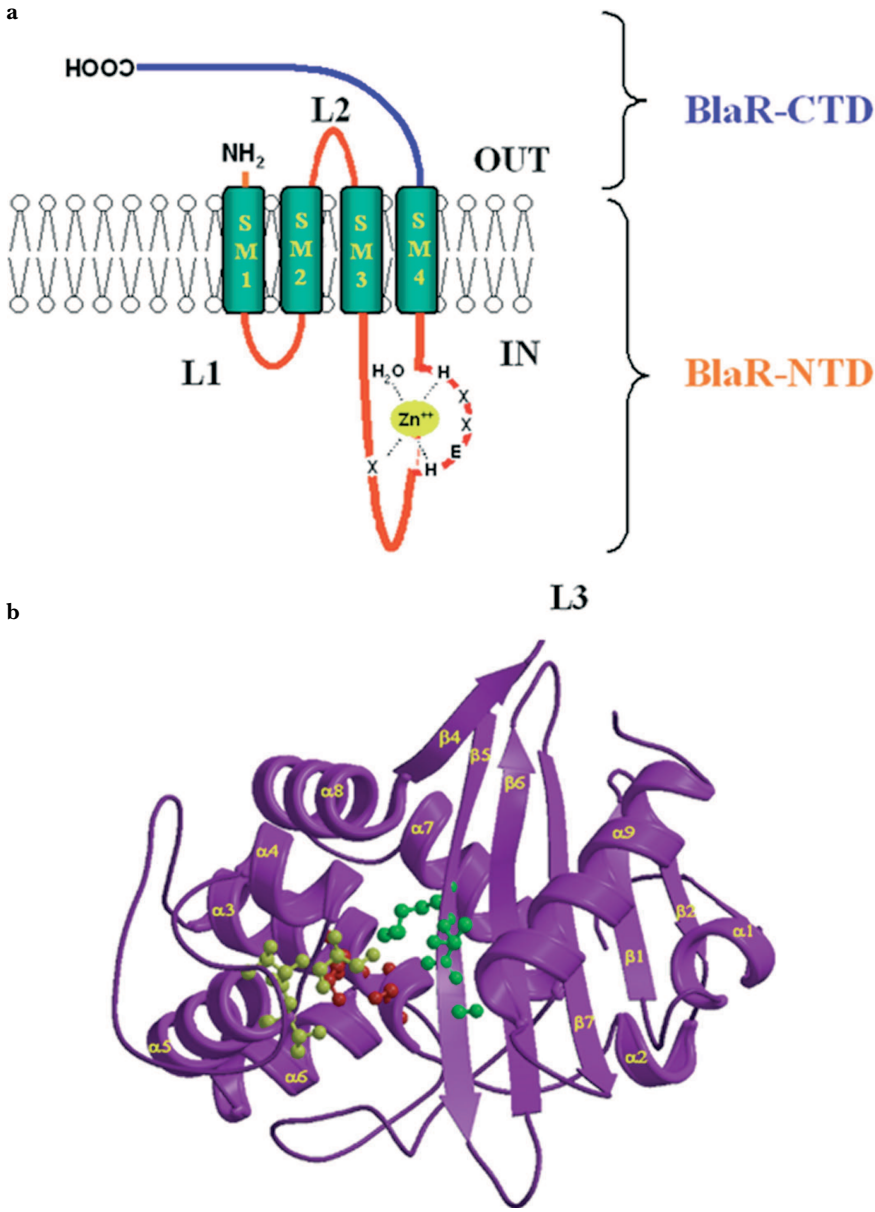


Fig. 10. The BlaR1 penicillin-receptor involved in the induction of the *B. licheniformis* β-lactamase: (**a**) membrane topology, (**b**) ribbon representation of the water-soluble carboxy terminal domain (BlaR-CTD), with the three conserved structural elements in the active site: the first motif (Ser402-Thr403-Tyr404-Lys405) in red, the second motif (Ser450-Thr451-Thr452) in yellow and the third motif (Lys539-Thr540-Gly541) in green.

The crystal structure of the extracellular penicillin receptor of BlaR (residues 346 to 601) has been determined. The thin needle-shaped crystals belong to the tetragonal form and space-group P41 were assigned to the crystal with unit cell axis $a = b = 45.85$ Å and $c = 130.20$ Å. The phasing procedure was the molecular replacement method with the AMORE package, using as search model the co-ordinates of one monomer of the OXA-2 structure (PDB code 1K38). The BlaR-CTD structure was refined to a resolution of 2.5 Å with R and R_{free} values of 20.4% and 25.7%, respectively. BlaR1-CTD and the class D β-lactamases have a very similar folding and distinguish mainly by the length and configuration of several loops, especially those surrounding the active site and connecting the strands of the seven stranded β-sheet and the relative positions of the terminal α-helices α1 [Nt] and α9 [Ct] (Fig. 10b). ??? The most striking differences in the active site of BlaR1-CTD is the absence of carbonatation of the motif 1 lysine residue (K205 in BlaR1-CTD and K70 in OXA-2), the replacement of V117 by T453 in motif 2 of BlaR1-CTD, and the absence of an arginine residue (R244 in OXA-2) [92].

The amino-terminal domain of the BlaR (BlaR-NTD, residues 1-345) exhibits four transmembrane segments (TM1-TM4) that form a four-α-helix bundle embedded in the plasma bilayer. The BlaR-CTD, which acts as a penicillin sensor, is fused at the carboxyl end of TM4. This membrane topology suggests that BlaR-CTD and the BlaR-NTD domain are responsible for signal reception and signal transduction, respectively. With the use of phage display experiments, we have highlighted an interaction between BlaR-CTD and the extracellular, 63-amino acid L2 loop connecting TM2 and TM3 [105]. This interaction does not occur in the presence of penicillin. This result suggests that binding of the antibiotic to BlaR1 might entail the release of the interaction between L2 and BlaR-CTD, causing a motion of the α-helix bundle and transfer of the information to the cytoplasm of the cell. In addition, fluorescence spectroscopy, CD, and Fourier transform IR spectroscopy experiments indicate that in contrast to the behavior of the corresponding *S. aureus* protein, the β-lactam antibiotic does not induce a drastic conformational change in *B. licheniformis* BlaR-CTD.

2.3.2. Induction of the AmpC β-lactamase in *Citrobacter freundii*

AmpD is a bacterial amidase involved in the recycling of cell-wall fragments in Gram-negative bacteria. Inactivation of AmpD leads to derepression of β-lactamase expression, presenting a major pathway for the acquisition of constitutive antibiotic resistance. We have reported the NMR structure of *C. freundii* AmpD [96]. A deep substrate-binding pocket explains the observed specificity for low molecular mass substrates. The fold is related to that of

bacteriophage T7 lysozyme. Both proteins bind zinc at a conserved site and require zinc for amidase activity, although the enzymatic mechanism seems to differ in detail. The structure-based sequence alignment identifies conserved features that are also conserved in the eukaryotic peptidoglycan recognition protein (PGRP) domains, including the zinc-coordination site in several of them. PGRP domains thus belong to the same fold family and, where zinc-binding residues are conserved, may have amidase activity. This hypothesis is supported by the observation that human serum N-acetylmuramyl-L-alanine amidase seems to be identical with a soluble form of human PGRP-L.

AmpD exhibits a strict specificity for 1,6-anhydromuropeptides and requires zinc for enzymic activity. In an attempt to assign the different zinc ligands and to probe the catalytic mechanism of AmpD amidase, molecular modelling based on the NMR structure and site-directed mutagenesis have been performed [106]. Mutation of the two residues presumed to act as zinc ligands into alanine (H34A and D164A) yielded inactive proteins which had also lost their ability to bind zinc. By contrast, the active H154N mutant retained the capacity to bind the metal ion. Three other residues which could be involved in the AmpD catalytic mechanism have been mutated (Y63F, E116A, K162H and K162Q). The E116A mutant was inactive, but on the basis of the molecular modelling this residue is not directly involved in the catalytic mechanism, but rather in the binding of the zinc by contributing to the correct orientation of His-34. The K162H and K162Q mutants retained very low activity (0.7 and 0.2% of the wild-type activity respectively), whereas the Y63F mutant showed 16% of the wild-type activity. These three latter mutants exhibited a good affinity for Zn ions and the substituted residues are probably involved in the binding of the substrate. We also describe a new method for generating the N-acetylglucosaminyl-1,6-anhydro-N-acetylmuramyl-tripeptide AmpD substrate from purified peptidoglycan by the combined action of two hydrolytic enzymes.

2.3.3. Induction of the BlaA and BlaB β-lactamases in *Streptomyces cacaoi*

Two class A β-lactamase genes called *blaL* and *blaU* have been identified in *Streptomyces cacaoi* and are inducible by β-lactam compounds. The two β-lactamase genes are controlled by two regulators named BlaA and BlaB. Whereas BlaA has been identified as a DNA-binding protein related to the LysR repressor/activator family, the function of BlaB is still unknown. Its primary structure is similar to that of the serine penicillin-recognizing enzymes (PREs). Indeed, the SXXK and KTG motifs are perfectly conserved in BlaB, whereas the common SXN element found in PREs is replaced by a SDG motif. Site-directed mutations were introduced in these motifs and they all disturb β-lactamase regulation. A water-soluble form of BlaB was also overexpressed

in the *Streptomyces lividans* TK24 cytoplasm and purified. To elucidate the activity of BlaB, several compounds recognized by PREs has been tested. BlaB could be acylated by some of them, and it can therefore be considered as a penicillin-binding protein. BlaB is devoid of β-lactamase, D-aminopeptidase, DD-carboxypeptidase or thiolesterase activity [99].

2.4. Carbone catabolite repression and substrate induction in *Streptomycetes*

Members of the genus *Streptomycetes* are soil bacteria that rely primarily on complex carbohydrates originating from plants for their carbon supply. These bacteria possess numerous genes that code for secreted hydrolytic enzymes involved in the degradation of cellulose, starch, chitin or xylan. The genes encoding several of these enzymes are usually inducible by the corresponding substrate (substrate induction, SI) and repressible by glucose or other simple carbon sources (carbon catabolite repression, CCR). We are studying the regulation of the expression of secreted enzymes from *Streptomyces* species specifically involved in the degradation of xylan, the second most abundant carbon polymer in plant cell walls after cellulose. Refined analyses of the promoter of *xlnC* from *Streptomyces* sp. EC3 has highlighted the principal *cis*-acting elements that participate in SI and CCR [94]. Different transcription factors presumed to be implicated in SI and CCR are also studied such as DasR. DasR is known to control the expression of all genes that constitute the phosphotransferase system responsible for the uptake of N-acetylglucosamine and probably the chitinase encoding genes responsible for degradation of N-acetylglucosamine polymer chitin. Computational motif prediction approaches has revealed about 40 new target genes of DasR in the genome of *S. coelicolor*. In the future, we intend to define the predicted DasR regulon using microarray techniques and identify the effector molecule responsible of modulating its DNA-binding ability.

2.5. Morphological differentiation in *Streptomyces coelicolor*

Streptomycetes are Gram-positive soil bacteria that undergo morphological ??? differentiation. Their life cycle includes germination, vegetative and aerial mycelial growth, secondary metabolite synthesis, and eventually spore morphogenesis. In order to restore vegetative growth once conditions become favourable, bacterial spores must be able to sense environmental changes that trigger the germination process. cAMP has been demonstrated to be one of these germination signalling molecules. Recently, we were focused on the study of the unique transcription factor of the CRP-FNR family in *Streptomyces*

coelicolor (Crp), which possesses a cyclic-nucleotide-monophosphate-binding domain and thus was considered as a good candidate to control the expression of genes involved in the germination process. We demonstrated that Crp was able to interact with cAMP [129] and its chromosomal inactivation led to a germination-defective mutant of which the phenotype is similar to the mutant of the adenylate cyclase gene (*cya*) unable to produce cAMP [130]. These similar phenotypes for both mutants and the observation of a peak of cAMP accumulation during germination strengthen the idea that a Cya/cAMP/Crp system centrally triggers the developmental process. In the future, we intend to identify the target genes and understand the regulatory network of Crp by means of computational motif predictions and proteomic approaches.

2.6. Deciphering the bacterial regulatory codes

With the monthly increasing amount of genome sequences, the challenge in genomics is no more sequencing but is to characterize and comprehend the functional elements within a genome. Transcription factors and their recognized *cis*-acting elements are two of the most important functional elements as their study permit a deeper understanding of the potential functions of individual genes regulated by newly identified DNA-binding sites. In this context, predicting gene transcription regulation is currently one of the greatest challenges as it provides a complementary analysis to genomic approaches to the discovering and the understanding of new regulatory systems. The first step in deciphering the bacterial regulatory codes is to refine the classification of the defined helix-turn-helix families by compiling data and knowledge on the three components involved in the transcriptional process: (i) the DNA-binding domain, (ii) the effector-binding domain, and (iii) the recognized *cis*-acting elements. So far, we have been focused on the GntR family for which we have defined different subfamilies according to the heterogeneity of the effector-binding domain [66, 127]. Our subfamily classification has been revealed appropriate as new regulatory codes were then predicted [66]. In addition, our *in silico* approach permitted to highlight the specific transcription factor (DasR) of the genes involved in the uptake of N-acetylglucosamine out of the hundreds of coding sequences annotated as "regulatory proteins" within the genome of *Streptomyces coelicolor* [127]. In the future, we intend to reiterate *in silico* analyses to other helix-turn-helix families in order to highlight new regulatory codes and present them to the scientific community as tools to predict new *cis/trans* relationships.

3. Protein folding and stability

3.1. Thermal stabilization of the *Staphylococcus aureus* PC1 β-lactamase by substrate

Although enzyme stabilization or destabilization upon ligand binding is a well-known phenomenon, quantitative data about the influence of substrates are rather scarce, since the enzyme destroys the potential stabilizing agent during the course of the experiments. In this work [36] we could monitor the thermal inactivation of the *S. aureus* PC1 β-lactamase by directly following the decrease of the rate of substrate utilisation, at temperatures above the melting point of the enzyme (~ 41.5 °C).

Not unexpectedly, substrate concentrations well below the K_m value have no stabilizing effect, but protection increases with substrate concentration and reaches a maximum under saturating conditions.

A combination of unfolding kinetic measurements and enzymatic studies, both under steady-state and non-steady state regimes, allowed most of the parameters characteristic of the two concurrent phenomena (i.e., substrate hydrolysis and enzyme denaturation) to be evaluated. Furthermore, molecular modelling studies show a good correlation between the extent of stabilization, and the magnitude of the energies of interaction with the enzyme.

Our analysis indicates that the enzyme is substantially stabilized towards heat-induced denaturation, independently of the relative proportions of non-covalent Henri-Michaelis complex (ES) and acyl-enzyme adduct (ES*).

The efficiency of the protection is strongly substrate-dependent and correlates well with the calculated energies of interaction of the various Henri-Michaelis complexes, which also appear to be valid for the corresponding covalent species.

Thus, for those substrates with which the two catalytic intermediates are expected to be significantly populated, both species (ES and ES*) appear to be similarly stabilized.

This is consistent with the view that the difference in stability between the free enzyme and the saturated ligand-enzyme complex quantitatively accounts for the non-covalent energetic complementarity between the ligand and the enzyme.

3.2. Folding and stability of lysozymes

3.2.1. Hen lysozyme

Hen lysozyme is one of the best characterised and most studied of all biological macromolecules. This small monomeric protein of 129 residues (M_r 14305)

is made up of two structural domains, referred to as the α- and β-domains. The kinetics of folding of this protein with its four disulphide bonds intact has been studied extensively using a variety of different techniques (C.M. Dobson, P.A. Evans and S.E. Radford (1994), Understanding how proteins fold: the lysozyme story so far, *Trends Biochem. Sci.* **19**, 31–37; A. Matagne and C.M. Dobson (1998), The folding process of hen lysozyme: a perspective from the "new view", *Cell. Mol. Life Sci.* **54**, 363–71). This folding process has been found to be a complex mechanism (Fig. 11), involving parallel events with distinct kinetic profiles. Following the very rapid formation (within $\sim$ 4 ms) of a hydrophobically collapsed state, kinetic heterogeneity is observed, and intermediate species become populated. The majority of the molecules ($\sim$ 75%) fold cooperatively to a well-defined intermediate in which the α-domain is persistently structured in the absence of a stable β-domain. The native state is then achieved by the subsequent formation of the β-domain ($\tau \approx 350$ ms). The remaining $\sim$ 25% of molecules acquire native-like structure in both α- and β-domains on a much shorter time-scale ($\tau \approx 10$ ms). This species, however, is not fully native and requires an additional step ($\tau \approx 100$ ms) associated with rearrangement and docking of the two partly structured domains to form a functional active site and the fully native state. A variety of techniques, including quenched-flow hydrogen exchange labelling monitored by electrospray ionization mass spectrometry, and stopped-flow optical measurements, have been used to investigate the refolding kinetics of hen lysozyme over a temperature range from 2°C to 50°C (*NB.* all these experiments [1] were performed at the Oxford Centre for Molecular Sciences, in the group of professor Christopher M. Dobson). Simple Arrhenius behaviour is not observed, and the overall refolding rate reaches a maximum at *ca* 40°C, i.e., much below the transition midpoint of the enzyme ($T_m = 70$°C). By using transition state theory, a global analysis of the kinetic data was performed [1], according to a sequential three-state model for the slow folding pathway. This analysis suggests that the temperature dependence of the rate constant for lysozyme folding largely relies on the heat-induced destabilization of the transient intermediate on the major folding pathway at 20°C, which results in a dramatic slowing down of the folding process above 40°C. Together with previous findings, these results indicate that the α-domain intermediate is a productive species on the folding route between the initial and final states, and which accumulates merely as a consequence of its intrinsic stability. This macroscopic description of the lysozyme folding process, based on classical pathways defined in terms of sequences of macrostates, is in good agreement with recent calculations of the exact dynamics of a simple folding model

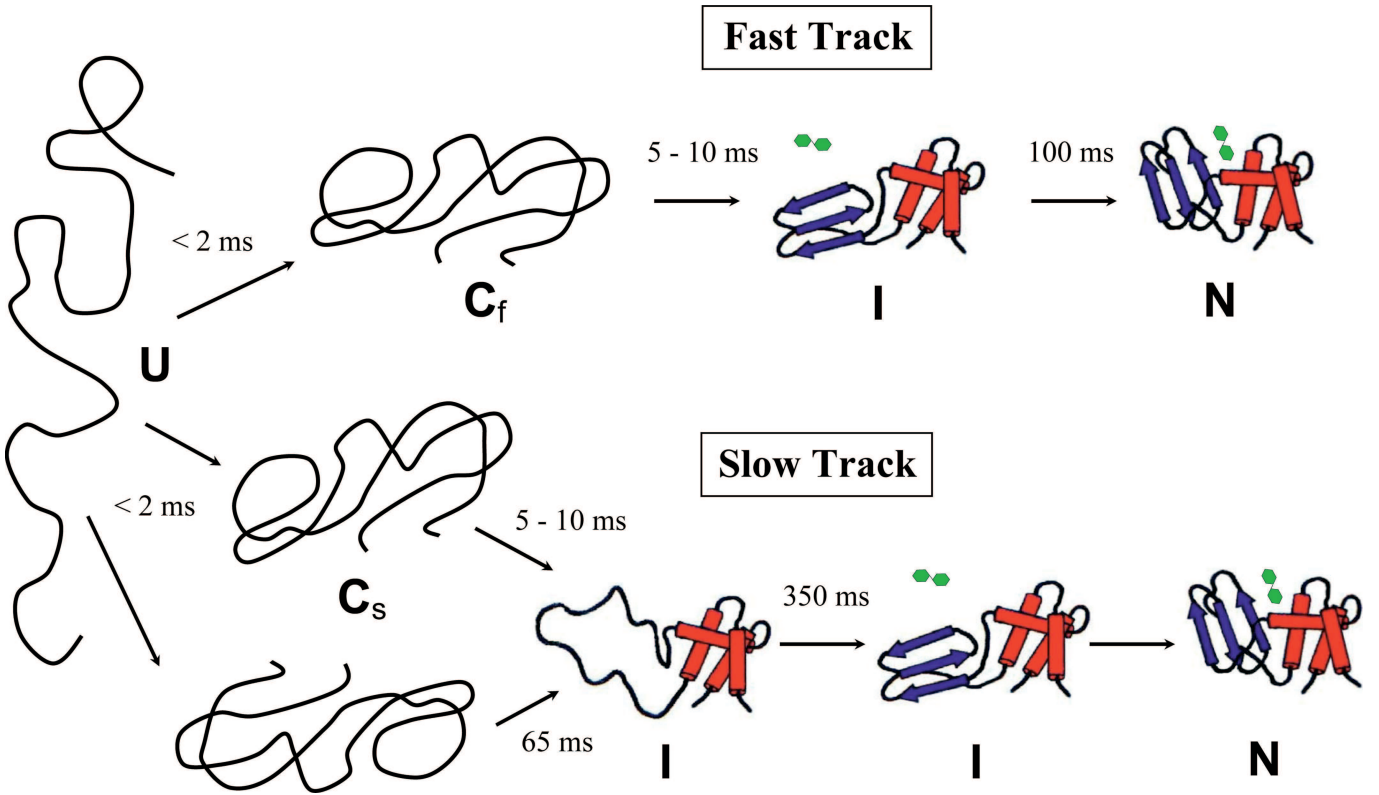


Fig. 11. Representation of the folding process of hen lysozyme. Starting from a highly heterogeneous unfolded state (U), collapse leads to various structural ensembles (C_f and C_s), which refold with substantially different kinetics. The parallel nature of the refolding process is highlighted, and both the fast and slow folding routes ($\sim 25\%$ and $\sim 75\%$ of molecules, respectively) are illustrated. Domains with native-like structures are represented in red (α-domain) or in blue (β-domain) and the enzyme with a functional active site with ligand (green) bound can be seen as the outcome of both folding tracks.

(S.B. Ozkan, K.A. Dill and I. Bahar (2002), Fast-folding protein kinetics, hidden intermediates, and the sequential stabilization model, *Prot. Sci.* **11**, 1958–1970).

3.2.2. Lambda lysozyme (Ludovic Bannwarth, master thesis)

Like other lysozymes, bacteriophage lambda lysozyme (λ lysozyme; 158 amino acid residues; M_r 17825) is involved in the digestion of bacterial cell walls. This enzyme, however, differs from other lysozymes with respect to its cleavage of the peptidoglycan; unlike other lysozymes, λ lysozyme is not a hydrolase but a transglycosylase. The enzyme X-ray crystal structure (C. Évrard, J. Fastrez and J.P. Declercq (1998), Crystal structure of the lysozyme from bacteriophage lambda and its relationship with V and C-type lysozymes, *J. Mol. Biol.*, **276**, 151–64) indicates that the general structural features of lysozymes are conserved, namely the organization into two structural domains connected by a helix, and the occurrence of the active site in a cleft at the interface between the two domains. From the point of view of protein evolution, λ lysozyme displays characteristics of lysozyme from different classes (Évrard *et al.*, 1998). Most remarkably, however, λ lysozyme is devoid of disulphide bonds, four of which are normally present in c-type lysozymes (e.g., from hen or human). With these ???
proteins, study of the oxidative refolding *in vitro* is quite challenging, at least in part because of the propensity of the fully reduced denatured protein to aggregate under most conditions used for efficient refolding. Hence, most refolding studies of c-type lysozymes have been performed with the four native ???
disulphide bonds intact (see 3.2.1). Although this model has provided valuable information on the principles that govern the folding of proteins, the presence of the native disulphide bonds at the beginning of the folding reaction is clearly not representative of the actual folding process *in vivo*. Thus, we believe that a comparison of the folding and stability properties of the λ lysozyme with those of the well-studied hen and human lysozymes may provide further insights into the general mechanisms of protein folding.

Preliminary studies have shown that the denaturation of λ lysozyme by guanidinium chloride and urea is a fully reversible phenomenon. Hence, unfolding of the enzyme could be investigated under equilibrium conditions, in the presence of various concentrations of denaturant. Optical measurements—i.e., intrinsic fluorescence emission, ANS binding and far UV circular dichroism (CD)—indicate that the enzyme is cooperatively unfolded in a single transition. In comparison with hen lysozyme ($\Delta G^o_{\mathrm{UN}} \approx -50\ \mathrm{kJ\,mol^{-1}}$) and with phage T4 lysozyme ($\Delta G^o_{\mathrm{UN}} \approx -52\ \mathrm{kJ\,mol^{-1}}$), a disulphide free v-type lysozyme, the λ lysozyme displays a significantly lower conformational stability ($\Delta G^o_{\mathrm{UN}} \approx -30\mathrm{kJ\,mol^{-1}}$). These data confirms that, in some respects, λ lysozyme

appears to be structurally different from the other members of both the c- and v-type lysozyme families (Évrard *et al.*, 1998). ??? ???

3.3. Single-domain antibody fragments (V_HHs)

3.3.1. High conformational stability of V_HHs

Camels, dromedaries and llamas generate functional antibodies formed by two heavy chains, but no light chains. Following immunization, recombinant antibody fragments (V_HHs) can be selected, which consist of a single domain only. These minimum-sized antigen-binders (~ 14 kDa) are produced as soluble monomers in bacteria and yeast (S. Muyldermans, C. Cambillau and L. Wyns (2001), Recognition of antigens by single-domain antibody fragments: the superfluous luxury of paired domains, *Trends Biochem. Sci.*, **26**, 230–35). A number of V_HH fragments have been generated against selected enzymes or haptens. Despite the absence of a light chain, these V_HHs display high affinities (in the nM range) for their antigens. Remarkably, it appears that a significant fraction of heavy-chain antibodies raised against enzymes interact directly with the active site. Thus, the immunization of dromedaries with the TEM-1 and BcII β-lactamases, two representatives of class A and class B enzymes, has led to the isolation of highly inhibitory single-domain V_HH antibody fragments [38].

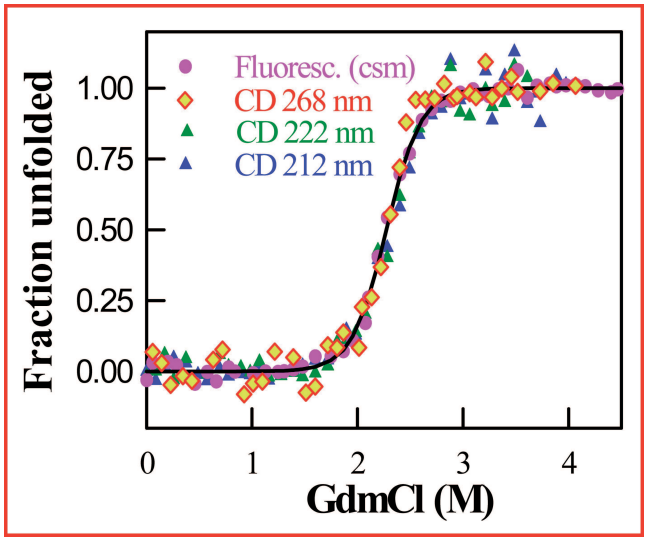


Fig. 12. GdmCl-induced unfolding transition of cAb-HuL6 followed by far and near UV CD, and fluorescence measurements.

A variety of techniques, including high pressure unfolding monitored by Fourier transform infrared spectroscopy, fluorescence, circular dichroism and surface plasmon resonance spectroscopy, have been used to investigate the equilibrium folding properties of six single-domain antigen-binders derived from camelid heavy-chain antibodies with specificities for lysozymes, β-lactamases, and a dye [72].

Various denaturing conditions (guanidinium chloride, urea, temperature and pressure) provided complementary and independent methods for characterizing the stability and unfolding properties of the antibody fragments. With all binders, chemical-induced unfolding is fully reversible, and occurs with a simple two-state transition (Fig. 12), where only the native and the denatured states are significantly populated. Thermally-induced denaturation, however, is not completely reversible, and the partial loss of binding capacity might be due, at least in part, to incorrect refolding of the long loops (CDRs), which are responsible for antigen recognition. Most interestingly, all the fragments are rather resistant to heat-induced denaturation (apparent $T_m = 60$–80°C), and display high conformational stabilities ($\Delta G^o_{298\mathrm{K}} = 30$–$60\ \mathrm{kJ\,mol^{-1}}$). In comparison with conventional antibody fragments, the reduced size, improved solubility and higher stability of the camelid heavy-chain antibody fragments are of special interest for biotechnological and medical applications.

3.3.2. Inhibition of amyloid fibrils by human lysozyme

Five mutants of human lysozyme (I56T, F57I, W64R, D67H and F57I/T70N) have so far been identified in connection with systemic amyloid disease ("protein folding disease"). The ability of at least two of these variants (I56T and D67H) to form amyloid deposits, is primarily a result of their reduced stability, which allows partial cooperative unfolding to take place under physiologically relevant conditions (D. Canet, A.M. Last, P. Tito, M. Sunde, A. Spencer, D.B. Archer, C. Redfield, C.V. Robinson and C.M. Dobson (2002), Local cooperativity in the unfolding of an amyloidogenic variant of human lysozyme, *Nature Struct. Biol.*, **9**, 308–15; M. Dumoulin, D. Canet, A.M. Last, E. Pardon, D.B. Archer, S. Muyldermans, L. Wyns, A. Matagne, C.V. Robinson, C. Redfield and C.M. Dobson (2005), Reduced global cooperativity is a common feature underlying the amyloidogenicity of pathogenic lysozyme mutations, *J. Mol. Biol.*, **346**, 773–88.). Both mutations cause a cooperative destabilization of a substantial segment of the polypeptide chain, which results in the transient population of a closely similar, partially unstructured species. In this distinct intermediate state, the β-domain and the adjacent C-helix are substantially unfolded, whereas the three remaining α-helices that form the core of the α-domain remain native-like.

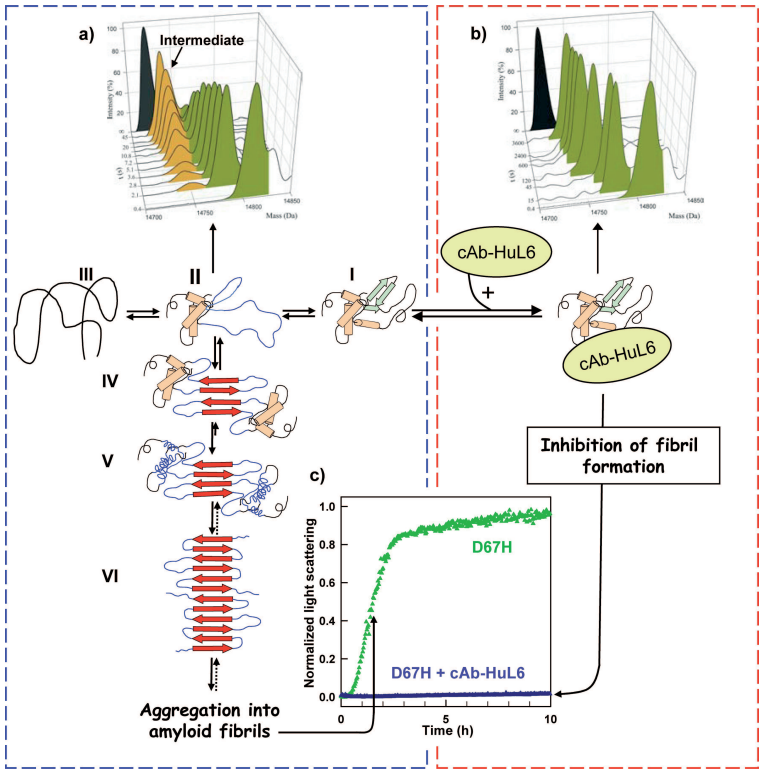


Fig. 13. **Blue box.** Schematic representation of the proposed mechanism for amyloid fibril formation by lysozyme. Under some physiological conditions, the variant proteins (I) transiently populate an intermediate species (II), as revealed by H/D exchange experiments analysed by mass spectrometry (panel a). In these intermediate species, the β-domain and the C-helix are cooperatively unfolded whereas the remaining of the α-domain is in its native like structure. These intermediate species then self-associate through the newly exposed aggregation prone regions (IV) *via* the formation of intermolecular interactions to initiate fibril formation. Further rearrangement (V and VI) is likely to occur in the remainder of the structure, including the recruitment of additional regions of the polypeptide chain into the β-sheet structure prior to the formation of mature fibrils. **Red box.** Proposed mechanism for the inhibition of fibril formation by a camelid antibody fragment. The electrospray mass spectra of D67H lysozyme in the presence of an equimolar concentration of the antibody fragment show a single peak (panel b), whose mass decreases with the length of time for which the exchange was allowed to proceed. The peaks of the species of lower mass observed in the spectra of the free D67H variant (peaks coloured yellow in panel a) and that result from a locally cooperative unfolding of the β-domain and the C-helix, are therefore not observed in the spectra of the D67H protein in the presence of the antibody fragment. This result indicates that the binding of the antibody fragment to the D67H variant restores the stability and global cooperativity that is characteristic of wild type lysozyme; similar results have been obtained for the I56T variant. Thus, in the presence of the antibody fragment the variant proteins do not populate the partially folded intermediate (species II, box on the left hand side) that otherwise can initiate the aggregation process. The result of antibody binding is therefore to prevent the ready conversion of the lysozyme variants into their aggregated states (panel c). [Adapted from M. Dumoulin & C.M. Dobson (2004), Probing the origins, diagnosis and treatment of amyloid diseases using antibodies, *Biochimie*, **89**, 589–600.]

One possible strategy for inhibiting and even reversing amyloid fibril formation is the use of specific antibodies. We have investigated the effects of the interaction of cAb-HuL6 [72], a fragment from a "heavy-chain" camel antibody with high specificity for native lysozyme and its I56T and D67H amyloidogenic variants. Due to the very favourable properties of this antibody fragment, including small size and high solubility and stability (see 3.3.1), conditions could be selected [98] under which the lysozyme variants readily aggregates *in vitro*, while the antibody fragment remains stable and able to tightly bind native lysozyme. We have shown that binding cAb-HuL6 to both the I56T (Dumoulin *et al.*, 2005) and D67H [98] lysozyme variants dramatically inhibits their ability to aggregate *in vitro* and form amyloid fibrils. H/D pulse-labelling experiments analysed by mass spectrometry and NMR indicate that the specific binding of the V_HH significantly stabilizes those regions of the protein involved in the locally cooperative unfolding events, and restores the high degree of global cooperativity that is characteristic of the wild-type protein. As a result of this binding, the ready conversion of the lysozyme variants into their aggregated states is prevented (Fig. 13). This provides further evidence that the formation of a partially unfolded species, resulting from the locally cooperative unfolding of the β-domain and the C-helix, is the critical event that triggers the aggregation process in the absence of the antibody fragment [98] (Dumoulin *et al.*, 2005). These data, combined with NMR and X-ray structural analyses of the complexes, suggest that the disruption of the interface region between the α- and the β-domains is a crucial event determining the amyloidogenicity of the variants. Moreover, the observation that binding of an antibody fragment restores the global cooperativity characteristic of the wild-type protein supports the idea that native state stabilization through antibody binding is a viable therapeutic strategy.

4. Xylanases: structure, stability and rational modification of properties

Family 11 *endo*-β-1,4-xylanases degrade xylan, the main constituent of plant hemicelluloses, and have many potential uses in biotechnology.

Three different xylanases were purified from culture filtrates of Streptomyces S38 [6]. One of the enzymes, named Xyl1 was particularly interesting for biodelignification processes.

On the basis of the known structures of related enzymes, the family 11 β-1,4-xylanases, structural models were built for the mesophilic *Streptomyces* S38 Xyl1 enzyme and for the thermophilic xylanase from *Thermomonospora*

fusca. A comparison of both models suggested mutations that might improve the stability of Xyl1 [7]. The Thr11Tyr mutation increased the optimum temperature from 60 to 70°C and the apparent T_m by 9°C. The models indicated that these increases in thermophilicity and thermostability were due to a novel aromatic interaction between Tyr11 and Tyr16. Other mutations increased the thermostability but not the thermophilicity.

Later, Xyl1 was crystallized and the structure solved at 2.0 Å by X-ray crystallography using the method of molecular replacement, with a final R factor of 18.5% ($R_{\text{free}} = 26.9\%$). Xyl1 has the overall fold characteristic to family 11 xylanases, with two highly twisted β-sheet defining a long cleft containing the two catalytic residues Glu87 and Glu177 [41].

By comparing this newly solved structure with those of alkalophilic or acidophilic xylanases, amino acid substitutions were identified which might explain the differences between the optimum pH values of the various enzymes. Mutations were performed in Xyl1 accordingly. The Trp20Tyr mutation decreased the optimum pH value from 6.0 to 5.0 [114] while Glu139Lys increased it to 7.5 (De Lemos *et al*, in press). The modified enzymes, at their optimum pH's retained activities similar to that of the wild-type at pH 6.0. Since Xyl1 exhibits one of the highest xylanase activities described so far, the respective activities of the two mutants at extreme pH values remained quite high: 600 IU/mg of protein at pH 3.0 for Trp20Tyr and 1300 IU/mg at pH 9.0 for Glu139Lys.

5. Protein hybrids

Enterotoxigenic *Escherichia coli* (ETEC) is responsible for significant economic losses in agriculture and in developing countries it causes diarrhoeal diseases in infants, in addition to traveller's diarrhoea. The fluid secretion, induced by ETEC infection, has been shown to be mediated by two classes of enterotoxins: the heat-labile enterotoxin (LT) and the heat-stable enterotoxin (ST). There are two subtypes of ST: STa and STb which differ in amino acid sequence and biological activities. The typical enterotoxin produced by bovine ETEC is the STa enterotoxin subtype. STa enterotoxins fall into two classes which differ only slightly. The first enterotoxin is composed of 18 amino acid residues (STp or STaI) and the other is composed of 19 amino acid residues (STh or STaII). They are designated STp and STh since they originate from porcine and human strains of enterotoxigenic *Escherichia coli*, respectively. Both enterotoxins have six cysteines which form three intramolecular disulfide bridges that are essential for the toxicity of the molecule.

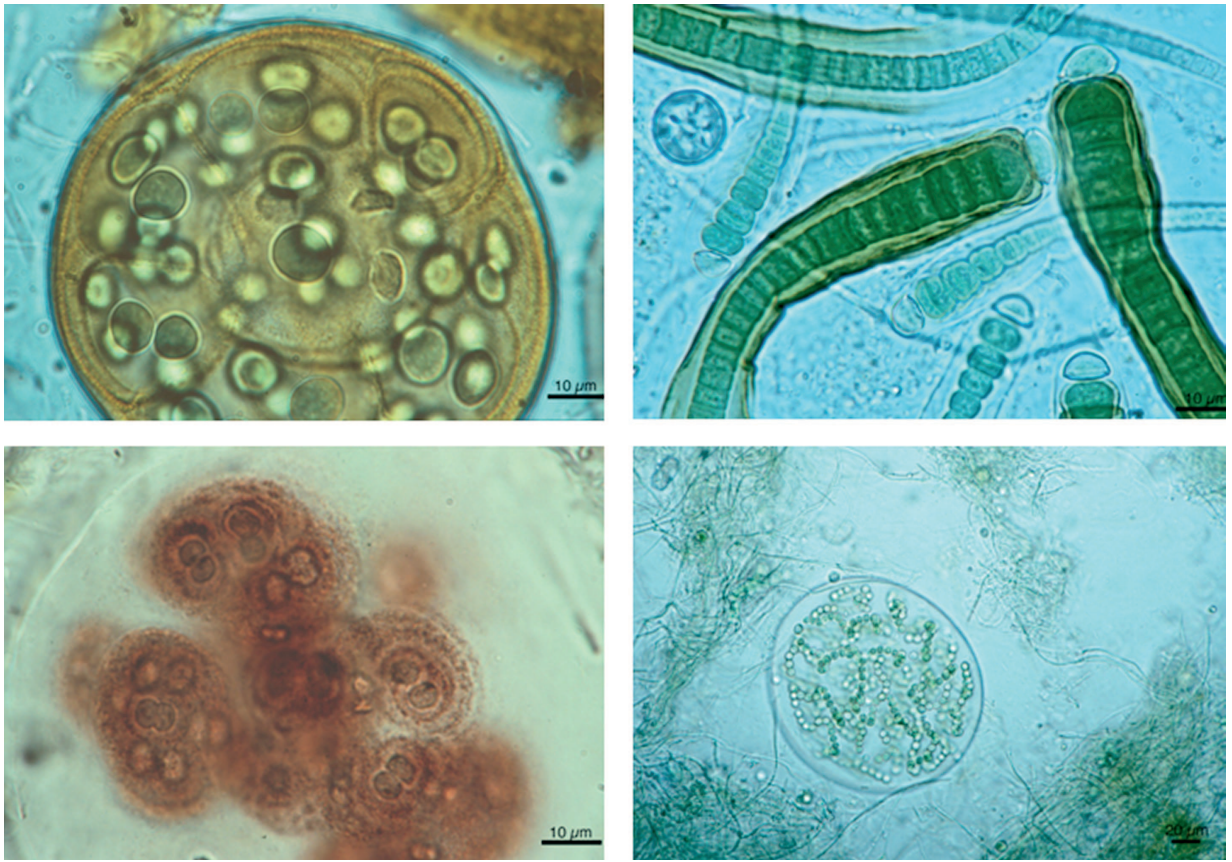


Fig. 14. Antarctic cyanobacteria from microbial mats of coastal lakes (see p. 66)

Because of its small size, STa is poorly antigenic and not immunogenic in its native form. However, STa can be made immunogenic when coupled to an appropriate carrier in a standard hapten-carrier configuration. Several approaches have been explored to obtain non-toxic immunogenic molecules for safe vaccine design. Several proteins have been coupled chemically to STa in order to increase the immunogenicity of the enterotoxin, including Bovine Serum Albumin, the B-subunits of cholera toxin (CTB), and the heat-labile enterotoxin (LT). Some hybrid fusion proteins have been constructed between ST and the major protein subunit ClpG of *E. coli* CS31A fimbriae (ClpG-ST), between ST and one or more subunits of the cholera toxin. Those constructions either failed to elicit neutralizing antibodies or retained some degree of STa-associated toxicity, suggesting that the immunogenic properties of the toxin are influenced by conformation associated with toxicity.

We choose to genetically insert the nucleotide sequence coding for the mature form of the STa I into the amp^{R} gene encoding the TEM-1β-lactamase. This enzyme is a 263-amino-acid protein that hydrolyses the β-lactam ring of antibiotics and renders bacteria resistant [20, 21] a.o. to penicillin. The ??? sequence encoding the STa oligopeptide was genetically inserted into two different surface loops of the β-lactamase TEM-1, in position 171 and 192 respectively named loop A and loop B. Three mutated STa sequences in which one or two cysteine residues were replaced by alanines, were also inserted in the TEM-1 molecule at position 171. These mutations disrupt one or two disulfide bridges and normally cause a complete loss of toxicity. The hybrid genes were cloned into an appropriate plasmid DNA expression vector. The plasmids were tested for their capacity to induce a humoral TEM-1 and STa specific response in BALB/c mice. In a second phase, an attempt was made to increase the STa specific antibody production, by boosting the DNA immunized mice with synthetic peptide.

6. Metagenomics

Metagenomics is an emerging field, which is based on the genomic analysis of microbial DNA that is extracted directly from communities environmental samples, a process that circumvents culturing. We are currently constructing DNA libraries from soil samples and screening them on the basis of functional (search for enzymatic activity) or sequence-based approaches. We are searching for novel β-lactamases produced by environmental bacterial and also for genetic mobile element that could be involved in the transfer of resistance gene from environment to clinic settings.

7. Genomic diversity in Cyanobacteria (Fig. 14)

Cyanobacteria are photoautotrophs that are very ancient and were responsible more than 2 billions years ago for the switch from a reducing atmosphere to the oxygenated one that was necessary for the apparition of modern life forms that need oxygen. Presently, they play an important biological role as primary producers in the food chains of many aquatic and terrestrial biotopes (also extreme ones), as atmospheric nitrogen fixers, and as producers of a wealth of secondary compounds.

In the past, their diversity has been assessed on the basis of morphological characters that might be quite plastic and, used alone, do not offer a sound basis for taxonomic identifications and phylogenetic inferences. For these reasons, the molecular diversity of cyanobacteria in different biotopes (microbial mats in Antarctic lakes, European and Belgian surface waters, symbiosis with the water fern *Azolla*, plankton of lake Tanganyika or the Arctic Sea, …) was studied. Because cyanobacteria may cause toxic blooms in surface waters, the use of molecular markers to detect the genetic potential for toxin production was also tested.

7.1. Diversity of cyanobacteria in microbial mats of Antarctic lakes

On the basis of the morphological identifications, it was believed that most Antarctic cyanobacteria were cosmopolitan. However, this resulted mainly from the use of identification keys that were made for temperate regions and a neglect of the ecological information. We have studied 6 samples from 5 lakes, of contrasted ecological features, from the Mc Murdo Dry Valleys [95] and two regions from Eastern Antarctica (Larsemann Hills and Vestfold Hills). Using the molecular taxonomic marker, the 16S rRNA gene, we showed that the genomic diversity is higher than the morphological one. To discuss the issues of diversity and distribution, we have defined 'Operational Taxonomic Units (OTUs)', or groups of sequences that could correspond to at least one species following the bacterial species definition. When a distance tree is constructed with all published Antarctic sequences, with our newly determined ones, a total of 53 OTUs were identified (Fig. 15), of which 38 have not been recorded for non-Antarctic sites (or at least were not present amongst the circa 3,400 cyanobacterial sequences now present in the databases). This suggests a high degree of endemism. Of the potentially endemic Antarctic OTUs, 28 were recorded in only one sample, and they may be limited to the area in which they were found. 5 other OTUs were found in several samples, but restricted to one region. The last 5 Antarctic OTUs were found in at least

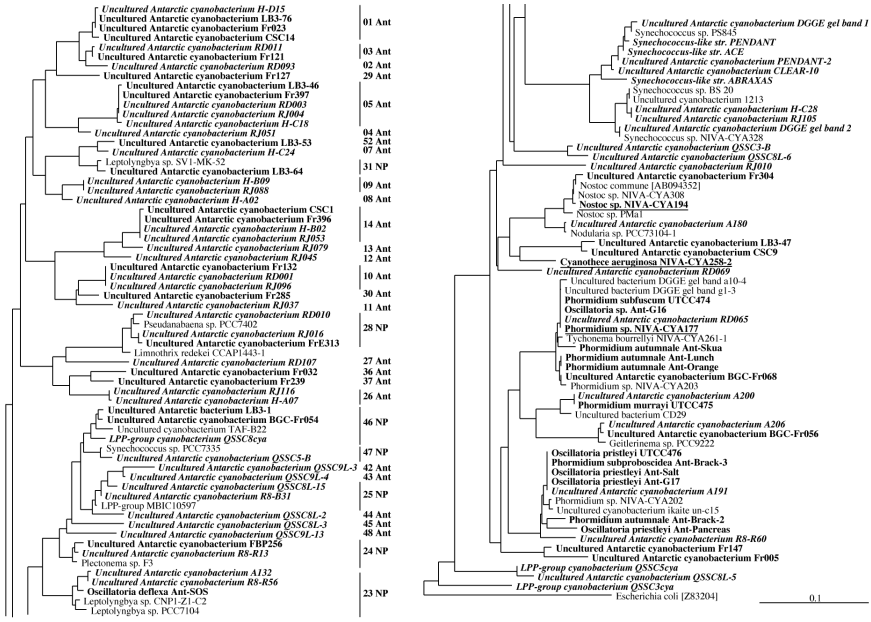


Fig. 15. Distance tree based on partial 16S rRNA gene sequences corresponding to *E. coli* positions 405 to 780. The tree was constructed by the neighbor-joining method with the software package ARB [http://www.arb-home.de/]. The dissimilarity values corrected with the equation of Jukes and Cantor (Jukes & Cantor 1969) were used to calculate the distance matrix. Indels and ambiguous nucleotides were not taken into account. The *E. coli* sequence is used as outgroup.

Antarctic sequences from Eastern Antarctica are in boldface italic type, these from Mc Murdo Dry Valleys are in boldface roman type and these from Dronning Maud Land are in boldface roman type and underlined. The evolutionary distance between two sequences is obtained by adding the lengths of the horizontal branches connecting them and using the scale bar (0.1 mutation per position).

2 regions, as were 9 non-polar OTUs. Thus, 60% of the non-polar OTUs were widely distributed, whereas this was the case of only 13% of the Antarctic ones. This would fit with the idea that non-polar OTUs had to be well adapted to transport and colonisation and thus were quite successful in spreading to new Antarctic habitats in different regions. Alternatively, it would be interesting to test whether the Antarctic genotypes are more specialised and less well disseminated.

Noteworthy is that in each sample studied several new OTUs were found. These results show that the bulk of the cyanobacterial genomic diversity in Antarctic lakes still remains to be discovered.

7.2. Diversity of planctonic cyanobacteria in European surface waters

This work was part of the EC project MIDI-CHIP and the BELSPO project B-BLOOMS (Algal proliferations: emerging problem for health and sustainable use of the surface waters). The planktonic cyanobacterial diversity in lakes in Belgium, Luxembourg, Czech Republic and Poland was determined on the basis of 16S rRNA gene sequences. Most frequently found were the genera *Microcystis*, *Anabaena*, *Aphanizomenon*, with sometimes *Planktothrix* present in summer and *Woronichinia* in autumn. The four first genera are potentially producing microcystins and neurotoxins. PCR primers based on the genes involved in the production of microcystins through a non ribosomal synthesis and described by other authors, were tested. They showed that most of the waterbodies contained organisms potentially toxic.

7.3. Diversity of picocyanobacteria in the Arctic Sea

This study contributes to the Canadian Arctic Shelf Exchange Study (CASES) (http://www.giroq.ulaval.ca/cases). Picocyanobacteria are abundant in polar lakes, but absent in the Arctic Sea and Southern Ocean. Their genomic diversity was studied alongside a gradient going from the Mackenzie river, its estuary and the Arctic Sea (15 samples) in autumn 2002. In the 6 clone libraries performed, 94 sequences of freshwater *Synechococcus* were obtained for the river sample, 49 from the estuary sample and 3 to 8 in the four seawater samples, that seemed to have been washed out from the coast because they are identical to the freshwater sequences. No genotypes from marine *Synechococcus* typical of temperate oceans were detected. Thus, it seems that the freshwater picocyanobacteria from the river are diluted in the Arctic Sea where the grazing pressure is high enough to eliminate them. The decrease of marine picocyanobacteria with decreasing water temperatures was observed by oceanographers during cruises from temperate to polar waters, and this probably explains the absence of this type of marine sequences in the Arctic Sea samples.

7.4. Genomic diversity of the cyanosymbionts in the water fern *Azolla*

Each leaf from the water fern *Azolla* presents a cavity containing symbiotic cyanobacteria. Thanks to an enzymatic digestion of the plant material, it was possible to isolate the cavities and to study the diversity of the symbionts in circa

50 cavities for 35 *Azolla* strains of all seven known *Azolla* species. Two sections are recognized by taxonomists of *Azolla*, Euazolla and Rhizosperma. The Denaturating Gradient Gel Electrophoresis (DGGE) method has shown that the symbionts found in one section were different from the ones in the other section. Thus, the cyanobionts diversity appears to follow the phylogeny of their host species. This is in agreement with the fact that this symbiosis is perpetual and obligate, since both partners have synchronized growth throughout the reproductive cycle of the fern and the isolated cyanobacteria can probably not be grown *in vitro*.

8. Theoretical chemistry and molecular modelling

Several topics have been studied including the exploration of energy hypersurfaces, the mechanism of enzymatic reactions and the interaction between the partners involved in the formation of molecular complexes.

8.1. Vicinity of several electronic states

8.1.1. Electron spectroscopy and mass spectrometry experiments

a) The vibrational analysis of CH_3Cl and CD_3Cl was determined by high level *ab initio* calculations and correlated very well to the experimental results obtained in UV photoabsorption and photoionization mass spectrometric investigation [33, 40].

b) The study of the complex formation involving polyammonium macrotricyclic cages and dicarboxylic acids was performed at the quantum chemistry level in order to be compared with electrospray mass spectra results [28].

8.1.2. Excited electronic states

In order to later investigate electronic transition in small peptide cations, a detailed study of the ionization energies of the natural a-amino acids was performed [110]. This study emphasized the influence of the conformation on the energy and the nature of the ionized state.

8.1.3. Hartree-Fock (HF) wave function instability

As emphasized by an extensive study on about one hundred molecules [5], it appears that the existence of an HF instability is a characteristic of most

unsaturated molecules. The triplet perturbation is related with spin properties of systems that possess electrons with nonnull orbital angular momentum, typically π electronic systems. Such kind of instabilities are already known in solid state physics [61] and were attributed to the peculiar properties of the highly valence correlated electrons. A detailed study is being performed at the quantum chemistry and multivariate statistical analysis levels [Christine Géron, PhD thesis].

The HF instability has also been related to the antioxydant character of a series of imidazolopyrazinones. These molecules behave as quenchers of superoxide anion [53].

8.2. Exploration of energy hypersurfaces

In her PhD thesis, Marie-Noëlle Ramquet has analyzed the mathematical scheme which conditions the appearance of bifurcations along the chemical reaction path. In some regions of the surface, the diagonalisation of the second derivative matrix can give rise to a zero eigenvalue which is associated to a flat region. At a "valley-ridge-inflexion" point (vri), the curvature of the surface changes and the intrinsic reaction path can bifurcate [8].

This feature can occur in several molecular rearrangements as the isomerisation of the methoxy radical. A two dimensional surfaces have been optimized in order to study the wave packets propagation in a dynamic study [76, 86].

8.3. Drug design and molecular modelling

This section concerns the study of molecular complexes and the enzymatic catalytic mechanism.

8.3.1. Molecular complexes

The ability of cyclodextrins to increase the solubility of several molecules has been demonstrated by phase-solubility and NMR experiments. Two types of compounds have been included in β and γ cyclodextrin: the miconazole [52] and the acetate of cyproterone [132]. The most striking feature in both cases is the importance of the entropy factor on the stability of the complexes. The calculations have been performed at the AM1 quantum chemistry level to estimate the force constant matrix.

8.3.2. Conformational analysis and relative energies

The highly selective imidazoline-preferring binding site PMS952 has been selected to study the incidence of intramolecular hydrogen bond and molecular

flexibility on its biological activity. The conformational space of the neutral and ionized forms present a great number of equilibrium structures, more than twenty, in a small energetic range lower than 20 Kcal/mole [22].

In another context, the energetically accessible conformational space of cinchona alcaloids plays an important role on the selection of enantiomers in the oxa Michaël reaction [103].

The total number of possible retrosynthetic bisections of C_{60} leads to nine different isometric C_{30} fragments. The energies, curvatures and homodesmotic stabilization energies of the C_{30} fragments have been evaluated at the *ab initio* 6-31G level [77].

RGD (Arg-Gly-Asp) peptidomimetics have been designed for covalent anchorage on biomaterials. Novel RGD-like molecules based on the tyrosine template have been studied. A modelling analysis has allowed to compare the various synthetic ligands of integrins and to propose novel pharmacophore structures [128].

8.3.3. Enzymatic reactions

8.3.3.1. Enzymes involved in the division and the regulation

Explaining the differences and similarities between the active serine β-lactamases classes on the basis of their 3D structure remains a challenge. In this viewpoint, a docking study of one good (benzylpenicillin) and one bad (ceftazidime) substrate of TEM (class A), P99 (class C) and OXA10 (class D) was realized ["Mécanistique des β-lactamases de classe A, C et D: approche par les méthodes de la chimie théorique", M. Gillon, TFE, ULg, 2002]. It pointed out that it could exist unusual orientations of the substrate that could open new reaction channels.

A study at the molecular mechanics and dynamics levels was performed to bring some explanations on the mutational experiments of the catalytic centre of the *Citrobacter freundii* AmpD N-acetylmuramyl-L-alanine amidase [96, 106] (Liepinsh *et al.*, 2003; Généreux *et al.*, 2004).

A molecular mechanics study of the transcription factor of *Streptomyces coelicolor*, Crp^{Sco}, was performed and compared to the results obtained for Crp^{Eco} [129].

8.3.3.2. Potential elastase inhibitors

Intrinsic reactivity of coumarins has been calculated using a model of a nucleophilic reaction between the ligand and the couple methanol-water.

These calculations aim to calibrate the differences in the reactivity of the lactonic carbonyl group [24].

A series of azetidin-2-ones has been designed and synthetised as potential suicide-inhibitors of porcine pancreatic elastases. The design has been also related to the reactivity which was estimated using a model a nucleophile involving the oxyanion hole environment and a imidazole as the model of histidine. Remarkably, the intrinsinc reaction path points out the incidence of the conformational adaptability during the opening of the C-N bond [55]. This feature has been a guide in the design of new compounds which could also act as inhibitors of human leukocyte elastase bond [109].

9. Other projects

9.1. The Mn-independent peroxidase from *Bjerkandera* sp.

A novel class of lignilolytic peroxidases has been recently described. These enzymes present a high affinity for manganese and dyes, they can also oxidise 2,6-dimethoxyphenol (DMP) and veratryl alcohol (VA) in a manganese-independent reaction and hence offer a high industrial interest. *Bjerkandera* sp. Strain B33/3, a white-rot fungus excretes such an enzyme (the RBP peroxidase). The cDNA corresponding to the mature RBP protein was first amplified and sequenced. The whole gene (named *rbpa* gene), except its promoter region, was also cloned and sequenced. The *open reading frame* appears interrupted by eleven introns. Both sequences were deposited in the EMBL database under the no. AF490538 and AY217015 respectively.

9.2. Biosynthesis of β-lactams

Our involvement in the TNA project principally concerned the development of assays for two key enzymes of the β-lactam biosynthetic pathway, namely the isopenicillin synthase (IPNS) and the deacetoxycephalosporin C synthase (DAOCS). DAOCS belongs to a family of 2-oxoacid-iron(II)-dependent dioxygenases, IPNS is structurally related to DAOCS but presents the exception that it does not require a 2-oxoacid co-substrate to carry out its catalytic process.

We have characterized the *in vitro* conditions for optimal catalytic activity for these two enzymes and our results have led to the establishment of simple and reliable continuous spectrophotometric assays.

These results also gave support to optimise the conditions for the more classical discontinuous HPLC assays and we also developed a novel HPLC assay for DAOCS based on phenylhydrazine derivatization of alpha-ketoglutarate (i.e., the 2-oxoacid co-substrate). This latter assay is general for any 2-oxoacid-utilising enzyme.

Using these new tools we have been able to better characterise and in some cases, determine steady-state kinetic parameters for wild-type and mutated enzymes, with IPNS and its natural substrate, L-α-aminoadipoyl-L-cysteinyl-D-valine substrate (ACV) and with DAOCS against a variety of penicillins and 2-oxoacids substrates.

This knowledge and skills have also been used to investigate other enzymes of the cephalosporin biosynthetic pathway (deacetylcephalosporin C synthase, cephalosporin 7α-hydroxylase) and on a protein of unknown function (ORF12) of the clavulanate biosynthetic pathway.

9.3. Optimization of gel filtration chromatography

Molecular sieve chromatography constitutes a powerful method for the purification of proteins. However, for optimal resolution, it requires a sample of relatively low initial volume, which does not exceed 2% of that of the column. In consequence, the protein solution must often be concentrated before it can be injected onto the column. We have developed a simple procedure [4], which results in an optimised resolution. Samples with a large initial volume, when compared to that of the molecular sieve column, are adsorbed on a small volume of ion exchange gel, and released by a short pulse of concentrated salt solution. The eluate is directly injected onto the molecular sieve column. The very low volume of the eluate ensures optimal conditions and resolution for the molecular sieving process. The method, which was applied as the polishing step in the purification of two β-lactamases (SHV-1 and PSE-2 [4]), could easily be scaled up for the treatment of large samples.

9.4. New tool to manipulate *Bacillus subtilis* chromosome

A novel method to manipulate the *Bacillus subtilis* 168 chromosome has been developed [126]. This method combines the use of *blaI*, which encodes a repressor involved in *Bacillus licheniformis* BlaP β-lactamase regulation, an antibiotic resistance gene, and a *B. subtilis* strain (BS1541) that is conditionally auxotrophic for lysine. We constructed a BlaI cassette containing blaI and the spectinomycin resistance genes and two short direct repeat DNA sequences, one at each extremity of the cassette. The BS1541 strain was obtained by replacing the *B. subtilis* P(lysA) promoter with that of the P(blaP) β-lactamase promoter. In the resulting strain, the cloning of the blaI repressor gene confers lysine auxotrophy to BS1541. After integration of the BlaI cassette into the chromosome of a conditionally lys-auxotrophic (BS1541) strain by homologous recombination and positive selection for spectinomycin resistance, the eviction of the BlaI cassette was achieved by single crossover between the two short

direct repeat sequences. This strategy was successfully used to inactivate a single gene and to introduce a gene of interest in the Bacillus chromosome. In both cases the resulting strains are free of selection marker. This allows the use of the BlaI cassette to repeatedly further modify the *Bacillus* chromosome.

9.5. New fluorescent band-shift assay

Gel retardation analysis, or band shift assay, is technically the simplest method to investigate protein-nucleic acid interactions. We have developed a new nonradioactive band shift assay using a fluorescent DNA target and an ALF-express automatic DNA sequencer in place of the current method that utilizes radioactively end-labeled DNA target and a standard electrophoresis unit [46]. In our study, the dsDNA targets were obtained by annealing two synthetic oligonucleotides or by PCR. In both cases, a molecule of indodicarbocyanine (CY5) was attached at the 5' OH end of one of the two synthetic oligonucleotides, with a ratio of one molecule of fluorescent dye per molecule of dsDNA. To demonstrate the feasibility of this new band shift assay method, the DNA-binding proteins selected as models were the BlaI and AmpR repressors, which are involved in the induction of the *Bacillus licheniformis* 749/I and *Citrobacter freundii* β-lactamases, respectively. The results show that the use of an automatic DNA sequencer allows easy gel retardation analysis and provides a fast, sensitive, and quantitative method. The ALFexpress DNA sequencer has the same limit of detection as a laser fluorescence scanner and can be used instead of a FluorImager or a Molecular Imager.

PUBLICATIONS 2000–2004

2000

[1] A. Matagne, M. Jamin, E.W. Chung, S.E. Radford, C.V. Robinson and C.M. Dobson. Thermal unfolding of an intermediate is associated with non-Arrhenius kinetics in the folding of hen lysozyme. *J. Mol. Biol.*, 2000, **297**, 193–210.

[2] C. Bompard-Gilles, V. Villeret, G.J. Davies, L. Fanuel, B. Joris, J.M. Frère and J. Van Beeumen. A new variant of the Ntn hydrolase fold revealed by the crystal structure of L-aminopeptidase D-Ala-esterase/amidase from *Ochrobactrum anthropi*. *Structure*, 2000, **8**, 153–162.

[3] D. Dehareng, G. Dive and A. Moradpour. An *ab initio* study of organic mixed valency. *Int. J. Quant. Chem.*, 2000, **76**, 552–573.

[4] F. Bouillenne, A. Matagne, B. Joris and J.-M. Frère. Technique for a rapid and efficient purification of the SHV-1 and PSE-2 β-lactamases. *J. Chromatography*, 2000, **B 737**, 261–265.

[5] D. Dehareng and G. Dive. Hartree-fock instabilities and electronic properties. *J. Comp. Chem.*, 2000, **21**, 483–504.

[6] J. Georis, F. Giannotta, E. De Buyl, B. Granier and J.M. Frère. Purification and properties of three endo-β-1,4-xylanases produced by *Streptomyces* sp. strain S38 which differ in their ability to enhance the bleaching of kraft pulps. *Enzyme Microb.Technol.*, 2000, **26**, 178–186.

[7] J. Georis, F. de Lemos Esteves, J. Lamotte-Brasseur, V. Bougnet, B. Devreese, F. Giannotta, B. Granier and J.M. Frère. An additional aromatic interaction improves the thermostability and thermophilicity of a mesophilic family 11 xylanase: structural basis and molecular study. *Protein Science*, 2000, **9**, 466–475.

[8] M.N. Ramquet, G. Dive and D. Dehareng. Critical points and reaction paths characterization on a potential energy hypersurface. *J. Chem. Phys.*, 2000, **112**, 4923–4934.

[9] A. Hoxha, R. Locht, B. Leyh, D. Dehareng, K. Hottmann and H. Baumgärtel. Photoelectron spectroscopy of vinylbromide and intramolecular dynamics of the ionic B state. *Chem. Phys.*, 2000, **256**, 239–249.

[10] M. Hernandez Valladares, M. Kiefer, U. Heinz, R. Paul Soto, W. Meyer-Klaucke, H.F. Nolting, M. Zeppezauer, M. Galleni, J.-M. Frère, G.M. Rossolini, G. Amicosante and H.W. Adolph. Kinetic and spectroscopic characterization of native and metal-substituted β-lactamase from *Aeromonas hydrophila* AE036. *FEBS Lett.*, 2000, **467**, 221–225.

[11] N.O. Concha, C.A. Janson, P. Rowling, S. Pearson, C.A. Cheever, B.P. Clarke, C. Lewis, M. Galleni, J.-M. Frère, D.J. Payne, J.H. Bateson, S.S. Abdel-Meguid. Crystal Structure of the IMP-1 Metallo β-Lactamase from *Pseudomonas aeruginosa* and its Complex with a Mercaptocarboxylate Inhibitor: Binding Determinants of a Potent, Broad-Spectrum Inhibitor. *Biochemistry*, 2000, **39**, 4288–4298.

[12] R. Locht, B. Leyh, A. Hoxha, D. Dehareng, H.W. Jochims and H. Baumgärtel. About the vacuum UV photoabsorption spectrum of methyl fluoride (CH_3F): the fine structure and its vibrational analysis. *Chem. Phys.*, 2000, **257**, 283–299.

[13] L. Chantalat, E. Duée, M. Galleni, J.M. Frère and O. Dideberg. Structural effects of the active site mutation Cysteine to Serine in *Bacillus cereus* Zinc-β-lactamase. *Protein Science*, 2000, **9**, 1402–1406.

[14] L. Boschi, P.S. Mercuri, M.L. Riccio, G. Amicosante, M. Galleni, J.-M. Frère and G.M. Rossolini. The *Legionella (Fluoribacter) gormanii* metallo-β-lactamase: a new member of the highly divergent lineage of molecular-subclass B3 β-lactamases. *Antimicrob. Agents Chemother.*, 2000, **44**, 1538–1543.

[15] C. Bompard-Gilles, H. Remaut, V. Villeret, T. Prange, L. Fanuel, M. Delmarcelle, B. Joris, J.-M. Frère and J. Van Beeumen. Crystal structure of a D-aminopeptidase from *Ochrobactrum anthropi*, a new member of the 'penicillin-recognizing enzyme' family. *Structure*, 2000, **8**, 971–980.

[16] A. Hoxha, R. Locht, B. Leyh, D. Dehareng, K. Hottmann, H.W. Jochims and H. Baumgärtel. The photoabsorption and constant ionic state spectroscopy of vinylbromide *Chem. Phys.*, 2000, **260**, 237–247.

[17] N. Franceschini, B. Caravelli, J.D. Docquier, M. Galleni, J.-M. Frère, G. Amicosante and G.M. Rossolini. Purification and biochemical characterization of the VIM-1 metallo-β-lactamase. *Antimicrob. Agents Chemother.*, 2000, **44**, 3003–3007.

[18] A. Cheggour, L. Fanuel, C. Duez, B. Joris, F. Bouillenne, B. Devreese, G. Van Driessche, J. Van Beeumen, J.-M. Frère and C. Goffin. The *dppA* gene of *Bacillus subtilis* encodes a new D-aminopeptidase. *Mol. Microbiol.*, 2000, **38**, 504–513.

[19] A. Dubus, M. Sami, T.J.N. Brown, C.J. Schofield, J.E. Baldwin and J.-M. Frère. Studies of isopenicillin N synthase enzymatic properties using a continuous spectrophotometric assay. *FEBS Letters*, 2000, **485**, 142–146.

[20] J. Lamotte-Brasseur, A. Dubus and R.C. Wade. pK(a) calculations for class C β-lactamases: the role of Tyr-150. *Proteins*, 2000, **40**, 23–28.

[21] E. Sauvage, G. Léonard, P. Charlier, R. Herman, P. Stefanic, J. Coyette, Y. Taburet, J. Dumas, D. Prévost, B. Schoot and J.P. Marquette. Structure of a low-affinity penicillin-binding protein from *Enterococcus faecium*. *ESRF* Highlights, 2000, 7.

[22] H.F. Ye, G. Dive, D. Dehareng, F. Heymans and J.J. Godfroid. Structure-activity relationships on adrenoceptors and imidazoline-preferring binding sites ($I_{1,2}$ – PBSs). Part 1. Weak intramolecular H-bond and conformational flexibility in a new I_1 – PBS – selective imidazoline analog, *trans* 1-(4',5'-dihydro-1'*H*-imidazol-2'-yl) methyl-2-hydroxyindane (PMS 952). *Bioorgan. Med. Chem.*, 2000, **8**, 1861–1869.

[23] M. Marrec-Fairley, X. Gallet, R. Brasseur, H. Hara, C. Fraipont, J.M. Ghuysen and M. Nguyen-Distèche. Differential functionalities of amphiphilic peptide segments of the cell-septation penicillin-binding protein 3 of *Escherichia coli*. *Mol. Microbiol.*, 2000, **37**, 1019–1031.

[24] 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.

2001

[25] C. Duez, M. Vanhove, X. Gallet, F. Bouillenne, J.D. Docquier, A. Brans and J.-M. Frère. Purification and characterization of PBP4a, a new low-molecular-weight penicillin-binding protein from *Bacillus subtilis*. *J. Bacteriol.*, 2001, **183**, 1595–1599.

[26] J. Wouters, P. Charlier, D. Monnaie, J.-M. Frère and E. Fonzé. Expression, purification, crystallization and preliminary X-ray analysis of the native class C β-lactamase from *Enterobacter cloacae* 908R and two mutants. *Acta Cryst.*, 2001, **D57**, 162–164.

[27] M. Galleni, J. Lamotte-Brasseur, G.M. Rossolini, J. Spencer, O. Dideberg and J.-M. Frère. Standard numbering scheme for class B β-lactamases. *Antimicrob. Agents Chemother.*, 2001, **45**, 660–663.

[28] C. Collette, D. Dehareng, E. De Pauw and G. Dive. Qualitative comparison between the quantum calculations and electrospray mass spectra of complexes of polyammonium macrotricyclic ligands with dicarboxylic acids. *J. Am. Soc. Mass. Spectrom.*, 2001, **12**, 304–316.

[29] F. Danel, J.-M. Frère and D.M. Livermore. Evidence of dimerisation among class D β-lactamases: kinetics of OXA-14 β-lactamase. *Biochimica et Biophysica Acta*, 2001, **1546**, 1132–1142.

[30] P.S. Mercuri, F. Bouillenne, L. Boschi, J. Lamotte-Brasseur, G. Amicosante, B. Devreese, J. Van Beeumen, J.-M. Frère, G.M. Rossolini and M. Galleni. Biochemical characterization of the FEZ-1 metallo-β-lactamase of *Legionella gormanii* ATCC33297^{T} produced in *Escherichia coli*. *Antimicrob. Agents Chemother.*, 2001, **45**, 1254–1262.

[31] A. Dubus, M.D. Lloyd, H.-J. Lee, C.J. Schofield, J.E. Baldwin and J.-M. Frère. Probing the penicillin sidechain selectivity of recombinant deacetoxycephalosporin C synthase. *Cell. Mol. Life Sci.*, 2001, **58**, 835–843.

[32] H.-J. Lee, M.D. Lloyd, I.J. Clifton, K. Harlos, A. Dubus, J.E. Baldwin, J.-M. Frère and C.J. Schofield. Alteration of the Co-substrate selectivity of deacetoxycephalosporin C synthase. The role of Arginine 258. *J. Biol. Chem.*, 2001, **276**, 18290–18295.

[33] R. Locht, B. Leyh, A. Hoxha, D. Dehareng, H.W. Jochims and H. Baumgärtel. The vacuum UV photoabsorption spectrum of methyl chloride (CH_3Cl) and its perdeuterated isotopomer CD_3Cl. II. A vibrational analysis. *Chem. Phys.*, 2001, **272**, 277–292.

[34] C. Bebrone, C. Moali, F. Mahy, S. Rival, J.D. Docquier, G.M. Rossolini, J. Fastrez, R.F. Pratt, J.-M. Frère and M. Galleni. CENTA as a chromogenic substrate for studying β-lactamases. *Antimicrob. Agents Chemother.*, 2001, **45**, 1868–1871.

[35] H. Remaut, C. Bompard-Gilles, C. Goffin, J.-M. Frère and J. Van Beeumen. Structure of the *Bacillus subtilis* D-aminopeptidase. DppA reveals a novel self-compartimentalizing protease. *Nat. Struct. Biol.*, 2001, **8**, 674–678.

[36] A. Lejeune, M. Vanhove, J. Lamotte-Brasseur, R.H. Pain, J.-M. Frère and A. Matagne. Quantitative analysis of the stabilization by substrate of *Staphylococcus aureus* PC1 β-lactamase. *Chemistry & Biology*, 2001, **8**, 831–842.

[37] M. Vilar, M. Galleni, T. Solmajer, B. Turk, J.-M. Frère and A. Matagne. Kinetic study of two novel enantiomeric tricyclic β-lactams which efficiently inactivate class C β-lactamases. *Antimicrob. Agents Chemother.*, 2001, **45**, 2215–2223.

[38] K.E. Conrath, M. Lauwereys, M. Galleni, A. Matagne, J.-M. Frère, J. Kinne, L. Wijns and S. Muyldermans. β-lactamase inhibitors derived from single-domain antibody fragments elicited in the *Camelidae*. *Antimicrob. Agents Chemother.*, 2001, **45**, 2807–2812.

[39] A. Zervosen, M.H. Valladares, B. Devreese, C. Prosperi-Meys, H.W. Adolph, P.S. Mercuri, M. Vanhove, G. Amicosante, J. Van Beeumen, J.-M. Frère and M. Galleni. Inactivation of *Aeromonas hydrophila* metallo-β-lactamase by cephamycins and moxalactam. *Eur. J. Biochem.*, 2001, **268**, 3840–3850.

[40] R. Locht, B. Leyh, A. Hoxha, D. Dehareng, K. Hottmann, H.W. Jochims and H. Baumgärtel. About the photoionization of methyl chloride. A treshold photoelectron spectroscopic and photoionization mass spectrometric investigation. *Chem. Phys.*, 2001, **272**, 293–313.

[41] J. Wouters, J. Georis, D. Engher, J. Vandenhaute, J. Dusart, J.-M. Frère, E. Depiereux and P. Charlier. Crystallographic analysis of family 11 endo-β-1,4-xylanase Xyl1 from *Streptomyces* sp. S38. *Acta Cryst.*, 2001, **D57**, 1813–1819.

[42] C. Mollard, C. Moali, C. Papamicael, C. Damblon, S. Vessilier, G. Amicosante, C.J. Schofield, M. Galleni, J.-M. Frère and G.C.K. Roberts. Thiomandelic acid, a broad spectrum inhibitor of Zinc β-lactamases. Kinetic and spectroscopic studies. *J. Biol. Chem.*,2001, **276**, 45015–45023.

[43] D. de Seny, U. Heinz, S. Wommer, M. Kiefer, W. Meyer-Klaucke, M. Galleni, J.-M. Frère, R. Bauer and H.W. Adolph. Metal ion binding and coordination geometry for wild type and mutants of metallo-β-lactamase from *Bacillus cereus* 569/H/9 (BcII). A combined thermodynamic, kinetic and spectroscopic approach. *J. Biol. Chem.*, 2001, **276**, 45065–45078.

[44] N. Franceschini, L. Boschi, S. Pollini, R. Herman, M. Perilli, M. Galleni, J.-M. Frère, G. Amicosante and G.M. Rossolini. Characterization of OXA-29 from *Legionella (Fluoribacter) gormanii*: molecular class D β-lactamase with unusual properties. *Antimicrob. Agents Chemother.*, 2001, **45**, 3509–3516.

[45] M. Dinant, D. Baurain, N. Coosemans, B. Joris and R.F. Matagne. Characterization of two genes encoding the mitochondrial alternative oxidase in *Chlamydomonas reinhardtii*. *Curr. Genet.*, 2001, **39**, 101–108.

[46] P. Filée, M. Delmarcelle, I. Thamm and B. Joris. Use of an ALFexpress DNA sequencer to analyze protein-nucleic acid interactions by band shift assay. *Biotechniques*, 2001, **30**, 1044–8, 1050–1.

[47] A. Amoroso, D. Demares, M. Mollerach, G. Gutkind and J. Coyette. All detectable HMM PBPs are modified in a high-level β-lactam resistant clinical of *Streptococcus mitis*. *Antimicrob. Agents Chemother.*, 2001, **45**, 2075–2081.

[48] C. Duez, W. Zorzi, F. Sapunaric, A. Amoroso, I. Thamm and J. Coyette. The penicillin resistance of *Enterococcus faecalis* JH2-2r results from an overproduction of the low-affinity penicillin-binding protein 4 and does not involve a *psr*-like gene. *Microbiology*, 2001, **147**, 2561–2569.

[49] A. Zervosen, V. Nieder, R.G. Gallego, J.P. Kamerling, J.F. Vliegenthart and L. Elling. Synthesis of nucleotide-activated oligosaccharides by β-galactosidase from *Bacillus circulans*. *Biol. Chem.*, 2001, **382**, 299–311.

[50] C. Prosperi-Meys, J. Wouters, M. Galleni and J. Lamotte-Brasseur. Substrate binding and catalytic mechanisms of class B β-lactamases: a molecular study. *Cellular and Molecular Life Sciences*, 2001, **58**, 2136–2143.

[51] M. Frederich, M.C. De Pauw, C. Prosperi, M. Tits, V. Brandt, J. Penelle, M.P. Hayette, P. De Mol and L. Angenot. Strychnogucines A and B, two new antiplasmodial bisindole alkaloids from *Strychnos icaja*. *Journal of Natural Products*, 2001 **64**, 12–16.

[52] G. Piel, G. Dive, B. Évrard, T. Van Hees, S. Henry de Hassonville and L. Delattre. Molecular modelling study of β and γ-cyclodextrin complexes with miconazole. *Eur. Journal of Pharm. Sci.*, 2001, **13**, 271–279.

[53] I. Devillers, G. Dive, C. De Tollenaere, B. Falmagne, B. de Wergifosse, J.-Fr. Rees and J. Marchand-Brynaert. Imidazolopyrazinones as potential antioxidants. *Bioorg. Med. Chem. Letters*, 2001, **11**, 2305–2309.

[54] A. Matagne, M. Galleni, N. Laraki, G. Amicosante, G. Rossolini and J.-M. Frère. β-lactamases, an old but ever renascent problem. In: "Novel Frontiers in the Production of Compounds for Biomedical Use", A. Van Broekhoven *et al.*, Eds. (Kluwer Academic Publishers, Dordrecht, The Netherlands), 2001, pp. 117–129.

2002

[55] S. Gérard, G. Dive, B. Clamot, R. Touillaux and J. Marchand-Brynaert. Synthesis, hydrolysis, biochemical and theoretical evaluation of 1,4-bis(alkoxycarbonyl)azetidin-2-ones as potential elastase inhibitors. *Tetrahedron*, 2002, **58**, 2423–2433.

[56] E. Fonzé, M. Vanhove, G. Dive, E. Sauvage, J.-M. Frère and P. Charlier. Crystal structures of the *Bacillus licheniformis* BS3 class A β-lactamase and of the acyl-enzyme adduct formed with Cefoxitin. *Biochemistry*, 2002, **41**, 1877–1885.

[57] P. Filée, K. Benlafya, M. Delmarcelle, G. Moutzourelis, J.-M. Frère, A. Brans and B. Joris. The fate of BlaI repressor during the induction of the *Bacillus licheniformis* BlaP β-lactamase. *Molecular Microbiology*, 2002, **44**, 685–694.

[58] J.-D. Docquier, F. Pantanella, F. Giuliani, M.C. Thaller, G. Amicosante, M. Galleni, J.-M. Frère, K. Bush and G.M. Rossolini. CAU-1, a subclass B3 metallo-β-lactamase of low substrate affinity encoded by an ortholog present in the *Caulobacter crescentus* chromosome. *Antimicrob. Agents Chemother.*, 2002, **46**, 1823–1830.

[59] S. Vessilier, J.-D. Docquier, S. Rival, J.-M. Frère, M. Galleni, G. Amicosante, G.M. Rossolini and N. Franceschini. Overproduction and biochemical characterization of the *Chryseobacterium meningosepticum* BlaB metallo-β-lactamase. *Antimicrob. Agents Chemother.*, 2002, **46**, 1921–1927.

[60] D. de Seny, C. Prosperi-Meys, C. Bebrone, G.M. Rossolini, M.I. Page, P. Noël, J.-M. Frère and M. Galleni. Mutational analysis of the two zinc-binding sites of the *Bacillus cereus* 569/H/9 metallo-β-lactame. *Biochem. J.*, 2002, **363**, 687–696.

[61] D. Dehareng, G. Dive and C. Geron. Hartree-fock instabilities: relation between an artifact and some properties. *Recent Res. Devel. Chem. Physics*, 2002, **3**, 193–206.

[62] S. Wommer, S. Rival, U. Heinz, M. Galleni, J.-M. Frère, N. Franceschini, G. Amicosante, B. Rasmussen, R. Bauer and H.W. Adolph. Substrate-activated Zinc binding of metallo-β-lactamases. *J. Biol. Chem.*, 2002, **277**, 24142–24147.

[63] B. Lakaye, A. Dubus, B. Joris and J.-M. Frère. Method for estimation of low outer membrane permeability to β-lactam antibiotics. *Antimicrob. Agents Chemother.*, 2002, **46**, 2901–2907.

[64] J. Alba, Y. Ishii, M. Galleni, J.-M. Frère, M. Ito and K. Yamaguchi. Cefcapene inactivates chromosome-encoded class C β-lactamases. *J. Infect. Chemother.*, 2002, **8**, 207–210.

[65] P.S. Mercuri, Y. Ishii, L. Ma, G.M. Rossolini, F. Luzzaro, G. Amicosante, N. Franceschini, J-M. Frère and M. Galleni. Clonal diversity and metallo-β-lactamase production in clinical isolates of *Stenotrophomonas maltophilia*. *Microbial Drug Resistance*, 2002, **8**, 193–200.

[66] S. Rigali, A. Derouaux, F. Giannotta and J. Dusart. Subdivision of the helix-turn-helix GntR family of bacterial in the FadR, HutC, MocR, and YtrA subfamilies. *J. Biol. Chem.*, 2002, **15**, 12507–12515.

[67] D. Baurain, L. Renquin, P. Scheldeman, A. Belay and A. Wilmotte. Remarkable conservation of internally transcribed spacer sequences of *Arthrospira ("Spirulina")* (Cyanophycea, cyanobacteria) strains from four continents and a recent and 30-year-old dried samples from Africa. *Journal of Phycology*, 2002, **38**, 384–393.

[68] A. Wilmotte, C. Demonceau, A. Goffart, J.-H. Hecq, V. Demoulin and A.C. Crossley. Molecular and Pigments Studies of the picophytoplankton in a region of the Southern Ocean (47 to 54° S, 141 to 144° E) in March 1998. *Deep Sea Research II*, 2002, **49**, 3351–3363.

[69] E. Sauvage, B. Schoot, J.P. Marquette, G. Leonard, P. Stefanic, F. Kerff, E. Fonzé, Y. Taburet, D. Prevost, J. Dumas, J. Coyette and P. Charlier. The 2.4 Å Crystal structure of the penicillin-resistant penicillin-binding protein PBP5fm from *Enterococcus faecium* in complex with benzylpenicillin. *Cell. Mol. Life Sci.*, 2002, **59**, 1223–1232.

[70] V. Gabelica, C. Vreuls, P. Filée, V. Duval, B. Joris and E.D. Pauw. Advantages and drawbacks of nanospray for studying noncovalent protein-DNA complexes by mass spectrometry. *Rapid Common Mass Spectrom*, 2002, **16**, 1723–1728.

[71] A. Moreau, D.S. Wang, S. Forget, C. Duez and J. Dusart. GC-rich template amplification by inverse PCR. DNA polymerase and solvent effects. *Methods Mol. Biol.*, 2002, **192**, 75–80.

[72] M. Dumoulin, K. Conrath, A. Van Meirhaeghe, F. Meersman, K. Heremans, L.J.G. Frenken, S. Muyldermans, L. Wyns and A. Matagne. Single-domain antibody fragments with high conformational stability. *Protein Science*, 2002, **11**, 500–515.

[73] D. Laeckmann, F. Rogister, J.V. Dejardin, C. Prosperi-Meys, J. Geczy, J. Delarge and B. Masereel. Synthesis and biological evaluation of aroylguanidines related to amiloride as inhibitors of the human platelet Na+/H+ exchanger. *Bioorganic & Medicinal Chemistry*, 2002, **10**, 1793–1804.

[74] C. Prosperi-Meys, D. De Seny, G. Llabres, M. Galleni and J. Lamotte-Brasseur. Active-site mutants of class B β-lactamases: substrate binding and mechanistic study. *Cellular and Molecular Life Sciences*, 2002, **59**, 2136.

[75] B. Castiglioni, E. Rissi, A. Frosini, M.A. Mugnai, S. Ventura, K. Sivonen, P. Rajaniemi, A. Rantala, A. Wilmotte, C. Boutte, C. Consolandi, R. Bordoni, A. Mezzelani, E. Busti, L. Rossi Bernardi, C. Battaglia and G. De Bellis. Application of an universal DNA microarray to cyanobacterial diversity assessment. *Minerva Biotec*, 2002, **14**, 253–257.

[76] E. Baloïtcha, B. Lasorne, D. Lauvergnat, G. Dive, Y. Justum and M. Desouter-Lecomte. Cumulative reaction probability by constrained dynamics: H transfer in HCN, H_2CO and CH_3O. *J. Chem. Phys.*, 2002, **117**, 727–739.

[77] F. Geneste, A. Moradpour, G. Dive, D. Peeters, J. Malthête, J.-Fr. Sadoc. Retrosynthetic analysis of fullerene C60: structure, stereochemistry and calculated stability of C30 fragments. *J. Org. Chem.*, 2002, **67**, 605–607.

[78] C. Goffin and J.-M. Ghuysen. Biochemistry and comparative genomics of SxxK superfamily acyltransferases offer a clue to the mycobacterial paradox: presence of penicillin-susceptible target proteins versus lack of efficiency of penicillin as therapeutic agent. *Microbiol. Mol. Biol. Rev.*, 2002, **66**, 702–738.

[79] J. Coyette and L. Hancock. Enterococcal cell wall. In "The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance". M. Gilmore, P. Courvalin, D. Clewell, G. Dunny, B. Murray and L. Rice, Eds. American Society for Microbiology, Washington D.C., 2002, pp. 177–218.

2003

[80] I. Garcia-Saez, P.S. Mercuri, C. Papamicael, R. Kahn, J.-M. Frère, M. Galleni, G.M. Rossolini and O. Dideberg. Three-dimensional Structure of FEZ-1, a Monomeric Subclass B3 Metallo-β-lactamase from *Fluoribacter gormanii*, in Native Form and in Complex with D-Captopril. *J. Mol. Biol.*, 2003, **325**, 651–660.

[81] C. Moali, C. Anne, J. Lamotte-Brasseur, S. Groslambert, B. Devreese, J. Van Beeumen, M. Galleni and J.-M. Frère. Analysis of the importance of the metallo-β-lactamase active site loop in substrate binding and catalysis. *Chemistry & Biology*, 2003, **10**, 319–329.

[82] P. Filée, C. Vreuls, R. Herman, I. Thamm, T. Aerts, P.P. De Deyn, J.-M. Frère and B. Joris. Dimerization and DNA Binding Properties of the *Bacillus licheniformis* 749/I BlaI Repressor. *J. Biol. Chem.*, 2003, **278**, 16482–16487.

[83] I. Garcia-Saez, J. Hopkins, C. Papamicael, N. Franceschini, G. Amicosante, G.M. Rossolini, M. Galleni, J.-M. Frère and O. Dideberg. The 1.5 Å structure of *Chryseobacterium meningosepticum* Zn-β-lactamase in complex with the inhibitor D-captopril. *J. Biol. Chem.*, 2003, **278**, 23868–23873.

[84] J.W. Anderson, S.A. Adediran, P. Charlier, M. Nguyen-Distèche, J.-M. Frère, R.A. Nicholas and R.F. Pratt. On the substrate specificity of bacterial DD-peptidases: Evidence from two series of peptidoglycan-mimetic peptides. *Biochem. J.*, 2003, **373**, 944–955.

[85] N. Rhazi, P. Charlier, D. Dehareng, D. Engher, M. Vermeire, J.-M. Frère, M. Nguyen-Distèche and E. Fonzé. Catalytic Mechanism of the *Streptomyces* K15 dd-Transpeptidase/Penicillin-Binding Protein Probed by Site-Directed Mutagenesis and Structural Analysis. *Biochemistry*, 2003, **42**, 2895–2906.

[86] B. Lasorne, G. Dive, D. Lauvergnat and M. Desouter-Lecomte. Wave packet dynamics along bifurcating reaction paths. *J. Chem. Phys.*, 2003, **118**, 5831–5840.

[87] J.D. Docquier, J. Lamotte-Brasseur, M. Galleni, G. Amicosante, J.-M. Frère and G.M. Rossolini. On functional and structural heterogeneity of VIM-type metallo-β-lactamases. *J. Antimicrob. Agents Chemother.*, 2003, **51**, 257–266.

[88] V. Duval, M. Swinnen, S. Lepage, A. Brans, B. Granier, C. Franssen, J.-M. Frère and B. Joris. The kinetic properties of the carboxy terminal domain of the *Bacillus licheniformis* 749/I BlaR penicillin-receptor shed a new light on the derepression of β-lactamase synthesis. *Mol. Microbiol.*, 2003, **48**, 1553–1564.

[89] J. Wouters, E. Fonzé, M. Vermeire, J.-M. Frère and P. Charlier. Crystal structure of *Enterobacter cloacae* 908R class C β-lactamase bound to iodo-acetamido-phenyl boronic acid, a transition-state analogue. *Cell. Mol. Life Sci.*, 2003, **60**, 1764–1773.

[90] J. Alba, C. Bauvois, Y. Ishii, M. Galleni, K. Masuda, M. Ishiguro, M. Ito, J.-M. Frère and B. Joris. A detailed kinetic study of Mox-1, a plasmid-encoded class C β-lactamase. *FEMS Microbiol Lett.*, 2003, **225**, 183–188.

[91] A.S. Ibuka, Y. Ishii, M. Galleni, M. Ishiguro, K. Yamaguchi, J.M. Frère, H. Matsuzawa and H. Sakai. Crystal structure of extended-spectrum beta-lactamase Toho-I: insights into the molecular mechanism for catalytic reactions and substrate specificity extension. *Biochemistry*, 2003, **42**, 10634–10643.

[92] F. Kerff, P. Charlier, M.-L. Colombo, E. Sauvage, B. Brans, J.-M. Frère, B. Joris and E. Fonzé. Crystal structure of the sensor domain of the BlaR penicillin-receptor from *Bacillus licheniformis*. *Biochemistry*, 2003, **42**, 12835–12843.

[93] M.H. Lee, M. Schere, S. Rigali and J.W. Golden. PlmA, a new member of the GntR family, has plasmid maintenance functions in *Anabaena* sp. Strain PCC 7120. *J. Bacteriol.*, 2003, **15**, 4315–4325.

[94] F. Giannotta, J. Georis, S. Rigali, M.J. Virolle and J. Dusart. Site-directed mutagenesis of conserved inverted repeat sequences in the xylanase C promoter region from *Streptomyces* sp. EC3. *Mol. Genet. Genomics*, 2003, **270**, 337–346.

[95] A. Taton, S. Grubisic, E. Brambilla, R. De Wit and A. Wilmotte. Cyanobacterial Diversity in Natural and Artificial Microbial Mats of Lake Fryxell (McMurdo Dry Valleys, Antartica): a Morphological and Molecular Approach. *Applied and Environmental Microbiology*, 2003, **69**, 5157–5169.

[96] E. Liepinsh, C. Généreux, D. Dehareng, B. Joris and G. Otting. NMR structure of *Citrobacter freundii* AmpD, comparison with bacteriophage T7 lysozyme and homology with PGRP domains. *J. Mol. Biol.*, 2003, **327**, 833–842.

[97] H. Van Melckebeke, C. Vreuls, P. Gans, G. Llabres, P. Filée, B. Joris and J.-P. Simorre. Solution Structural Study of BlaI: Implication for the repression of genes involved in antibiotic resistance. *J. Mol. Biol.*, 2003, **333**, 711–720.

[98] M. Dumoulin, A.M. Last, A. Desmyter, K. Decanniere, D. Canet, G. Larsson, A. Spencer, D.B. Archer, J. Sasse, S. Muyldermans, L. Wyns, C. Redfield, A. Matagne, C.V. Robinson and C.M. Dobson. A camelid antibody fragment inhibits the formation of amyloid fibrils by human lysozyme. *Nature*, 2003, **424**, 783–788.

[99] C. Raskin, C. Gerard, F. Giannota and J. Dusart. BlaB, a protein involved in the regulation of *Streptomyces* cacaoi β-lactamases, is a penicillin-binding protein. *Cell. Mol. Life Sci.*, 2003, **60**, 1460–1469.

[100] B. El Amiri, B. Remy, N.M. Sousa, B. Joris, N. Gérardin-Otthiers, Z. Perenyl, H.B. Mboko and J.F. Beckers. Isolation and partial characterisation of three pregnancy-associated glycoproteins form the ewe placenta. *Molecular Reproduction and development*, 2003, **64**, 199–206.

[101] F. Sapunaric, C. Franssen, P. Stefanic, A. Amoroso, O. Dardenne and J. Coyette. Redefining the role of *psr* in β-lactam resistance and cell autolysis of *Enterococcus hirae*. *J. Bacteriol.*, 2003, **185**, 5925–5935.

[102] M. Vanhove, M. Zakhem, B. Devreese, N. Franceschini, C. Anne, C. Bebrone, G. Amicosante, G.M. Rossolini, J. Van Beeumen, J.M. Frère and M. Galleni. Role of Cys221 and Asn116 in the zinc-binding sites of the *Aeromonas hydrophila* metallo-β-lactamase. *Cell. Mol. Life Sci.*, 2003, **60**, 2501–2509.

2004

[103] A. Merschaert, P. Delbeke, D. Daloze and G. Dive. The rational design of modified Cinchona alkaloid catalysts. Application to a new asymmetric synthesis of chiral chromanes. *Tetrahedron Letters*, 2004, **45**, 4697–4701.

[104] M.-L. Colombo, S. Hanique, S. Baurin, C. Bauvois, K. De Vriendt, J. Van Beeumen, J.-M. Frère and B. Joris. The *ybxl* of *Bacillus subtilis* 168 encodes a class D β-lactamase of low activity. *Antimicrob. Agents Chemother.*, 2004, **48**, 484–490.

[105] S. Hanique, M.-L. Colombo, E. Goormaghtigh, P. Soumillion, J.-M. Frère and B. Joris. Evidence of an intra molecular interaction between the two domains of the BlaR1 penicillin receptor during the signal transduction. *J. Biol. Chem.*, 2004, **279**, 14264–14272.

[106] C. Généreux, D. Dehareng, B. Devreese, J. Van Beeumen, J.-M. Frère and B. Joris. Mutational analysis of the catalytic centre of the *Citrobacter freundii* AmpDN-cetylmuramyl-L-alanine amidase. *Biochem. J.*, 2004, **377**, 111–120.

[107] K. Valegård, A.C. Terwisscha van Scheltinga, A. Dubus, G. Ranghino, L.M. Öster J. Hadju and I. Andersson. The structural basis of cephalosporin formation in a mononuclear ferrous enzyme. *Nature Struct. Biol.*, 2004, **11**, 95–101.

[108] W.Y. Tsang, A. Dhanda, C.J. Schofield, J.-M. Frère, M. Galleni and M.I. Page. The inhibition of metallo-β-lactamase by thioxo-cephalosporin derivatives. *Bioorganic & Medicinal Chemistry Letters*, 2004, **14**, 1737–1739.

[109] S. Gérard, M. Galleni, G. Dive and J. Marchand-Brynaert. Synthesis and evaluation of N1/C4-substituted β-lactams as PPE and HLE inhibitors. *Bioorg. Med. Chem.*, 2004, **12**, 129–138.

[110] D. Dehareng and G. Dive. Vertical ionisation energies of α-L-amino acids as a function of their conformation: an ab initio study. *Int. J. Mol. Sci.*, 2004, **5**, 301–331 (open access: www.mdpi.net/ijms).

[111] C. Duez, S. Hallut, N. Rhazi, S. Hubert, A. Amoroso, F. Bouillenne, A. Piette and J. Coyette. The *ponA* gene of *Enterococcus faecalis* JH2-2 codes for a low-affinity class A penicillin-binding protein. *Journal of Bacteriology*, 2004, **186**, 4412–4416.

[112] B. Grisart, F. Farnir, L. Karim, N. Cambisano, J.J. Kim, A. Kvasz, M. Mni, P. Simon, J.-M. Frère, W. Coppieters and M. Georges. Genetic and functional confirmation of the causality of the DGAT1 K232A quantitative trait nucleotide in affecting milk yield and composition. *Proc. Natl. Acad. Sci.* USA, 2004, **101**, 2398–2403.

[113] A. Zervosen, W.P. Lu, Z. Chen, R.E. White, T.P. Demuth and J.-M. Frère. Interactions between penicillin-binding proteins (PBPs) and two novel classes of PBP inhibitors, arylalkylidene rhodanines and arylalkylidene iminothiazolidin-4-ones. *Antimicrob. Agents Chemother.*, 2004, **48**, 961–969.

[114] F. de Lemos Esteves, V. Ruelle, J. Lamotte-Brasseur, B. Quinting and J.-M. Frère. Acidophilic adaptation of family 11 endo-β-1,4-xylanases: Modeling and mutational analysis. *Protein Sci.*, 2004, **13**, 1209–1218.

[115] P. Douette, R. Navet, F. Bouillenne, A. Brans, C. Sluse-Goffart, A. Matagne and F. Sluse. Secondary-structure characterization by far-UV CD of highly purified uncoupling protein 1 expressed in yeast. *Biochem. J.*, 2004, **380**, 139–145.

[116] K. Sabbe, D.A. Hodgson, E. Verleyen, A. Taton, A. Wilmotte, K. Vanhoutte and W. Vyverman. Salinity, depth and the structure and composition of microbial mats in continental Antarctic lakes. *Freshwater Biology*, 2004, **49**, 296–319.

[117] G. Garau, I. Garcia-Saez, C. Bebronne, C. Anne, P. Mercuri, M. Galleni, J.-M. Frère and O. Dideberg. Update of the standard numbering scheme for class B β-lactamases. *Antimicrob. Agents Chemother.*, 2004, **48**, 2347–2349.

[118] P. Mercuri, I. Garcia-Saez, K. De Vriendt, I. Thamm, B. Devreese, J. Van Beeumen, O. Dideberg, G.M. Rossolini, J.-M. Frère and M. Galleni. Probing the specificity of the subclass B3 FEZ-1 metallo-β-lactamase by site-directed mutagenesis. *J. Biol. Chem.*, 2004, **279**, 33630–33638.

[119] L. Dreesen, C. Hubert, Y. Sartenaer, Y. Caudano, C. Volcke, A.A. Mani, A. Peremans, P.A. Thiry, S. Hanique et J.-M. Frère. Electronic and molecular properties of an adsorbed protein monolayer probed by two-color sum-frequency generation spectroscopy. *Langmuir*, 2004, **20**, 7201–7207.

[120] L.M. Öster, A.C. Terwisscha van Scheltinga, K. Valegård, A. MacKenzie Hose, A. Dubus, J. Hajdu and I. Andersson. Conformational flexibility of the C terminus with implications for substrate binding and catalysis revealed in a new crystal form of deacetoxycephalosporin c synthase. *J. Mol. Biol.*, 2004, **343**, 157–171.

[121] C. Vreuls, P. Filée, H. Melckebeke, T. Aerts, P. De Deyn, G. Llabres, A. Matagne, J.P. Simorre, J.-M. Frère and B. Joris. Guanidine hydrochloride denaturation of the dimeric *B. licheniformis* BlaI repressor highlights an independent domain unfolding pathway. *Biochem. J.*, 2004, **384**, 179–190.

[122] J.D. Docquier, T. Lopizzo, S. Liberatori, M. Prenna, M.C. Thaller, J.-M. Frère and G.M. Rossolini. Biochemical characterization of the THIN-B metallo-β-lactamase of *Janthinobacterium lividum*. *Antimicrob. Agents Chemother.*, 2004, **48**, 4778–4783.

[123] A. Piette, C. Fraipont, T. den Blaauwen, M.E.G. Aarsman, S. Pastoret and M. Nguyen-Distèche. Structural determinants required to target penicillin-binding protein 3 to the septum of *Escherichia coli*. *J. Bacteriol.*, 2004, **186**, 6110–6117.

[124] B. Castiglioni, E. Rizzi, A. Frosini, K. Sivonen, P. Rajaniemi, A. Rantala, M.A. Mugnai, S. Ventura, A. Wilmotte, C. Boutte, S. Grubisic, P. Balthasart, C. Consolandi, R. Bordoni, A. Mezzelani, C. Battaglia and G. De Bellis. Development of a universal microarray based on the ligation detection reaction and 16S rRNA gene polymorphism to target diversity of cyanobacteria. *Applied and Environmental Microbiology*, 2004, **70**, 7161–7172.

[125] S. Pastoret, C. Fraipont, T. Den Blaauwen, B. Wolf, M.E.G. Aarsman, A. Piette, A. Thomas, R. Brasseur and M. Nguyen-Distèche. Functional analysis of the cell division protein FtsW of *Escherichia coli*. *J. Bacteriol.*, 2004, **186**, 8370–8379.

[126] A. Brans, P. Filée, A. Chevigné, A. Claessens and B. Joris. A new integrative method to generate *Bacillus subtilis* recombinant strains free of selection marker. *Applied and Environmental Microbiology*, 2004, **70**, 7241–7250.

[127] S. Rigali, M. Schlicht, P. Hoskisson, H. Nothaft, M. Merzbacher, B. Joris and F. Titgemeyer. Extending the classification of bacterial transcription factors beyond the helix-turn-helix motif as an alternative approach to discover new cis/trans relationships. *Nucleic Acids Res.*, 2004, **32**, 3418–3426.

[128] S. Biltresse, M. Attolini, G. Dive, A. Cordi, G.C. Tucker, J. Marchand-Brynaert. Novel RGD-like Molecules Based on the Tyrosine Template: Design, Synthesis and Biological Evaluation on Isolated Integrins $\alpha_V\beta_3/\alpha_{IIb}\beta_3$ and in Cellular Adhesion Tests. *Bioorg. Med. Chem.*, 2004, **12**, 5379–5393.

[129] A. Derouaux, D. Dehareng, E. Lecocq, S. Halici, H. Nothaft, F. Giannotta, G. Moutzourelis, J. Dusart, B. Devreese, F. Titgemayer, J. Van Beeumen and S. Rigali. Crp of *Streptomyces coelicolor* is the third transcription factor of the large CRP-FNR superfamily able to bind cAMP. *Biochem. Biophys. Res. Comm.*, 2004, **325**, 983–990.

[130] A. Derouaux, S. Halici, H. Nothaft, T. Neutelings, G. Moutzourelis, J. Dusart, F. Titgemeyer and S. Rigali. Deletion of a cyclic AMP receptor protein homologue diminishes germination and affects morphological development of *Streptomyces coelicolor*. *J. Bacteriol.*, 2004, **186**, 1893–1897.

[131] V. Van Lierde, C.C. Chéry, K. Strijkmans, M. Galleni, B. Devreese, J. Van Beeumen, L. Moens and F. Vanhaecke. Capillary electrophoresis hyphenated to inductively coupled plasma-sector field-mass spectrometry for the stoichiometric determination of Zn bound to *Aeromonas hydrophila* Zn beta-lactamase. *J. Anal. At. Spectrom.*, 2004, **19**, 888–893.

[132] S. Henry de Hassonville, G. Dive, B. Évrard, P. Bertholet, L. Delattre, G. Piel. Application of molecular modeling to the study of cyproterone acetate stability in the presence of cyclodextrin derivatives. *J. Drug. Del. Sci. Tech.*, 2004, **14**, 357–362.

[133] P. Charlier, J.-P. Wery, O. Dideberg and J.-M. Frère. *Streptomyces albus G* D-Ala-D-Ala carboxypeptidase. In: "Handbook of Metalloproteins, Volume 3", A. Messerschmidt, W. Bode, M. Cygler, eds. (J. Wiley & Sons Ltd), 2004.

[134] H. Remaut, C. Goffin, J.-M. Frère and J. Van Beeumen. *Bacillus subtilis* D-aminopeptidase DppA. In: "Handbook of Metalloproteins, Volume 3", A. Messerschmidt, W. Bode, M. Cygler, eds. (J. Wiley & Sons Ltd), 2004.

[135] J.M. Ghuysen. Zinc D-Ala-D-Ala carboxypeptidase (*Streptomyces*). In: "Handbook of Proteolytic Enzymes, 2nd edition", A.J. Barrett, N.D. Rawlings and J.F. Woessner, eds. (Elsevier Academic Press), 2004, chap. 254.

[136] H. Remaut and C. Goffin. D-Aminopeptidase DppA. In: "Handbook of Proteolytic Enzymes, 2nd edition", A.J. Barrett, N.D. Rawlings and J.F. Woessner, eds. (Elsevier Academic Press), 2004, chap. 301.

[137] J.M. Wilkin and M. Nguyen-Distèche. Penicillin-binding protein 5, a serine type D-Ala-D-Ala carboxypeptidase. In: "Handbook of Proteolytic Enzymes, 2nd edition", A.J. Barret, N.D. Rawlings and J.F. Woessner, eds., (Elsevier Academic Press), 2004, chap. 606.

[138] J.M. Ghuysen. *Streptomyces* K15 D-Ala-D-Ala transpeptidase. In: "Handbook of Proteolytic Enzymes, 2nd edition", A.J. Barrett, N.D. Rawlings and J.F. Woessner, eds. (Elsevier Academic Press), 2004, chap. 608.

[139] J.-M. Frère. *Streptomyces* R61 D-Ala-D-Ala carboxypeptidase. In: "Handbook of Proteolytic Enzymes, 2nd edition", A.J. Barrett, N.D. Rawlings and J.F. Woessner, eds. (Elsevier Academic Press), 2004, chap. 609.

[140] J.-M. Frère. *Actinomadura* R39 D-Ala-D-Ala carboxypeptidase. In: "Handbook of Proteolytic Enzymes, 2nd edition", A.J. Barrett, N.D. Rawlings and J.F. Woessner, eds. (Elsevier Academic Press), 2004, chap. 614.

[141] J.-M. Frère and J. Van Beeumen. DmpA L-aminopeptidase D-Ala-D-Ala esterase/amidase of *Ochrobactrum anthropi*. In: "Handbook of Proteolytic Enzymes, 2nd edition", A.J. Barrett, N.D. Rawlings and J.F. Woessner, eds. (Elsevier Academic Press), 2004, chap. 644.

Coordonnées déposées dans la 'Protein Data Bank'

(http://www.rcsb.org/pdb/)

06–04–2000	code d'accès 1EQS	(1.65 Å)	E. Fonzé, P. Charlier
07–04–2000	code d'accès 1ES2	(1.55 Å)	E. Fonzé, P. Charlier
07–04–2000	code d'accès 1ES3	(2.20 Å)	E. Fonzé, P. Charlier
07–04–2000	code d'accès 1ES4	(1.90 Å)	E. Fonzé, P. Charlier
10–04–2000	code d'accès 1ES5	(1.40 Å)	E. Fonzé, P. Charlier
11–04–2000	code d'accès 1ESI	(1.80 Å)	E. Fonzé, P. Charlier
20–04–2000	code d'accès 1ESU	(2.00 Å)	E. Fonzé, P. Charlier
05–01–2001	code d'accès 1HIX	(2.00 Å)	J. Wouters, P. Charlier
12–02–2001	code d'accès 1I2S	(2.00 Å)	E. Fonzé, P. Charlier
12–02–2001	code d'accès 1I2W	(2.00 Å)	E. Fonzé, P. Charlier
28–05–2001	code d'accès 1J9M	(1.65 Å)	E. Fonzé, P. Charlier
02–10–2001	code d'accès 1K38	(1.60 Å)	F. Kerff, E. Fonzé, P. Charlier
08–10–2001	code d'accès 1K4E	(2.00 Å)	F. Kerff, E. Fonzé, P. Charlier.
08–10–2001	code d'accès 1K4F	(1.60 Å)	F. Kerff, E. Fonzé, P. Charlier.
17–10–2001	code d'accès 1K6R	(2.30 Å)	F. Kerff, E. Fonzé, P. Charlier.
17–10–2001	code d'accès 1K6S	(2.00 Å)	F. Kerff, E. Fonzé, P. Charlier.
24–01–2003	code d'accès 1NRF	(2.45 Å)	F. Kerff, P. Charlier, E. Fonzé.
27–01–2004	code d'accès 1S6R	(2.24 Å)	J. Wouters, E. Fonzé, P. Charlier.
06–08–2004	code d'accès 1W5D	(2.10 Å)	E. Sauvage, P. Charlier.
31–08–2004	code d'accès 1W79	(1.80 Å)	E. Sauvage, P. Charlier.
01–09–2004	code d'accès 1W7F	(2.00 Å)	S. Petrella, E. Sauvage, P. Charlier
02–09–2004	code d'accès 1W7G	(1.65 Å)	J. Remiche, E. Sauvage, P. Charlier

28–09–2004	code d'accès 1W8Q	(2.80 Å)	E. Sauvage, P. Charlier.
01–10–2004	code d'accès 1W8Y	(2.40 Å)	E. Sauvage, P. Charlier.
02–12–2004	code d'accès 1Y54	(2.40 Å)	C. Michaux, P. Charlier, J. Wouters

PATENTS

Novel use of lipopeptide preparations

A. Brans, S. Dufour, B. Joris, M. Paquot, R. Brasseur, M. Deleu, H. Legros, J. Destain, J.-M. Frère, P. Jacques, P. Thonart
Patent number: WO2004002510 (08/01/04)
Application number: WO2003EP06817 20030627

Hybrid proteins of beta-lactamase class A

M. Galleni, P. Filée, F. Giannotta, B. Quinting, N. Ruth, P. Mercuri, A. Zervosen, C. Jérôme, R. Jérôme, J.-M. Frère
Brevet n° 04075403-1 (11/02/04)

Method for detecting toxic and non-toxic cyanobacteria

University of Helsinki, University of Liège and Consiglio Nazionale delle Ricerche
A. Wilmotte, C. Boutte, S. Grubisic, P. Balthasart
International patent WO04104211A2, (May 21 2004)

COLLABORATIONS

ARGENTINA

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

BELGIUM

FUNDP — Laboratoire d'Écologie des Eaux Douces, Namur (J.P. Descy).

FUNDP — Département de Pharmacie, Namur (B. Masereel, L. Pochet).

FUNDP — Laboratoire de Spectroscopie moléculaire de surface, Namur (P. Thiry).

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

FUNDP — Laboratoire de Chimie Biologique Structurale, Namur (J. Wouters).

FUSAGx — Section de Biophysique moléculaire numérique, Gembloux (R. Brasseur, A. Thomas, L. Lins).

FUSAGx — Unité de bio-industries, Gembloux (J.L. Arpigny, P. Thonart).

KUL — Laboratorium voor Biomoleculaire Dynamica, Leuven (Y. Engelborgs).

KUL — Afdeling van Chemische en Biologische Dynamica, Leuven (K. Heremans).

KUL — Laboratorium voor Medicinale Chemie, Leuven (P. Herdewyn).

RUG — Department of Biochemistry, Physiology and Microbiology, Gent (B. Devreese, J. Van Beeumen).

RUG — Vakgroep Organische Chemie, Gent (J. Van der Eycken).

RUG — Laboratory of Protistology and Aquatic Ecology, Gent (W. Vijverman, K. Sabbe).

SCKCEN — Laboratory of Microbiology, Mol (M. Mergeay).

UCL — Unité de Microbiologie, Louvain-la-Neuve (A.M. Corbisier).

UCL — Laboratoire de biochimie physique et des biopolymères, Louvain-la-Neuve, (J. Fastrez, P. Soumillon).

UCL — Laboratoire de Chimie organique de synthèse, Louvain-la-Neuve (J. Marchand-Brynaert).

UCL — Pharmacologie cellulaire et moléculaire, Louvain-la-Neuve (D. van Ackeren, F. van Bambeke, P. Tulkens).

UCL — Laboratoire de Chimie quantique, Louvain-la-Neuve (D. Peeters).

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

UCL — Unité de Chimie structurale, Laboratoire de Cristallographie, Louvain-la-Neuve (J.P. Declercq).

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

ULB (CERIA) — Service de Microbiologie, Bruxelles (V. Stalon, J. Wouters).

ULB (IBMM) — Service de Génétique Appliquée, Bruxelles (E. Godfroid, A. Jacquet).

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

ULg — Centre de Recherches en Neurobiologie Cellulaire et Moléculaire, Liège (L. Bettendorff).

ULg — Service de Pharmacie galénique, Liège (L. Delattre).

ULg — Algologie, Mycologie et Systématique expérimentale (V. Demoulin).

ULg — Laboratoire de Spectrométrie de Masse, Liège (E. De Pauw).

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

ULg — Centre d'Étude et de Recherche sur les Macromolécules, Liège (R. Jérôme).

ULg — Département de Physique, Groupe de Physique biomédicale, Laboratoire de Biophysique structurale, Liège (G. Llabrès).

ULg — Laboratoire de Dynamique moléculaire, Liège (R. Locht, B. Leyh).

ULg — Centre de Recherches du Cyclotron, Liège (A. Luxen).

ULg — Service de Microbiologie, Liège (J. Mainil).

ULg — Département des Maladies Infectieuses et Parasitaires, Liège (B. Mignon).

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

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

ULg — Laboratoire de Virologie, Épidémiologie et Pathologie des Maladies virales, Liège (E. Thiry).

ULg — Laboratoire de Chimie Analytique, Liège (L. Thunus).

ULg — Centre de Recherches sur les Protéines Prion, Liège (W. and D. Zorzi).

UMH — Laboratoire de Chimie Biologique, Mons (R. Wattiez).

VUB — Instituut voor Moleculaire Biologie en Biotechnologie, Brussel (S. Muyldermans, L. Wyns).

CAMEROON

Université de Yaoundé — Laboratoire de Pharmacie, Yaoundé (J.P. Gangoue).

CANADA

Université Laval — Département de Biologie et Centre d'études nordiques, Québec, (W. Vincent).

CZECH REPUBLIC

University of South Bohemia — Department of Botany, České Budějovice (J. Komarek).

DENMARK

Den Kongelige Veterinær-og Landbohøjskole — Department of Mathematics and Physics, Copenhagen (R. Bauer).

FINLAND

Finnish Environment Institute, Helsinki (P. Kuuppo).

University of Helsinki — Department of Applied Chemistry and Microbiology, Helsinki (K. Sivonen).

FRANCE

CNRS — Institut de Biologie Structurale, Laboratoire de Cristallographie Macromoléculaire, Grenoble (O. Dideberg, A. Dessen, T. Vernet).

CNRS — Institut de Biologie Structurale, Laboratoire de Résonance Magnétique Nucléaire, Grenoble (J.-P. Simorre).

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

ESRF (FIP) — Laboratoire de Cristallographie et Cristallogenèse des Protéines, Grenoble (C. Bauvois, J.-L. Ferrer).

Hôpital Cochin, Paris (A. Philippon).

INRA — Unité de Recherches Laitières et de Génétique Appliquée, Jouy-en-Josas (S. Kulakauska).

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

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

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

Université de Nantes — ProtNeteomix, Nantes (V. Sakanyan).

Université de Paris V — Laboratoire de Chimie et Biochimie, Paris (Y. Le Merrer, C. Gravier-Pelletier).

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

Université de Paris VI (LRMA) — Bactériologie, Paris (W. Sougakoff, S. Petrella).

Université de Paris VII — Laboratoire de Pharmacochimie Moléculaire, Paris (J.J. Godfroid, F. Heymans).

Université de Paris XI — Laboratoire de Chimie Théorique, Orsay (M. Desouter).

Université de Paris XI — Département de Biochimie Moléculaire et Cellulaire, Orsay (D. Mengin-Lecreulx, D. Blanot).

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

Université de Pau et des Pays de l'Adour — Département de Biochimie (S. Davail).

Université de Technologie de Compiègne — Laboratoire de Génie Enzymatique et Cellulaire, Compiègne (B. Bihan-Avalle, A. Friboulet).

GERMANY

EMBL Hamburg Outstation (W. Meyer-Glaucke).

Friedrich-AlexanderUniversität Erlangen-Nürnberg — Lehrstuhl für Mikrobiologie, Nürnberg (F. Titgemeyer).

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

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

Universität Kaiserslautern — Abteilung Mikrobiologie, Kaiserslautern (R. Hakenbeck).

Universität des Saarlandes — Technische Biochemie, Saarbrücken (H.W. Adolph, E. Heinzle, A. Tholey).

INDIA

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

ITALY

Consiglio Nationale delle Ricerche — Istituto di Tecnologie Biomediche, Segrate (G. De Bellis).

Consiglio Nationale delle Ricerche — Istituto per lo Studio degli Ecosistemi, Firenze (S. Ventura).

Università degli Studi di Siena — Dipartimento di Biologia Molecolare, Siena (G.M. Rossolini).

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

JAPAN

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

Toho University Medical School — Department of Microbiology (Y. Ishii).

LUXEMBOURG

Public Research Centre Gabriel Lippmann — Environment and Biotechnologies Research Unit, Luxemburg (L. Hoffmann).

POLAND

University of Gdansk and Medical University of Gdansk — Intercollegiate Faculty of Biotechnology, Gdansk (M. and K. Waleron).

University of Lodz — Department of Applied Ecology, Lodz (J. Mankiewicz).

PORTUGAL

Instituto Nacional de Engenharia e Biotecnologia Industrial, Lisbonne (J. Duarte, P. Moreira).

Universidade Católica Portuguesa — Escola Superior de Biotecnologia, Porto (F.X. Malcata, P. Moreira).

SLOVENIA

Lek — Pharmaceutical and Chemical Company, Ljubljana (U. Urleb).

Jozef Stefan Institute — Department of Biochemistry and Molecular Biology, Ljubljana (R. Pain).

University of Ljubljana — Faculty of Pharmacy, Ljubljana (S. Gobec).

SPAIN

Universidad Autónoma de Madrid — Centro de Biología Molecular, Madrid (J.A. Ayala, J. Berenguer).

Universidad Autónoma de Madrid — Departamento de Biología, Madrid (A. Quesada).

Universidad de Salamanca — Instituto de Microbiología Bioquímica, Salamanca (R. Santamaria, J.M. Fernandez-Abalos, M. Diaz, S. Rodriguez).

SWEDEN

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

Stockholm University — Department of Botany, Stockholm (U. Rasmussen).

SWITZERLAND

Université de Lausanne — Unité de Génétique et de Biologie Microbiennes, Lausanne (D. Karamata, P. Margot, P. Moreillon).

THE NETHERLANDS

Leiden Institute of Chemistry — Department of Biochemistry, Leiden (G. Van Wezel).

University of Amsterdam — Department of Molecular Cell Biology, Amsterdam (T. Den Blaauwen, N. Nanninga).

The University of Utrecht — Department of Biochemistry of Membranes, Utrecht (E.J. Breukink).

TUNISIA

Université de Tunis — Laboratoire de Biochimie et de Technobiologie, Tunis (O. Belhadj).

UNITED KINGDOM

Bristish Antarctic Survey, NERC, Cambridge (D. Hodgson).

John Innes Centre — Department of Molecular Microbiology, Norwich (P. Hoskisson and M. Buttner).

Pisces Conservation Ltd, Lymington (R. Seaby, P. Henderson).

University of Cambridge — Department of Chemistry, Cambridge (C.M. Dobson, M. Dumoulin).

University of Huddersfield — Biomolecular Sciences Research Centre, Huddersfield (M. Page).

University of Leeds — School of Biochemistry and Molecular Biology, Leeds (I. Chopra, C. Fishwick).

University of Leicester — Biological NMR Centre, Leicester (C. Damblon, G. Roberts).

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

University of Sheffield — Department of Molecular Biology and Biotechnology, Sheffield (E. Hayhurst, S. Foster).

USA

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

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

University of Connecticut Health Centre — Department of Biochemistry, Farmington, CT (B. and P. Setlow).

University of Iowa — Department of Microbiology, Iowa City, IA (D. Weiss).

Wesleyan University — Department of Chemistry, Middletown, CT (R. Pratt).

PhD THESES

26/01/00 — Mohammed Terrak (Biochemistry).
Relation structure-fonction des différents modules de la protéine 1b liant la pénicilline d'*Escherichia coli*.

28/01/00 — Sylvie Bossrez (Biochemistry).
Étude comparative de la capacité des parois isolées de souches d'Entérocoque à piéger les éléments en trace.

22/03/00 — Noureddine Rhazi (Biochemistry).
Étude du mécanisme catalytique des DD-peptidases bactériennes.

30/03/01 — Birgit Quinting (Biochemistry).
Construction de protéines hybrides comme vaccins potentiels.

28/09/01 — Patrice Filée (Biochemistry).
Étude du répresseur BlaI de *Bacillus licheniformis* 749/I impliqué dans la régulation de la β-lactamase BlaP.

29/09/01 — Dominique De Seny (Biochemistry).
Étude spectroscopique de la β-lactamase de *Bacillus cereus* II.

07/12/01 — Marie-Noëlle Ramquet (Chemistry).
Recherche de structures à l'équilibre sur une surface d'énergie potentielle.

07/01/02 — Kamal Benlafya (Biochemistry).
Étude du récepteur BlaR1 impliqué dans la regulation de la β-lactamase BlaP chez *Bacillus licheniformis* 749/I.

11/01/02 — Frédéric Sapunaric (Zoology).
Rôle régulateur de la protéine Psr sur la synthèse de la protéine PBP5 impliquée dans la résistance d'*Enterococcus hirae* aux β-lactamines.

20/02/02 — Paola Sandra Mercuri (Biochemistry).
Étude de la métallo-β-lactamase FEZ-1.

15/03/02 — Frédéric Kerff (Physics).
Étude structurale par radiocristallographie de trois pénicilloyl sérine transférases originales : le domaine C-terminal du récepteur BlaR de *Bacillus licheniformis*, nécessaire à l'induction des

β-lactamases et les β-lactamases de classe D de *Salmonella typhimurium* et *Pseudomonas aeruginosa*.

13/05/02 — Sébastien Rigali (Biology).
XlnR, un nouveau facteur de transcription impliqué dans la répression glucose chez *Streptomyces lividans*.

07/06/02 — Serge Leimanis (Biochemistry).
Étude du rôle compensatoire de la protéine PBP5 chez des mutants de division conditionnels d'*Enterococcus hirae*.

14/09/02 — Florence Mahy (Biology).
Étude par mutagenèse dirigée de la carbapénèmase de classe A NMCA.

09/04/03 — Catherine Généreux (Biochemistry).
Étude de l'anhydro-N-acétylmuramyl-L-alanine amidase d'AmpD de *Citrobacter freundii*.

10/04/03 — Séverine Hallut (Biochemistry).
Caractérisation de la protéine membranaire bifonctionnelle codée par le gène *ponA* d'*Enterococcus faecalis*.

30/06/03 — Catherine Raskin (Biochemistry).
Étude de la protéine BlaB impliquée dans la régulation des β-lactamases de *Streptomyces cacaoi*.

12/05/04 — Christina Franssen (Biology).
Origine de la surproduction de la protéine PBP5 responsable de la résistance aux β-lactamines d'*Enterococcus hirae* R40.

21/06/04 — Sophie Hanique (Biochemistry).
Étude du récepteur membranaire BlaRl impliqué dans l'induction de la β-lactamase BlaP de *Bacillus licheniformis* 749/I.

20/09/04 — Maria Colombo (Biochemistry).
Étude du domaine C-terminal du récepteur BlaR1 de *Bacillus licheniformis* et de son homologue YbxI de *Bacillus subtilis*.

17/12/04 — Christelle Vreuls (Chemistry).
Caractérisation du répresseur BlaI impliqué dans la régulation de la β-lactamase BlaP chez *B. licheniformis* 749/I.

FINAL YEAR LICENCE PROJECTS

1999-2000	DEC Biochemistry	Hamid Bensmida
	DES Biotech	Abdelmounaim El Abdellaoui
	Biology (Erasmus)	Neide Frango de Gounveia
	Zoology	Séverine Hubert
	DES Biotech	Btissam Nayjib
	Biochemistry	Kathy Ridremont
	Chemistry	Nicolas Willet
2000–2001	DES Protein Engineering	Stéphane Baurin
	Zoology	Mélanie Laschet
	Biochemistry	Vincent Lauria
	Engineering in Physical Sciences	Lara Vigneron
2001–2002	Zoology	Arnaud Bataille
	Biochemistry	Michaël Gillon
	Biochemistry	Thierry Gouders
	Botany	Julia Horion
	Biochemistry	Benoît Wolf
2002–2003	Maîtrise Paris VI	Ludovic Banwarth
	Zoology	Renaud Berlemont
	Biochemistry	Mounir Djama
	Zoology	Anne Famerie
	Zoology	Anne Gougnard
	Zoology	Thibaud Neutelings
2003–2004	Zoology	François Bastien
	Biochemistry	Alexandre Di Paolo
	Biochemistry	Bernadette Lamsoul
	Biochemistry	Cédric Lemaire
	Chemistry	Julie Vandenameele

COURSES

P. Charlier

Biophysique, *partim* cristallographie des macromolécules, 2L Sciences physiques ;

Structure et dynamique des macromolécules biologiques, *partim* cristallographie, 2L Sciences biochimiques ;

Cristallographie appliquée aux biomolécules, DEA en Sciences et DES en Biochimie : Ingénierie des protéines et modélisation moléculaire.

Propriétés structurales des protéines, DES en Biochimie : Ingénierie des protéines et modélisation moléculaire.

J. Coyette

Biochimie des microorganismes, 2L Biochimie ;

Biologie I, 1C Chimie ;

Biologie II, 2C Chimie ;

Bactériologie, 1L Biochimie, 1L Biologie végétale, 1L Biologie animale, DES en Biotechnologie ;

Génie génétique des bactéries (y compris plasmides et éléments transposables), DES en Biotechnologie.

G. Dive, D. Dehareng

Approche théorique et expérimentale des spectroscopies non magnétiques, 3^{e} cycle interuniversitaire en chimie physique moléculaire, Louvain-la-Neuve, 2–6 juin 2001.

Approche théorique et expérimentale des spectroscopies magnétiques, 3^{e} cycle interuniversitaire en chimie physique moléculaire, Louvain-la-Neuve, 19–28 mai 2003.

D. Dehareng

Aide à l'utilisation de la chimie quantique, cours à option, 2L Sciences chimiques ;

Interactions inter et intra-moléculaires, DEA en Sciences.

J.-M. Frère

Biochimie générale, 1L Sciences chimiques;

Chimie générale biologique, *partim* enzymologie, 2L Sciences chimiques;

Propriétés fonctionnelles des macromolécules biologiqes, 1L Sciences biochimiques;

Introduction aux principes généraux de la biologie et de la biochimie, 3^{e} épreuve Ingénieur Civil Chimiste;

Purification des protéines, 2L Sciences biochimiques, DEA en Sciences biochimiques;

Compléments d'enzymologie, 2L Sciences biochimiques, DEA en Sciences biochimiques.

M. Galleni

Biochimie et thermodynamique des systèmes biologiques, 2C Sciences biologiques, épreuve préalable à la licence en sciences biochimiques, épreuve préalable à la licence en sciences biologiques;

Biochimie et thermodynamique des systèmes biologiques, *partim* thermodynamique, épreuve préalable à la licence en sciences biochimiques, épreuve préalable à la licence en sciences biologiques;

Biochimie des macromolécules, 1L Sciences biologiques (biologie animale);

Chimie des macromolécules biologiques, 1L Sciences biochimiques;

Biochimie comparée et adaptative, *partim* animal, 2L Sciences biochimiques.

B. Joris

Génomique et bioinformatique, 1L Sciences biochimiques.

A. Matagne

Travaux pratiques d'Enzymologie (50 h), 1L Sciences biochimiques, University of Liège, 1997–;

Advanced course on "Protein Purification: What to do and how (under the supervision of Prof. Jean-Marie Frère), University of Liège, 6–9 June 2000, 11-14 June 2002, 6-8 May 2003, 11-13 May 2004;

Réacteurs biochimiques, *partim* cinétique chimique et enzymatique et phénomènes de transfert des masses (12 h), DEA en Biotechnologie, University of Liège, 2002–;

Structure et dynamique des macromolécules biologiques, *partim* dynamique (40 h), 2L Sciences biochimiques, University of Liège, 2003–.

S. Rigali

Bioinformatique, Haute École André Vésale de la Province de Liège, 2001–2002, 2003–2004, 2004–2005.

A. Wilmotte

Approches moléculaires de la diversité des microorganismes, 2L Sciences biochimiques, 2L Biologie végétale, 2L Biologie animale.

VISITORS

Alcocer, Marcos (University of Nottingham, UK)	08/10/02
	10/03/03 to 12/03/03
Amoroso, Ana (Universidad de Buenos Aires, Argentina)	11/01/00 to 15/05/00
	20/11/00 to 13/02/01
	12/11/01 to 19/12/01
	01/02/03 to 22/03/03
	01/05/04 to 30/04/05
Anne, Christine (Université Paris V, France)	06/2002 to 05/2003
Archer, David (University of Nottingham, UK)	08/10/02 to 09/10/02
Badarau, Adriana (University of Huddersfield, UK)	25/01/04 to 01/02/04
Berthet, Nicolas (Université de Paris VII, France)	05–07/04
Chedad, Allel (KULAK, Belgium)	06/11/03
Chouchani, Chedly (Université de Tunis, Tunisie)	03–06/04
Collet, Jean-François (Université Catholique de Louvain, Belgium)	13/06/00
	29/08/03
	19/05/04
Conrath, Katja (Vrije Universiteit Brussel, Belgium)	05/02/01 to 10/02/01
Calusinska, Magdalena (University of Gdansk, Poland)	15/09/04 to 15/02/05
Damblon, Christian (University of Leicester, UK)	19/05/03
De Genst, Erwin (Vrije Universiteit Brussel, Belgium)	22/10/04
De Jongh, Harmen (Wageningen Centre for Food Sciences, The Netherlands)	26–27/08/04
Dobson, Christopher (University of Cambridge, UK)	8–9/10/02
Dumoulin, Mireille (University of Cambridge, UK)	08/10/02 to 12/10/02
	10/03/03 to 17/03/03
	06/04/03 to 14/04/03
	19/09/03 to 05/10/03
	24/08/04 to 12/09/04
	29/11/04 to 06/12/04
Engelborghs, Yves (Katholieke Universiteit Leuven, Belgium)	25/10/02
	07/02/03
Forge, Vincent (CEA, Grenoble, France)	03/11/04 to 07/11/04
Gardini, Francesco (Università degli studi di Siena, Italy)	04/04

Goormaghtigh, Éric (Université Libre de Bruxelles, Belgium)	24/10/03
Gutkind, Gabriel (Universidad de Buenos Aires, Argentina)	09/10/01 to 19/10/01 03/08/02 to 17/08/02 09/12/02 to 17/12/02
Heremans, Karel (Katholieke Universiteit Leuven, Belgium)	11/09/01 30/08/02
Itzhaki, Laura (University of Cambridge, UK)	29–30/08/02
Lasorne, Benjamin (Université de Paris-Sud, France)	one week in April 03
Leung, Thomas (The Hong Kong Polytechnic University, Hong-Kong)	9–10/07/01
Meersman, Filip (Katholieke Universiteit Leuven, Belgium)	29/11/02
Moali, Catherine (CNRS, Lyon)	01/02 to 07/02
Moreira, Patricia (Universidade Católica Portuguesa, Portugal)	03/09/00 to 03/12/00 01/09/01 to 01/12/01
Muylderman, Serge (Vrije Universiteit Brussel, Belgium)	14/02/00 8–9/10/02
Pain, Roger (Jožef Stefan Institute, Ljubljana, Slovenia)	19/04/00 to 23/04/00 23/04/01 to 27/04/01 29/08/02 to 05/09/02 20/09/04 to 22/09/04
Papaefthimiou, Dimitra (University of Stockholm, Sweden)	24/06/03 to 04/09/03
Pardon, Els (Vrije Universiteit Brussel, Belgium)	06/04/03 to 14/04/03
Petrella, Stéphanie (Université Paris VI, France)	01/01/04 to 01/01/05
Phichith, Ping (CNRS, Compiègne, France)	11–12/10/04
Pieboji, Joseph (Université de Yaoundé, Cameroun)	2002–2003
Pimentel, Elisangela (Erlangen-Nürnberg University, Germany)	29/11/04 to 10/12/04
Plückthun, Andreas (Universität Zurich, Switzerland)	18/01/00
Radford, Sheena (University of Leeds, UK)	28–29/08/03
Redfield, Christina (University of Oxford, UK)	8–9/10/02 26-27/08/04
Robinson, Carol (University of Cambridge, UK)	8–9/10/02
Rossolini Gianmaria (University of Siena, Italy)	10/01/2004 to 17/01/2004
Rousseau, Frédéric (Vrije Universiteit Brussel, Belgium)	27/08/04
Selevsek, Nathalie (Universität des Saarlandes, Germany)	06–07/03
Soumillon, Patrice (Université Catholique de Louvain, Belgium)	18/10/02
Titgemeyer, Fritz (Erlangen-Nürnberg University, Germany)	10/12/04
Van Hove, Patrick (Université de Laval, Québec, Canada)	21/03/02 to 20/06/02
Van Nuland, Nico (University of Utrecht, The Netherlands)	28–29/08/03
Vogel, Andreas (EMBL-Hamburg, Germany)	14–16/10/02

Waleron, Krzysztof (University of Gdansk, Poland)	05/01/03 to 31/01/04
Waleron, Malgorzata (University of Gdansk, Poland)	20/01/03 to 20/04/03 01/06/03 to 31/01/04
Wyns, Lode (Vrije Universiteit Brussel, Belgium)	8–9/10/02 10/04/03
Yoshikazu, Ishii (University of Toho, Japan)	01/10/99 to 01/11/00
Yribarren, Anne-Sophie (CNRS, Compiègne, France)	04/02/02 to 24/02/02
Zawadzka, Joanna (University of Warsaw, Poland)	01/04/04 to 30/06/04
Zéba, Boukaré (Université de Ouagadougou, Burkina Faso)	01/04/03 to 15/07/03

CONFERENCES, CONGRESSES*

Belgian Branch of the Royal Society of Chemistry, Tervueren, Belgium, March 8, 2000 (**J.M. Frère**).

EC sectorial meeting "Controlling the proliferation of the microbial cell factory", Verona, Italy, April 19–21, 1999 (M. Nguyen-Distèche).

EC Biotechnology Consortium, Edimburgh, UK, September 27–28, 1999 (**M. Nguyen-Distèche**).

2nd Symposium on Protein Structure Analysis for Biomedical Research, Murnau, Germany, March 30–April 1, 2000 (E. Fonzé, E. Sauvage).

671st Meeting of the Biochemical Society "From Protein Folding to New Enzymes", University of Leeds, UK, April 11–13, 2000 (A. Matagne).

"Mechanisms of bacterial resistance related to the cell wall", Universidad de Buenos Aires, Buenos Aires, Argentina, May 12–21, 2000 (**J. Coyette**).

9th Biennal UA-UC Conference on Infectious Diseases, Banff, British Columbia, Canada, May 14–17, 2000 (**F. Sapunaric**).

10th International Congress of Quantum Chemistry, Menton, France, June 5–9, 2000 (D. Dehareng).

Gordon Research Conference "Bacterial Cell Surfaces", New London, NH, USA, June 25–30, 2000 (**M. Nguyen-Distèche**).

14th Symposium of the Protein Society, San Diego, CA, USA, August 5–9, 2000 (A. Matagne).

SmithKline Beecham Biologicals, Rixensart, Belgium, September 11, 2000 (**J.-M. Frère**).

40th ICAAC meeting, Toronto, Canada, September 17–20, 2000 (**F. Sapunaric**).

"Non-Arrhenius Kinetics in the Folding of Hen Lysozyme", I.C.P. Brussels, Belgium, September 21, 2000 (**A. Matagne**).

SMR Symposium "Conquering Antibacterial Resistance", London, UK, September 28, 2000 (**J.-M. Frère**).

---

* Speakers are indicated in bold letters.

Biological Physics and Synchrotron Radiation, Grenoble, France, October 11–14, 2000 (P. Charlier, E. Sauvage).

SmithKline Beecham Biologicals, Rixensart, Belgium, November 6, 2000 (**J.-M. Frère**).

Faculté des Sciences, Université de Nancy, France, November 22, 2000 (**J.-M. Frère**).

"Non-Arrhenius Kinetics in the Folding of Hen Lysozyme", Leicester, UK, November 22, 2000 (**A. Matagne**).

Symposium « Résistance et virulence des cocci à Gram positif ? », Pasteur Institute, Paris, France, December 6, 2000 (A. Amoroso, C. Franssen, F. Sapunaric, S. Hallut, J. Coyette).

"Crystallographic structure of the PBP5fm, the protein responsible for resistance to β-lactam antibiotics in *Enterococcus faecium*", Aventis Pharma, Romainville, Paris, France, December 17, 2000 (**E. Sauvage**).

Pharmaconférences, Université de Liège, Belgium, January 11, 2001 (**J.-M. Frère**).

"Protons in Proteins: Biological applications of neutron diffraction", Grenoble, France, January 25–27, 2001 (P. Charlier, E. Fonzé).

Anti-Infective Scientific Advisory Board, Ortho-McNeil-Pharmaceuticals, New York, USA, February 1–2, 2001 (**J.-M. Frère**).

11th ESRF User's meeting "High Throughput Structural Biology", Grenoble, France, February 19–21, 2001 (P. Charlier).

Toho University School of Medicine (for pharmaceutical scientists), Tokyo, Japan, February 26, 2001 (**J.-M. Frère**).

Toho University School of Medicine (for β-lactamase specialists), Tokyo, Japan, February 27, 2001 (**J.-M. Frère**).

Japanese Association of Microbiology Technologists, Tokyo, Japan, February 28, 2001 (**J.-M. Frère**).

4th European Symposium of the Protein Society, Paris, France, April 18–22, 2001 (A. Matagne).

101st Annual meeting of the Am. Soc. for Microbiology, Orlando, Florida, USA, May 20–24, 2001 (C. Franssen, M. Colombo, J. Coyette).

The "International Functional Genomics of Gram-Positive Microorganisms", San Diego, USA, June 24–28, 2001 (B. Joris).

Contact Forum WOG-Density Functional Theory, K.U. Leuven, Belgique, September 17, 2001 (D. Dehareng).

41st ICAAC Meeting, Chicago, USA, September 22–25, 2001 (**S. Leimanis**).

"Protein Folding and Misfolding from Molecular Diseases", Kortrijk, Belgique, September 26, 2001 (A. Matagne, E. Fonzé, E. Sauvage).

International Congress of the Argentinian Society for Microbiology, Buenos Aires, Argentina, October 5–8, 2001 (**M. Galleni**).

Rencontres biotechnologiques Midi-Pyrénées, Toulouse, France, October 18, 2001 (**J.-M. Frère**).

2nd Belgian Crystallography Symposium, Bruxelles, Belgique, October 24, 2001 (P. Charlier, E. Sauvage).

1st conference of the FNRS Structural Biology Contact Group "Structure/Function Relationships in Proteins", Gembloux, Belgique, October 31, 2001 (P. Charlier, D. Dehareng, J.-M. Frère, M. Nguyen-Distèche, E. Fonzé, N. Rhazi, **E. Sauvage**).

Workshop on Natural Communities of Nitrogen-Fixing Cyanobacteria: New Techniques for Field Studies, Bertinoro, Italy, November 6–10, 2001 (**A. Wilmotte**, C. Boutte).

5e rencontre des quanto-chimistes belges, Liège, Belgique, November 16, 2001 (**G. Dive**).

"Collisions: an international conference on collisions in the universe", Facultés Universitaires Notre-Dame de la Paix, Namur, Belgique, November 21–22, 2001 (D. Dehareng).

FNRS contact group for Synchrotron Radiation, Namur, Belgique, November 23, 2001 (P. Charlier, E. Fonzé, E. Sauvage).

Joint meeting of the Belgian Society of Biochemistry and Molecular Biology and the Belgian Biophysical Society on "Current Advances in Biospectroscopies", Leuven, Belgium, December 7, 2001 (organized by A. Matagne).

NEMS 2001, International meeting on Carbapenems, Venise, Italy, December 13–15, 2001 (**J.-M. Frère**).

41st ICAAC meeting, Chicago, USA, December 16–19, 2001 (**M. Galleni**).

« Apport de la chimie quantique en pharmacochimie : de la molecule isolée à la catalyse enzymatique, Paris, France, February 22, 2002 (**G. Dive**).

Eight β-lactamase workshop, Holy Island, UK, March 24–28, 2002 (**J.-M. Frère**).

Seminar at the Life Science Dept at the UCL, "Biosynthesis and turnover of the bacterial peptidoglycan", Louvain-la-Neuve, Belgique, March 13, 2002 (**J. Coyette**).

ASCMAP meeting on "Analysis of Single Cells in the Marine Phytoplankton, Bremerhaven, Germany, April 15–20, 2002 (A. Wilmotte).

Symposium "Self-Organization and Structure-Function Relation of Biomolecules, Leucorea Wittenberg, Germany, April 17–20, 2002 (A. Matagne).

BAGECO-7 (Bacterial Genetics and Ecology), Bergen, Norway, June 15–19, 2002 (C. Boutte).

Gordon Research Conference "Bacterial Cell Surfaces", New London, NH, USA, June 23–28, 2002 (**M. Nguyen-Distèche**).

« Étude de la stabilité conformationnelle de fragments d'anticorps constitutes ??? d'un seul domaine", UTC Compiègne, France, June 24, 2002 (**A. Matagne**).

ESF-CYANOFIX Summer School "Cyanobacteria and Nitrogen Fixation in Extreme Environments", Longyeargyen, Norway, June 25–July 3, 2002 (**A. Taton**, A. Wilmotte).

"The World of Microbes", Xth International Congress of Bacteriology and Applied Microbiology, Paris, France, July 27–August 1, 2002 (**J.-M. Frère**). New tool to manipulate *Bacillus subtilis* chromosome.

16th Symposium of the Protein Society, San Diego, CA, USA, August 17–21, 2002 (A. Matagne).

Joint meeting of the Belgian Biophysical Society and the FNRS contact group "Structural Biology" on "Protein Folding and Stability", Liège, Belgium, August 30, 2002 (organized by A. Matagne).

SWAP (Science for Water Policy) 2002, Norwich, UK, September 2, 2002 (A. Wilmotte).

Symposium "Cyanobacterial Nitrogen Fixation: from Molecules to Ecological Systems", Tomar, Portugal, September 25–29, 2002 (**A. Wilmotte**, C. Boutte, A. Taton).

Friedrich-Alexander University, Erlangen-Nürnberg, Germany, November 27, 2002 (**S. Rigali**).

J. March Workshop "Manufacturing bacteria: design, production and assembly of cell division bacteria", Madrid, Spain, December 15–18, 2002 (M. Nguyen-Distèche).

Third International Symposium of Ecological Genetics, KUL Leuven, Belgium, February 5–7, 2003 (A. Wilmotte, C. Boutte, C. Schmoker).

5th European Symposium of The Protein Society, Florence, Italy, March 29–April 2, 2003 (A. Matagne).

"Equilibrium Folding Properties of Single-Domain Antibody Fragments from Camelids", Siena, Italy, April 3, 2003 (**A. Matagne**).

13th ECCMID (European Society for Clinical Microbiology and Infectious Diseases) meeting, Glasgow, United Kingdom, May 8–10, 2003 (**J.-M. Frère**, **M. Galleni**).

Departamento de Microbiología y Genetica, Salamanca, Spain, May 21, 2003 (**S. Rigali**).

EURESCO Conference "Molecular Bioenergetics of Cyanobacteria", Aquafredda di Maratea, Italy, May 17, 2003 (**A. Wilmotte**).

184th meeting of the Belgian Society of Biochemistry and Molecular Biology on "Protein Folding and Misfolding", Gembloux, Belgium, May 23, 2003 (A. Matagne).

Joint meeting of Belgian and German Biophysicists on "Folding, Dynamics and Interaction of Biomolecules", Hünfeld, Germany, May 29–June 1, 2003 (**A. Matagne**).

University of Surrey, Guilford, UK, June 5, 2003 (**S. Rigali**).

Congress "Metagenomics 2003, from Microbial Diversity to Function", Darmstadt, Germany, June 12–14, 2003 (A. Wilmotte).

"A Highly Stable Single-Domain Antibody Fragment that Inhibits Fibril Formation by Stabilizing Two Human Lysozyme Variants", Dyax, Liège, Belgium, June 17, 2003 (**A. Matagne**).

The "International Conference on Functional Genomics of Gram-Positive Microorganisms", Baveno, Italy, June 22–27, 2003 (B. Joris).

RiSCC (Regional Sensitivity to Climate Change in Antarctic Terrestrial and Limnetic) Workshop, Varese, Italy, July 2–7, 2003 (**C. Schmoker**).

6th Girona Seminar on Molecular Similarity, Girona, Spain, July 24–30, 2003 (**G. Dive**).

Aquatic Biodiversity: Past, Present and Future, Antwerp, Belgium, August 11–13, 2003 (A. Wilmotte, C. Boutte).

2nd meeting of the Belgian Biophysical Society on "Protein Folding and Stability", Liège, Belgium, August 29, 2003 (organized by A. Matagne).

10th International Conference on the Application of Density Functional Theory in Chemistry and Physics, Vrije Universiteit Brussel, Belgique, September 7–12, 2003 (D. Dehareng).

5th conference of the FNRS Structural Biology Contact Group "Biophysical methods for protein structure analysis", Liège, Belgique, September 12, 2003 (P. Charlier).

43rd annual ICAAC (American Society for Microbiology), Chicago, USA, September 14–17, 2003 (**J.-M. Frère**).

16th Falterage at the Leucorea Wittenberg, Germany, September 19–21, 2003 (**A. Matagne**).

Symposium on Extremophiles "Happy Microbes in Hostile Niches", Brussels, Belgium, September 27, 2003 (A. Wilmotte, C. Schmoker).

Workshop on "Cyanobacterial Diversity and Toxicity", Brussels, Belgium, October 20, 2003 (**A. Wilmotte**, C. Boutte).

Predicting evolution of β-lactamase mediated bacterial resistance. A micro symposium. The Danish Veterinary Institute, Copenhagen, Denmark, November 7, 2003 (**J.-M. Frère**).

5th meeting of Quantum Chemistry in Belgium, Universiteit Gent, Belgium, November 21, 2003 (D. Dehareng, G. Dive, **C. Géron**).

Meeting of the Belgian Society for Microbiology "Microbial Immune Evasion Strategies", Brussels, Belgium, November 21, 2003 (A. Wilmotte).

Meeting of the European Platform for Biodiversity Research Strategy "Genetic Biodiversity in Agricultural and Natural Systems: Measurement, Understanding, Management", Firenze, Italy, November 21–24, 2003 (**A. Wilmotte**).

European Conference on the Role of Research in Combating Antibiotic Resistance, Rome, Italy, November 28–30 (**J.-M. Frère**).

International Conference on Arctic Microbiology, Rovianemi, Finland, March 22–28, 2004 (C. Schmoker).

Seminar of the Biology Department, Programme de la Chaire de Recherche du Canada en Études des Écosystèmes Aquatiques, Université de Laval, Québec, Canada, March 25, 2004 (**A. Wilmotte**).

Euresco Conferences, University of Exeter, UK, April 13–18, 2004 (D. Dehareng).

International Symposium CSSD: Cyanobacteria for Health, Science and Development, Embiez Island, France, May 2–4, 2004 (**A. Wilmotte**).

6th International Conference on Toxic Cyanobacteria, Bergen, Sweden, June 21–27, 2004 (C. Boutte).

Gordon Research Conference "Bacterial Cell Surfaces", New London, NH, USA, June 27–July 2, 2004 (**J.-M. Frère**, **M. Nguyen-Distèche**, **B. Joris**).

μ-Theochem: Modelling and Understanding in Theoretical Chemistry, Lucca, Italy, August 1–4, 2004 (**G. Dive**).

3rd Meeting of the Belgian Biophysical Society on "Protein Folding and Stability", University of Liège, Belgium, August 27, 2004 (**A. Matagne**).

16th IAC (International Association for Cyanophyte research) Symposium, Luxembourg, August 30–September 3, 2004 (**A. Wilmotte**, **C. Boutte**, **C. Schmoker**).

Workshop on Electronic Recognition of DNA Molecules, Université de Liège, Belgique, September 1–3, 2004 (D. Dehareng).

6th International Conference on Protein Stabilization ProtStab 2004, Bratislava, Slovakia, September 26–29, 2004 (**A. Matagne**).

Mini-Symposium on "Bacterial Cell Division and Cell Wall", Tübingen, Germany, October 12–13, 2004 (**M. Nguyen-Distèche**, **B. Joris**).

Friedrich-Alexander University, Erlangen-Nürnberg, Germany, October 15–16, 2004 (**S. Rigali**).

A highly stable single-domain antibody fragment that inhibits fibril formation by stabilizing two human lysozyme variants, C.I.P., Brussels, Belgium, November 4, 2004 (**A. Matagne**).

STAYS ABROAD

Different stays as invited professor at the University of Paris VII, Laboratoire de Pharmacologie Moléculaire, Paris, France, one month in 2001–2002 and 2003–2004 (G. Dive).

Different stays as invited professor at the University of Paris XI, Laboratoire de Chimie Théorique, Paris, France, one month in 2000–2001 and 2002–2003 (G. Dive).

Different stays at the ESRF Laboratory (European Synchrotron Radiation Facilities), Grenoble, France, February 7–9, April 7–9, May 8–12, June 6–9, November 13–18, 2000, March 2–3, 2001, February 26–28, September 6–9, 2002, April 11–14, October 8–9, November 20–21, 2003, March 10–12, June 19–21, September 25–27, 2004 (Crystallography team).

Different stays at the Oxford Centre for Molecular Sciences, University of Oxford, UK, May 15–19, 2000; September 26–29, 2000; October 20–22, 2000, June 25–29, 2001 (A. Matagne).

Department of Bacterial Resistance, University of Buenos Aires, Argentina, May 6–20, 2000 (M. Galleni).

Departamento de Microbiología, Universidad de Buenos Aires, Buenos Aires, Argentina, Third cycle lectures on the "Mechanisms of bacterial resistance related to the cell wall"; May 12–21, 2000 (J. Coyette).

Department of Bacterial Resistance, University of Buenos Aires, Argentina, October 5–19, 2001 (M. Galleni).

John Innes Centre, Norwich, UK, March 11–15, 2002 (A. Derouaux).

Different stays at the Department of Chemistry, University of Cambridge, UK, January 7–9, 2002; May 23–25, 2002; January 7–10, 2003 (A. Matagne).

CNRS — Institut de Biologie Structurale, Grenoble, France, June 12, 2003 (M. Nguyen-Distèche).

Université de Pau et des Pays de l'Adour, Mont de Marsan, France, November 4–8, 2003 (M. Galleni).

Campement Byers, Livingston Island, Péninsule Antarctique, February 1–19, 2004 (A. Wilmotte).

Département de Biologie et Centre d'études nordiques, Université de Laval, Canada, March 20–27, 2004 (A. Wilmotte).

Department of Biochemistry, Biological NMR Centre, University of Leicester, UK, July 25–31, 2004 (O. Jacquin).

AWARDS

Dominique de Seny	DSM Award (2002)
Jean-Denis Docquier	ESCMID Research Fellowship 2004
Jean-Marie Frère	Chaire Francqui au titre belge, KUL, année 2001–2002 Chaire Francqui au titre belge, ULB, année 2002–2003
Moreno Galleni	Prix Léon et Henri Fredericq – Académie Royale des Sciences de Belgique 2003
Michael Gillon	Prix Marcel Florkin 2002

LIST OF THE ORGANISMS AND SOCIETIES HAVING SUPPORTED THE CIP

— Les Pôles d'attraction interuniversitaires
— BELSPO : Politique scientifique fédérale
— Les Actions de Recherche concertées
— La Région wallonne
— Le Commissariat général aux Relations internationales
— Le Fonds pour la Formation à la Recherche dans l'Industrie et l'Agriculture
— Le Fonds National de la Recherche Scientifique
— Le Fonds de la Recherche fondamentale collective
— Le Fonds de la Recherche scientifique médicale
— L'Université de Liège
— La Loterie Nationale
— L'Union européenne
— L'Organisation du Traité de l'Atlantique Nord
— Aventis (France)
— 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
— CNRS/INSERM : Molécules et cibles thérapeutiques
— Johnson Pharmaceutical, (USA)
— Eli Lilly (Mont-Saint-Guibert)
— L'Agence spatiale européenne (ESA)

COMPOSITION OF THE CENTRE

Managing committee

Director: J.-M. Frère

Codirector: G. Dive

Executive committee: J. Coyette, M. Galleni, B. Joris

Other Members: A. Brans, P. Charlier, D. Dehareng, C. Duez, J. Dusart, C. Fraipont, F. Giannotta, C. Goffin, A. Matagne, M. Nguyen-Distèche, E. Sauvage, A. Wilmotte

http://www.cip.ulg.ac.be/

Senior Scientists

1. Microbial biochemistry and physiology

Prof. J. Coyette

Drs. A. Amoroso, A. Derouaux, C. Duez, V. Duval, C. Fraipont, C. Goffin, M. Nguyen-Distèche, N. Rhazi, M. Terrak

Ph.D. students: X. Henry, S. Hubert, S. Pastoret, A. Piette, B. Wolf

2. Molecular biology and genetics

Drs. A. Brans, P. Filée, F. Giannotta, B. Joris, S. Rigali

Ph.D. students: C. Raskin, R. Berlemont, A. Chahboune, M. Delmarcelle, A. Famerie, S. Grubisic, G. Moutzourelis

3. Protein chemistry and enzymology

Profs. J.-M. Frère, M. Galleni

Drs. A. Matagne, C. Bebrone, F. de Lemos Esteves, P. Mercuri, P. Filée, C. Prosperi, N. Ruth, A. Zervosen

Ph.D. students: S. Baurin, C. Beauvois, F. Bouillenne

4. Protein crystallography

Drs. P. Charlier, E. Sauvage, E. Fonzé

Ph.D. student: F. Kerff

5. Applied quantum chemistry and Protein modelling

Drs. G. Dive, D. Dehareng

6. Cyanobacteria

Dr. A. Wilmotte

Ph. D. C. Boutte, C. Schmoker, A. Taton

Administrative and technical assistance

P. Balthasart, C. Bortuzzo, P. Catanzaro, A. Claessens, A. Dernier, G. Gaspard, R. Herman, F. Julemont, A.-M. Matton, N. Otthiers, I. Thamm, O. Verlaine, N. Yilmaz.

Achevé d'imprimer

Dépôt légal