Discussion: Session 2

DR. P. M. BENNETT. May I ask Dr. Nicholas if the penicillin-binding proteins have been mutated either naturally or in the laboratory to increase their ability to hydrolyze penicillins—as distinct from the data we showed that indicates reduced ability?

DR. R. NICHOLAS. I have not done it myself, but I have spoken to people who have, like Jeremy Knowles and others. He said he had also tried it by chemical mutagenesis and then looked for increased activity. Although the results have been negative, this does not mean that it will not work but that it has not worked yet. I think that it may take more than one or two mutations. It may possibly even require having a helix move or something a little bit more drastic than just a single amino acid change.

DR. N. A. C. CURTIS. Has anyone any idea what the enzymic activity of PBP (penicillin-binding protein) 7 might be?

DR. B. G. SPRATT. I can illuminate you negatively. I don't think anybody knows.

DR. G. A. JACOBY. May I show a slide? (Figure 1.) Classifiers can be divided into "lumpers" and "splitters." Having been a splitter, I would like to present a little data that lumps [1]. This has to do with the deduced amino acid sequence of several plasmid-mediated β-lactamases that have recently been determined. We sequenced PSE-2 in our laboratory and think we have part of PSE-1. OXA-2 is J. W. Dale's work [2], which has been in the literature for several years, and OXA-1 comes from M. Ouellette at Laval University. You can see that all of these β-lactamases have SXXK, where XX is identical. There is a good deal more amino acid homology among the top four β-lactamases than with TEM-1. That is, the PSE sequences are related to the OXA sequences. Thus, even though they hydrolyze somewhat different substrates, at an amino acid level they are related. Also, the immediate vicinity of the active site cannot be responsible for the substrate differences between the OXA and the PSE enzymes and TEM-1 because the same four amino acids are found there. Thus, other regions of the molecule must help to determine which particular substrates are best hydrolyzed. It does appear that these four enzymes at least, and probably other OXA- and carbenicillin-hydrolyzing enzymes, make up a family of enzymes (class D?) that are distinct from the other plasmid-determined enzymes examined so far.

DR. R. C. LEVESQUE. We have completed the sequence of OXA-5. It has the four basic STFK amino acids that you have shown, so there you have another one in the class D. One important point you made was that even though this region is conserved, apparently other regions would be involved, presumably for determination of substrate profile. Why for example does SHV-2 hydrolyze cefotaxime, and why does SHV-1 not? I guess one will have to look at the sequence and do what we call "protein engineering" to determine what other regions of the protein are important for substrate-profile hydrolysis. The plasmid-mediated enzymes would be a nice model on which to base this engineering.

DR. A. TOMASZ. May I ask a question of Dr. Ghuysen? I do not know anything about the way one derives the conclusions from the kind of work that you describe, but I nevertheless venture a question. When you do a forced alignment analysis, you said you use the PBP/DD-peptidase of *Streptomyces* R61 as a template and sacrifice up to 25% of the peptide. Do you not in this process of alignment also sacrifice the three-dimensional structure?

DR. J.-M. GHUYSEN. This is of course an important point. For pair-wise comparison of the primary structure of each of the penicillin-interactive proteins with that of the *Streptomyces* R61 DD-peptidase used as a template, the highly conserved tetrad Ser-X-X-Lys, which is located close to the amino terminus and possesses the active-site serine, and the highly conserved triad His-Thr-Gly, Lys-Thr-Gly, or Lys-Ser-Gly, which is located close to the carboxy terminus of the proteins, were used as calibration marks. These two groups were selected because they are known to occupy critical positions in the active site of the *Streptomyces* R61 DD-peptidase. From this starting point, adjustments were made such that the two calibration marks effectively aligned, the deletions or insertions that were needed to obtain an optimal match were restricted to stretches possessing residues known to favor loop or turn formation, and the α-helix and β-strand potentials, as predicted by the Robson-Garnier procedure, were not or were only slightly affected. These alignments highlighted, in addition to the selected calibration marks, several other conserved boxes consisting of strict identities or homologous residues. Subsequently, the three-dimensional structure of the β-lactamase of *Staphylococcus aureus* and that of the β-lactamase of *Streptomyces albus* G were elucidated independently

ß-Lactamase	Sequence
OXA-1	P D S T F K I A L S L M A F D A E I I - D Q K T I F K W D
OXA-2	P A S T F K I P H T L F A L D A G A V R D E F Q I F R W D
PSE-1	A A S T F K V L N T L I A L E E G A I S G E N Q I L
PSE-2	P A S T F K I P N A I I G L E T G V I K N E H Q G F K W D
TEM-1	M M S T F K V L L C G A V L S R V D A G Q E Q L V R R I H

Figure 1. Comparison of β-lactam sequences. Data are adapted from [1].

by Herzberg and Moult in Edmonton [3] and Dideberg in Liège [4], yielding a clear picture of the whole connectivity between the secondary structures. These structural data then allowed us to position the known secondary structures along the amino acid alignments and the conserved boxes in the known three-dimensional structures. This showed us that, indeed, the alignments had not introduced any gap in the secondary structures of the two β-lactamases and that the proposed deletions occurred in loops connecting particular α-helices.

DR. TOMASZ. From the central position of the *Streptomyces* R61 PBP in your comparisons, would you speculate that this would be the progenitor of β-lactamases? In other words, is it an additional example that antibiotic producers may be the origin of antibiotic resistance?

DR. GHUYSEN. My firm belief is that all the active-site serine, penicillin-interactive proteins are related in an evolutionary sense and form a superfamily of enzymes. This is another example of divergent evolution. Of course, depending on the evolutionary distance, these enzymes have acquired different amino acid sequences and distinct functionalities and specificities. Yet they would have conserved the same pattern of polypeptide scaffolding. Divergent evolution means that these enzymes have a common ancestor, and Dr. Tomasz asked me to speculate on that.

A few principles and observations can be taken into consideration. First, the DD-peptidases/PBPs are important or even essential bacterial enzymes, whereas the β-lactamases are dispensable, unless β-lactam antibiotics are present in the environment. Second, *Streptomyces* are soil bacteria and one may assume that they were among the first bacteria to be exposed to β-lactam molecules. Third, *Streptomyces* are the only known bacteria that spontaneously excrete some low-molecular-weight DD-peptidases/PBPs during growth. Fourth, the exocellular DD-peptidase/PBP of *Streptomyces* R61 occupies an important position in the family tree of the interactive-penicillin proteins, where it serves as a bridge between the β-lactamases of class A and the β-lactamases of class C (figure 2).

On this basis, one may propose a possible mechanism for the emergence of β-lactamases. The primary response of soil *Streptomyces* to exposure to β-lactam compounds produced by other microorganisms was to develop an excretion mechanism permitting release of a membrane-bound PBP in the environment and immobilization of the β-lactam molecules in the form of stable acyl enzymes. Further improvement of this detoxication mechanism was the conversion of this water-soluble β-lactam-binding enzyme into a β-lactam-hydrolyzing enzyme by remodeling of the active site. Experiments to reproduce in the laboratory this proposed evolutionary transition from the *Streptomyces* R61 DD-peptidase to β-lactamase are on their way.

DR. P. COURVALIN. According to your theory then, the ancestor protein is the carboxypeptidase, right? Is this because you feel you must find a physiologic role for these enzymes? We have heard that mutants of PBPs 5 and 6 replicate normally. Thus, these enzymes now appear to be nonessential, and their role today is a complete mystery.

DR. GHUYSEN. But Dr. Tomasz has almost convinced us that PBP7 is important. Is it possible to put antibiotic pressure on *Streptomyces* in the laboratory to see if it can be forced to produce a soluble form of its PBP? This would essentially be a speeding up of events that I think occurred slowly in nature.

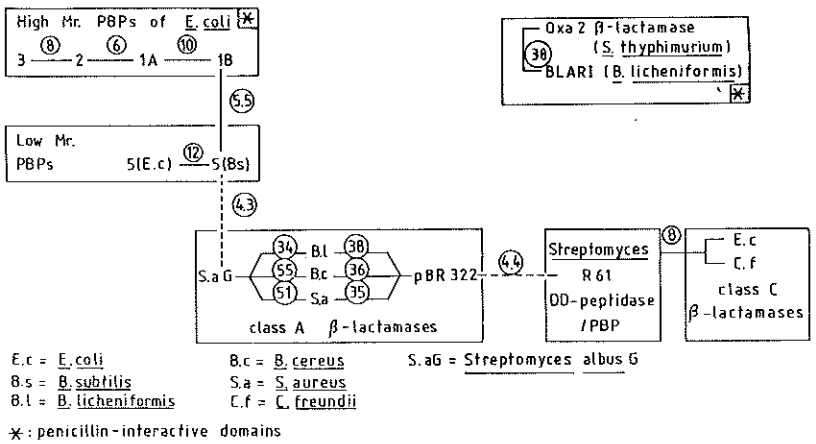

Figure 2. Family tree of penicillin-interactive proteins. Homology searches were made using the Goad-Kanehisa procedure. The significance of the comparison between pairs of sequences was assessed using the SEQDP program. A standard deviation unit (SDU) of 5 or higher indicates a statistically significant homology. The SDU values are circled. BLAR1 refers to the carboxy terminal 247 amino acid region of the penicillin receptor involved in β-lactamase induction in *Bacillus licheniformis*.

DR. TOMASZ. May I ask a question about the PBP 5 and 6 mutants? Were these deletion mutants? Did you look at the cell wall composition in these bacteria?

DR. J. BROOME-SMITH. We simply asked whether the double mutant strain grew normally and whether it showed any significant increase in sensitivity to a range of antibiotics. We have never looked at the cell wall composition. It may be very different. We are not claiming that those bacteria would survive in a person, for example, but they survive well in the laboratory.

DR. B. WIEDEMANN. I would like to come back to the evolution of the β-lactamase. Dr. Ghuysen, do you really believe that a β-lactam antibiotic was a necessary selective pressure for the evolution of β-lactamase? Don't you think, for example, that in Enterobacteriaceae, these β-lactamases existed for a very long time before the organisms were ever exposed to any antibiotic? Or do you believe that their ancestral soil bacteria evolved this β-lactamase, and this was then stable in all the other bacteria coming afterwards?

DR. GHUYSEN. Really, I cannot answer your question. You should ask God!

DR. S. MITSUHASHI. In Japan after the war, penicillin was available for study only in the laboratory. I was studying soil bacteria at that time and found a bacterium isolated from potato that quite easily destroyed penicillin. By this time Abraham and Chain had already published their findings of a penicillinase in *Escherichia coli*. However, I discovered soil bacteria that easily produced penicillinase, although penicillin had never been used in Japan to that time.

DR. GHUYSEN. Maybe I should add something. If you compare the *Streptomyces* R61 PBP sequence with the sequences of the class C β-lactamases, there is an obvious homology. To get homology between the *Streptomyces* R61 penicillin-binding protein and class A β-lactamases, you have to make some manipulations via the forced alignment that I showed you before. However, in comparisons of the DD-peptidase/PBP of *Streptomyces* R61 and the *Bacillus licheniformis* β-lactamase, there is no similarity between primary structures.

Despite this, all of the secondary structures present the same special disposition. So I think that all these observations are really striking and all agree at present with a common evolutionary origin. I was also very happy to hear you say, Dr. Mitsuhashi, that soil organisms might be the origin of what have now become the β-lactamases.

SIR MARK H. RICHMOND. On this business of whether penicillinase is a consequence of man's evolution of penicillin, I think categorically that the enzyme existed long before the drug. The experiments that Sneath did when he went to the British Museum and got soil samples from the plants that Banks brought back in the eighteenth century revealed bac-

teria that produced penicillinase as much and of the same type as now. So there is no doubt that the enzymes predate human activities in this area.

I think there is a part of the puzzle that one must not forget. I am struck by the widening range of bacteria that themselves are found to produce β-lactams. One can point to the work that has been done by the Squibb group, where many organisms now have been shown to produce immensely small amounts of β-lactams, primarily monobactams. Thus the hypothesis has to emerge that these monobactams are endogenous regulators of cell wall biosynthesis. You have to realize that you are not only looking at the synthesis of peptidoglycan, you are looking at the synthesis of peptidoglycan in an extremely complex, organized system where things have to be switched on and switched off very precisely in a very small structure. It seems to me that the missing part of the puzzle and one that must be examined concerns the question, Are β-lactams of one kind or another very, very widely distributed in bacteria as endogenous regulators?

One final comment I would like to make is that the fungi, which do produce β-lactams, do not have targets. Thus, maybe the source of the selective pressure that Dr. Ghuysen is looking for involves the fungi. Now, whether they got the ability to produce β-lactams endogenously or whether they got it from bacteria or *Streptomyces*, I don't know. But I think there is a whole area of endogenous regulators that must interact with all of these enzymes that we are considering at the moment. These regulators, almost by definition, are likely to be β-lactams or β-lactam-like molecules.

DR. GHUYSEN. However, there is no experimental evidence that these monobactams are regulatory molecules.

SIR MARK. I agree.

DR. T. J. FRANKLIN. I would like to follow up what Sir Mark has said. My colleagues David Allison and Robert Nolan have obtained evidence that a monobactam-like molecule is produced by *Pseudomonas aeruginosa*. Interestingly, this molecule seems to be confined to the cytoplasmic domain; it is not secreted by the organism. Our conclusion from that – and it has to be tentative at the present time – is that the monobactam may indeed have some regulatory role in the cell. Although it is not possible at this stage to conclude that it regulates cell wall biosynthesis, that would be an attractive proposition. Having heard Dr. Spratt's account, it is a little difficult to see how an intracellular β-lactam could gain access to the β-lactam receptor site in the PBPs, which project into the periplasmic space, unless there is some specific transport process that carries minute quantities of the β-lactam out into the periplasm.

SIR MARK. One could have the hypothesis – and I stress it is only a hypothesis – that the cell wall–synthesizing enzyme should be inactive at the point that it is made. It could then be activated by the removal of such a small molecule at the place in the cell where it has to work. So it could be that you have the cytoplasmic synthesis of the polypeptide, which picks up its inhibitor in the cytoplasm or in the cytoplasmic membrane. It then passes through the membrane, and the inhibitory molecule is removed as it comes into the periplasmic domain. Such an activation mechanism is possible.

DR. K. BUSH. I would like to make a comment about the monobactams. At this point the monobactams that we have discovered do not appear to be cell wall–synthesis regulators or any other kind of endogenous regulator. However, we do not know what kind of monobactams may have been produced at the time of occurrence of the evolution from cell wall–binding proteins or cell wall–synthesizing proteins into β-lactamases. So I think we have to go back several thousand years or before that to find out.

DR. FRANKLIN. Do you have evidence then that monobactams are not regulators of cell wall biosynthesis?

DR. BUSH. We have no evidence that says that they are.

SIR MARK. The real regulator is going to be present in very, very small amounts.

References

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