

Chapter 17

THE INDUCTION OF β -LACTAMASES IN EUBACTERIA

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ABSTRACT

The production of a β -lactamase can be induced by the presence of a β -lactam in the culture medium. Three distinct mechanisms have been discovered. In *Bacillus licheniformis* and *Staphylococcus aureus*, a penicillin receptor whose properties are similar to those of a PBP detects the presence of the antibiotic. In *Citrobacter freundii*, the damage caused by β -lactams to the peptidoglycan is detected by an intracellular protein and a similar system might prevail in *Streptomyces cacaoi*. Finally, in *Aeromonas sp* regulation involves a two-component system.

1. INTRODUCTION

In some Gram-positive or -negative bacteria, the presence of a β -lactam antibiotic in the external medium induces the biosynthesis of a β -lactamase that hydrolyses this antibiotic and allows bacterial survival [1]. In *Bacillus licheniformis* and *Staphylococcus aureus*, the mechanism of induction involves similar genes and results in the induction of a class A β -lactamase [2]. This mechanism is also related to that which leads to the induction of the resistant PBB2a in methicillin resistant *S. aureus* (MRSA) [3]. In *Citrobacter freundii*, the presence of penicillin induces the production of a class C β -lactamase. It is one of the two mechanisms observed in Gram-negative bacteria [4]. The second induction mechanism was described in *Aeromonas sp.* and it is not related to the *C. freundii* one [5-6]. On the contrary,

an induction mechanism which seems to be related to the *C. freundii* mechanism was also found in *Streptomyces cacaoi*. Finally, it is reported in literature that the two β -lactamases present in *Bacillus cereus* are inducible and that an extracytoplasmic function sigma factor and its anti-sigma factor control their expression [7].

The physico-chemical properties of the bacterial cytoplasmic membrane and those of the β -lactam antibiotics make the passage of these antibiotics through this membrane very difficult. As a consequence, in all induction phenomena listed above, the different microorganisms have developed systems that detect the antibiotic in the external medium and transmit into the cytoplasm a signal targeting the regulatory protein that controls the expression of the gene coding for the β -lactamase. The following paragraphs give a synthesis of our current knowledge about these induction mechanisms.

2. INDUCTION MECHANISM OF THE BLAP β -LACTAMASE IN *BACILLUS LICHENIFORMIS* AND OF THE BLAZ β -LACTAMASE AND PBP2A IN *STAPHYLOCOCCUS AUREUS*

2.1. *Bacillus Licheniformis*

At least three genes, *blaI*, *blaR1* and *blaR2*, are involved in the induction of the expression of the BlaP β -lactamase gene of *B. licheniformis*. The *blaP*, *blaI* and *blaR1* genes form a divergeon (*bla* divergeon) in which the latter two genes constitute an operon (Figure 1A). The *blaI* gene codes for a cytoplasmic protein of 128 amino acids that binds to the DNA, and the *blaR1* gene for a membrane protein of 601 amino acids, a sensor receptor able to detect penicillin. The *blaR2* gene, situated in another locus distant from the *bla* divergeon, remains to be isolated, but it has been postulated that the BlaR2 protein would play a role in the cytoplasmic signal between the BlaR1 sensor and the regulatory protein BlaI [1].

In the absence of inducing antibiotic, BlaI, as a dimer ($K_d = 25 \mu\text{M}$, ref 8), recognises and binds specifically to three inverted repeats (operator regions or BlaI operator) located in the intergenic region between the *blaP* gene and the *blaI-blaR1* operon. Two of these operator regions are localised in the *blaP* promoter (*PblaP*) and the third one is in the promoter of the operon (*PblaI-R1*) (Figure 1A). When bound to DNA, BlaI acts as a repressor and regulates the expression of *blaP* and of the *blaI-blaR1* operon. However, the expression of the β -lactamase gene is repressed more tightly than that of the operon. This is easily explainable by the presence of two operator regions in *PblaP* instead of one in *PblaI-R1*, and by a higher affinity of BlaI for the operator regions of *PblaP* [8]. So, in the absence of inducer, "leakage" from the *PblaI-R1* promoter repressed by BlaI is more important than leakage from *PblaP* and the amount of BlaI is sufficient to maintain the production of β -lactamase at a basal level, and allow the presence of the BlaR1 sensor in the membrane [8]. The tridimensional structure of the DNA-recognising N-terminal domain of BlaI (Bla-NTD, residues 1 to 82) was determined by RMN and showed that this repressor belongs to the winged helix family of repressors. The 2D NMR spectrum of N15-labeled BlaI shows that only the N-terminal domain (1-82) gives a well dispersed picture, whereas the C-terminal domain (83-128, BlaI-CTD) is poorly defined and appears in the same 2D spectrum zone as an unstructured polypeptidic chain [9]. The circular dichroism spectrum of the native protein under the same conditions or of BlaI-CTD

purified in the presence of detergent shows that in solution, this domain is well structured. The observation by NMR of a non structured C-terminal domain could be explained by a more dynamic structure of this part of the molecule. Study of the denaturation of BlaI by guanidium chloride (GdnHCl) confirms that BlaI is indeed composed of two domains which are denatured independently and that the C-terminal domain is denatured at a lower concentration of the denaturing agent ($[\text{GdnHCl}]_{1/2} = 1.75 \text{ M}$) than the N-terminal domain ($[\text{GdnHCl}]_{1/2} = 4 \text{ M}$). This property is in agreement with a more dynamic C-terminal domain, as suggested by NMR experiments [10]. Another peculiar feature of the dimerisation domain is its importance in the affinity of BlaI for its operator sequence. In the case of purified BlaI-NTD, the affinity for the DNA target is decreased 10,000 fold ($K_d = 200 \mu\text{M}$) and in the whole BlaI protein, denaturation of the C-terminal domain by GdnHCl leads to the loss of the high affinity for DNA. These results support the importance of the C-terminal domain for the inactivation of BlaI during the induction process [11].

By determining the organisation of BlaR1 in the membrane, it was shown that this protein is constituted of two domains, an N-terminal domain containing four transmembrane segments (BlaR-NTD, residues 1 to 350, the NH_2 terminal end of which is exposed at the outside of the cell) and a C-terminal domain (BlaR-CTD, residues 351-601) localised exclusively outside the cell (Figure 2). The amino acid sequence of BlaR-CTD is homologous to those of the class D β -lactamases. The four transmembrane segments of BlaR-NTD delimit three loops; L1 (9 aa), L2 (63 aa) and L3 (189 aa), L1 and L3 being intracellular, and L2 extracellular. In L3, an $\text{H}^{212}\text{-E-X-X-H}$ motif has been discovered which is the signature of the zinc proteases of the thermolysin family [12]. Directed mutagenesis and radioactive zinc binding experiments demonstrated that this motif binds a Zn^{2+} ion and that it is important for the mechanism of induction of the β -lactamase (K. Benlafya, S. Berzigotti and Joris unpublished data). When the part of the gene coding for BlaR-CTD (separated from BlaR-NTD by genetic engineering) is introduced in *E. coli*, and placed under the control of transcription and export signals proper to this host, the BlaR-CTD domain is produced under a soluble form in the periplasm of the host. Moreover, BlaR-CTD produced and purified from this source is able to recognise and form very stable covalent adducts with β -lactam antibiotics [13]. The topological description of BlaR1 corresponds to the general organisation of a receptor in which BlaR-CTD plays the role of a sensor, able to detect penicillin. The transmembrane segments and the L2 loop constitute the transducing module, the L3 loop acting as a transmitter that amplifies in the cytoplasm the signal given by BlaR-CTD, when acylated by penicillin. Interaction between BlaR-CTD, immobilised in an ELISA test well, and a phage displayed L2 loop was observed in the absence of penicillin. It was also shown that this interaction is weakened in the presence of the inducing antibiotic [14]. The tridimensional structure of BlaR-CTD from *B. licheniformis* has been determined and, as expected, it was found to be very similar to the 3D-structure of a class D β -lactamase (Figure 3, [15]).

Cloning of the *blaP-blaI-blaR1* divergeon on a *Bacillus sp* plasmid and its transfer into *Bacillus subtilis* 168 (a strain which produces no β -lactamase) have shown that in this recombinant strain, the induction of BlaP β -lactamase is possible. The existence of the *blaR2* gene being well established in *B. licheniformis*, it must be assumed that this gene is also present in *B. subtilis* 168, and that it exerts a cellular function in all *Bacillus* species. Given that the *B. licheniformis* strain is hardly transformable, all the studies of the induction mechanism of the BlaP β -lactamase were carried out mainly in *B. subtilis* [16].

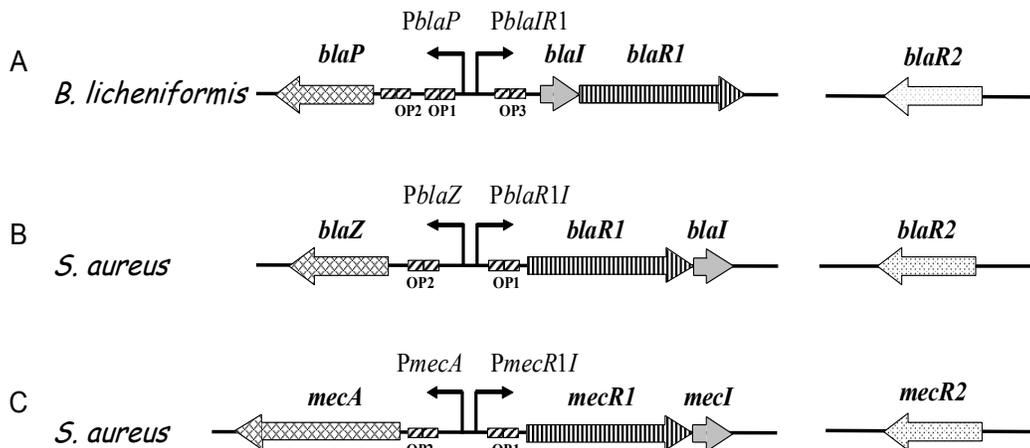


Figure 1. Organisation of the chromosomal genes involved in the induction of the BlaP β -lactamase of *B. licheniformis* (A) and the BlaZ beta-lactamase (*blaZ* gene)/PBP2a (*mecA* gene) in *S. aureus* (B and C).

A: in *B. licheniformis*, the expression of the gene coding for the BlaP β -lactamase is regulated by the products of at least three genes: *blaI*, *blaR1* and *blaR2*. *blaI* and *blaR1* form an operon. The BlaI protein is a cytoplasmic protein, that binds DNA and recognises specifically three operator regions (OP1, OP2 and OP3), situated in the intergenic region of the *blaP*-*blaI*-*blaR1* divergeon. The *blaR2* gene is not identified yet and its inactivation gives rise to a non inducible mutant. See text for more details.

B and C: on the *S. aureus* chromosome, *blaI/mecI* and *blaR1/mecR1* are organised in an operon and form a divergeon with the gene encoding the β -lactamase or the resistant PBP. In this strain the order of the genes in the operons differs from that in *B. licheniformis*. Two operator sequences (OP1 and OP2) recognised by BlaI/MecI are present in the intergenic region between *blaZ/mecA* and *blaR1/mecR1*.

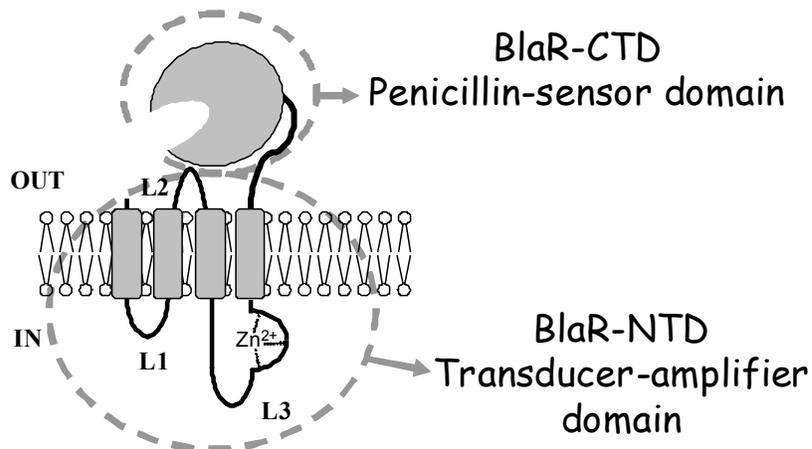


Figure 2. Membrane organisation of the *B. licheniformis* BlaR1 penicillin receptor.

BlaR-CTD acts as a penicillin sensor, whereas BlaR-NTD plays the role of a transducer-transmitter of the signal generated by the acylation of BlaR-CTD in the presence of the inducing antibiotic. SM1, SM2, SM3 and SM4: transmembrane segments. L1, L2 and L3: BlaR-NTD loops. See text for more details.



Figure 3. Surimposed ribbon diagrams of BlaR-CTD (magenta) and the class D beta-lactamases OXA-10 (green) and OXA-1 (red) [Kerff, 2003 #599].

Presently, the induction mechanism of the BlaP β -lactamase of *B. licheniformis* can be summarized as follows. When a β -lactam antibiotic is present in the external medium, it is detected by BlaR-CTD. The acylation of the active serine of BlaR-CTD results in a change of the interaction between BlaR-CTD and the L2 loop, which would lead to a reorganisation of the transmembrane segments and to a conformational change of the cytoplasmic L3 loop, activating the enzyme activity of this latter. The activation of L3 would be obtained by a cleavage of its polypeptidic chain, between residues R²⁹³ and R²⁹⁴ [20], as in the case of the induction of the BlaZ β -lactamase of *S. aureus* (see point 2.2, Figure 4). It is not clearly established yet if this cleavage is due to an autolysis or to the action of another cytoplasmic protease. This type of enzymic activation is found in several proteases, which are produced first as a proenzyme (for example thermolysin [17]). In the case of the induction of the BlaP β -lactamase in *B. licheniformis*, the activated L3 loop would cleave a pro-coactivator, still unknown, generating a coactivator of BlaI. When complexed by its coactivator, the latter, would be unable to bind DNA thus allowing the induction of BlaP. The coactivator of BlaI has not been identified yet, however, its presence has been indirectly demonstrated by following the fate of BlaI during the induction, in both *B. subtilis* and *B. licheniformis*. In the latter strain, BlaI was degraded during the induction, whereas in *B. subtilis*, BlaI is not proteolysed nor modified, but loses its DNA-binding capacity. These observations in *B. subtilis* are the basis of the assumption that a coactivator is involved in the inactivation of BlaI [18]. The proteolysis of BlaI in the cytoplasm of *B. licheniformis* would be the consequence of the formation of the BlaI-coactivator complex, followed by its cleavage by some cytoplasmic proteases.

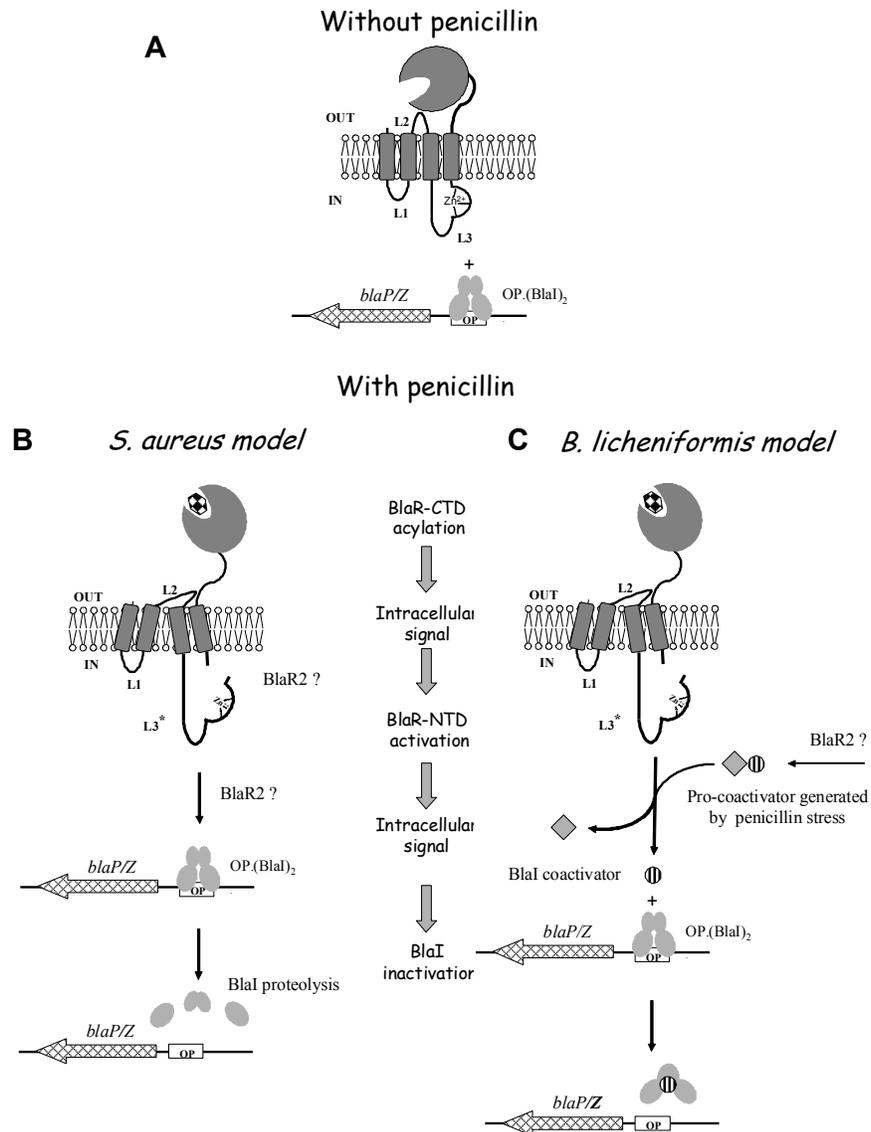


Figure 4. Induction mechanism of the BlaP and BlaZ β -lactamases in *B. licheniformis* and *S. aureus*, respectively.

In both microorganisms, products of the *blaI* and *blaR1* genes involved in the induction of the BlaP and BlaZ β -lactamases are similar and play the same role in the induction mechanism. BlaI is a DNA-binding protein acting as a repressor and BlaR1 is a membrane protein that plays the role of penicillin receptor.

A: in absence of penicillin outside the cell

B and C: the largest difference between the two induction models resides in the mode of inactivation of the BlaI repressor. In *B. licheniformis*, it is proposed that BlaI is inactivated by the linking of a coactivator, whereas in *S. aureus*, the proposal is that BlaI is inactivated by proteolysis.

In both organisms, the *blaR2* gene remains unidentified. It is proposed that the protein BlaR2 could play a role in the cleavage of the large cytoplasmic loop of the BlaR receptor when the latter is acylated by the inducer or could alternatively be involved in the production of the proactivator in *B. licheniformis* or in the proteolysis of BlaI in *S. aureus*.

A difficulty in the study of the induction process of a β -lactamase resides in the fact that the inducing antibiotic is also a substrate of the induced enzyme. That is why the inducer disappears more or less rapidly depending on the hydrolytic efficiency of the enzyme towards the inducing antibiotic. Consequently, in order to increase the half-life of the inducer, a poor substrate of the induced β -lactamase can be used as inducer in the study of the induction phenomenon. In the case of the BlaR-CTD receptor, the kinetic data obtained about the acylation of BlaR-CTD show that the 7-cephalosporanic acid (7-ACA), a cephalosporin deprived of C7 acyl side chain, is both a good acylating agent for BlaR-CTD and a very poor substrate for the β -lactamase. The analysis of the results obtained by using 7-ACA as a substrate, leads to the conclusion that two conditions are necessary to induce the β -lactamase: firstly, the BlaR receptor must be acylated by the inducer, and secondly, a cellular stress caused by the presence of this antibiotic inducer must be generated (2). At concentrations in 7-ACA allowing the acylation of BlaR (around 2 μ M) but having no effect on the growth of *B. licheniformis*, no induction of β -lactamase was observed. When the concentration of 7-ACA affects the bacterial growth ([7-ACA] = 18,5 μ M), the β -lactamase is induced. The kinetic constants of acylation of the PBP in *B. licheniformis* indicate that at a 7-ACA concentration allowing the induction of the β -lactamase PBP1, the lethal target of this bacterial strain, is more than 70 % inhibited which would be the cause of the cellular stress. Similar experiments carried out with cephalixin, an antibiotic specific of the PBP3 of *B. licheniformis*, lead to the same conclusion: induction only occurs when PBP1 is acylated. This is obtained at a concentration higher than that necessary to inhibit PBP3 [2]. The cellular stress generated by the presence of the inducing antibiotic would result in the production and the accumulation in the bacterial cytoplasm of a molecule which would be the pro-coactivator of BlaI and whose hydrolysis by BlaR1 would yield the coactivator necessary for interfering with the interaction between BlaI and its nucleic operator.

2.2. Staphylococcus Aureus

In *S. aureus*, the products of the *blaI* and *blaR1* genes involved in the induction of the *blaZ* β -lactamase gene, are homologous to those described in *B. licheniformis*, and possess similar properties [3, 19]. The largest genetic difference resides mainly in the organisation of the divergeon. In *S. aureus*, the order of the genes in the operon is inverted compared to that in the *B. licheniformis* operon. Moreover the *S. aureus* intergenic region between *blaZ* and the *blaR1-blaI* operon contains two operator sequences, one in each operator region, instead of three in *B. licheniformis* (Figure 1B). In *S. aureus*, the presence of the *blaR2* gene is postulated, but this gene remains to be identified. In the staphylococcal strains harbouring the resistant PBP2a, this protein can also be induced and the genes responsible for this induction are located in the *mec* divergeon, including *mecA*, *mecI* and *mecR1*, which code for the resistant PBP, the repressor and the penicillin receptor respectively (Figure 1 C). Concerning the induction mechanism in *S. aureus*, Zhang et al. suggested that the inactivation of the BlaI or MecI repressors during induction was due to the cleavage of the peptide bond linking the residues N¹⁰¹ and F¹⁰² (*S. aureus* BlaI numbering), directly or indirectly, by the L3 loop of the activated BlaR1 receptor. These authors have also shown that acylation of BlaR1 by the inducing antibiotic leads to the cleavage of the receptor, between residues R²⁹³ and R²⁹⁴ in the L3 loop (*S. aureus* BlaR1 numbering). This would activate its metalloproteolytic activity

(Figure 4, ref 20) . It should be noticed that the proteolysis of the BlaI repressor in *S. aureus* does not exclude the presence of a coactivator, as in *B. licheniformis*. Indeed, proteolysis of the repressor could also result from binding of the coactivator and its release in the cytoplasm, rendering it more susceptible to cytoplasmic proteases. This possibility cannot be excluded because in vitro proteolysis of BlaI by BlaR1 or by a protease activated by the action of the activated receptor has not been demonstrated yet. Moreover, in the tridimensional structures of BlaI and MecI of *S. aureus* the cleavage site is not accessible to the solvent [21]. However, as was shown by the 2D NMR spectrum and chemical denaturation experiments of *B. licheniformis* BlaI, the C-terminal domains of these repressors should be very mobile [9]. This result is in agreement with those obtained by 3D analysis of both *S. aureus* repressors in which two cavities can be observed [22-23]. These cavities display the poorly compact aspect of the C-terminal domains of both repressors. Do they allow the binding with a coactivator? To answer this question, identification of the coactivator suggested in *B. licheniformis* is needed. So far, four BlaR-CTD tertiary structures are available in the structure banks. The first to be published corresponds to that of the C-terminal domain of the non acylated *B. licheniformis* receptor [15]. The other three correspond to the apo-BlaR-CTD of *S. aureus* and to the same C-terminal domain acylated by penicillin G or by ceftazidime [24-25]. MecR-CTD has been also crystallized and its structures with or without penicillin G or oxacillin have been obtained [26]. As quoted above, the tertiary structures of BlaR/MecR-CTD are very close to those of class D serine β -lactamases (Figure 3). In the active sites of these β -lactamases, a lysine residue is carboxylated by the CO_2 dissolved in the buffer as in the OXA-10 β -lactamase for which this modification is essential for the catalytic activity [27]. For BlaR/MecR-CTD, carboxylation of the corresponding lysine is debated. Indeed, it could not be demonstrated, except in solution and it is absent in the structures obtained by X-ray diffraction. To explain this difference, Birk *et al* propose that in the apo-BlaR-CTD, Lys 426 (*S. aureus* BlaR1 numbering) is carboxylated, and that acylation of BlaR-CTD by the antibiotic leads to its decarboxylation. So, these authors explain why BlaR-CTD catalyses the opening of the β -lactam ring as do the class D β -lactamases and why the formed acylenzyme is stable. Indeed, in the class D β -lactamases, carbonation of the lysine is essential for the formation and hydrolysis of the acylenzyme [24].

Acylation by penicillin G does not induce important conformation changes in the tertiary structure of the *S. aureus* BlaR-MecR-CTD [24-26], in contrast to the proposal of Golemi *et al* based on circular dichroism studies [28]. These crystallographic results are in agreement with those obtained with *B. licheniformis* BlaR-CTD. In this case, no conformational change could be detected upon acylation of the sensor by penicillin G by circular dichroism or by isotopic hydrogen/deuterium exchange in the protein peptide bonds [14].

3. INDUCTION MECHANISM IN *CITROBACTER FREUNDII*

In *C. freundii*, expression of the *ampC* gene coding for a class C β -lactamase is both repressed and activated by the product of the *ampR* gene, that plays a repressor-activator role. Comparison of the primary structure of the AmpR protein with the sequences available in protein libraries shows that its N-terminal domain, which recognises DNA, belongs to the LysR regulator family. In the *C. freundii* chromosome, the *ampC* and *ampR* genes are

organised as a divergeon (Figure 5). AmpR recognises an operator sequence, located in the intergenic region between *ampC* and *ampR*, and controls expression of both *ampC* and its own gene. Generally, in Gram-negative bacteria possessing an inducible β -lactamase whose gene is controlled by AmpR, the induction mechanism is directly linked to the peptidoglycan (PG) biosynthesis, to its turnover and to a cellular stress induced by the inducing antibiotic. Indeed, AmpR recognises two groups of ligands. The first group consists of the UDP-MurNAc-pentapeptide, the most abundant PG cytoplasmic precursor, the second one contains the 3 anhydro-MurNAc-peptides (tri, tetra and pentapeptides) which originate from peptidoglycan turnover [29]. In the absence of inducer, AmpR which always interacts with DNA, binds the UDP-MurNAc-pentapeptide and the complex acts as a repressor. During growth, a large proportion of pre-existing PG is degraded yielding mainly periplasmic GNAc-anhydro-MurNAc-tetrapeptide which reenters the cytoplasm via the AmpG permease. There, it is degraded to GNAc, anhydro-MurNAc, D-alanine and the LAla—Dglu- γ →m-A₂pm tripeptide by the successive actions of the cytoplasmic NagZ, LdcA and AmpD enzymes (Figure 5). The tripeptide can subsequently be reintroduced into the PG precursor by Mpl. The concentrations of anhydro-MurNAc-tetra- and -tripeptides are never significant in the presence of an active AmpD. In *ampD*⁻ mutants, the anhydro-MurNAc- tripeptide accumulates, binds to AmpR and transforms it into an activator. The anhydro-MurNAc-tetra- and pentapeptides have similar effects on AmpR. It is not known if the UDP-MurNAc-pentapeptide is released from the new complex. Mutations responsible for the disappearance of AmpD activity are rather common and in consequence class C β -lactamase overproducers appear with a high frequency in the hospital setting.

In the presence of an inducing β -lactam, the inactivation of the PBPs disturbs the balance between PG biosynthesis and degradation. By an unknown mechanism, this also increases the activity of the autolytic system and in consequence results in the formation of large amounts of peptidoglycan turnover products. This modifies the quantity and the composition of the mixture of degradation products which reenter the cytoplasm via AmpG [30]. For instance, in the presence of benzylpenicillin, the GNAc-anhydro-MurNAc-pentapeptide becomes the major component [30]. The successive actions of NagZ and AmpG degrade this compound into GNAc, anhydro-MurNAc and pentapeptide and the latter is coupled to UDP-MurNAc by Mpl [31]. However, AmpD cannot cope with the increased amount of substrate and the anhydro-MurNAc pentapeptide accumulates, binds to AmpR and transforms it into an activator. Interestingly, the anhydro-MurNAc-tetrapeptide never seems to be present in significant quantities. In fact, its reintroduction into the biosynthesis pathway is lethal.

In addition to the induction phenomenon, this model nicely explains why *ampG*⁻ mutants are non-inducible while *ampD*⁻ mutants are constitutive overproducers.

4. INDUCTION MECHANISM IN *STREPTOMYCES CACAOI*

In the chromosome of *S. cacaoi*, two genes coding for two class A β -lactamases are present and induced by the same mechanism [1]. The *blaL* gene is located upstream of regulatory *blaA* and *blaB* genes with which it constitutes a divergeon (Figure 6). The *blaU* gene is situated in another chromosomal region. The *blaA* gene codes for a protein whose N-terminal DNA-binding module belongs to the LysR family, as the AmpR protein [32].

However, in the proteins of this family, the C-terminal modules are highly variable, being adapted to the structure of a regulator. Thus, the C-terminal domain of BlaA differs from that of AmpR. The BlaB protein is cytoplasmic and of unknown function. However, BlaB exhibits some similarities of sequence with some class A β -lactamases and forms stable acylenzymes with some β -lactam antibiotics and with N-acetyl-L-Lys-D-Ala-D-Ala-OH [33]. So far, the induction mechanism of BlaU and BlaL is not elucidated and the link between the antibiotic present outside the cell and the two regulatory cytoplasmic proteins remains to be identified.

5. INDUCTION MECHANISM IN *AEROMONAS HYDROPHILA*

In several *Aeromonas* species, the expression of one or more β -lactamases is inducible. In *Aeromonas hydrophila*, three β -lactamases AmpH (class D), CepH (class C) and ImiH (class B) are coded by unlinked genes and are regulated by the same couple of proteins (BlrA and BlrB) belonging to the two component system (TCS) family. These two proteins are coded by an operon localised upstream to the *ampH* gene. BlrB is a membrane protein possessing two transmembrane segments and a cytoplasmic segment containing the histidine kinase signature. BlrA is a cytoplasmic protein which would be phosphorylated by BlrB [5]. As in the three other induction mechanisms, the inducer in *Aeromonas* is also a β -lactam antibiotic. The mechanism by which the presence, of this antibiotic outside the cell is signalled to the cytoplasm remains unknown. BlrA and BlrB are homologous to the TCS CreB and CreC of *Escherichia coli* which is a general metabolic regulator, controlling the expression of many genes, in response to nutrient variations in the external medium. Avison *et al* have shown that the *CreBC* system of *E. coli* can regulate the expression of the *A. hydrophila* β -lactamases [34].

6. CONCLUSION

The induction of the AmpC β -lactamase in *C. freundii* is coupled to the peptidoglycan biosynthesis, to the peptidoglycan turnover and to the stress due to the action of the inducing antibiotic on the peptidoglycan biosynthesis. This stress leads to the accumulation in the cytoplasm of peptidoglycan fragments to be recycled and to the activation of the β -lactamase gene transcription. In *B. licheniformis*, we postulate that both a similar cellular stress and the acylation of the BlaR1 receptor are necessary for the induction of the BlaP β -lactamase. These conclusions differ from those proposed in the literature to describe the inactivation of BlaI/MecI repressor, during the induction of the BlaZ β -lactamase and of the resistant PBP2a in *S. aureus*. However, the similarity between the products of the regulatory genes present in both organisms is such that different mechanisms of repressor inactivation are unexpected. Nevertheless, the inactivation mechanism proposed for *B. licheniformis* could also describe the one which prevails in *S. aureus*, if the release of BlaI/MecI from the DNA and its proteolysis by cytoplasmic proteases in *S. aureus* is due to the binding of a coactivator. Identification of the coactivator of BlaI in *B. licheniformis* and the verification that the same coactivator does not exist in *S. aureus* should allow to decide if the two mechanisms are

different. In *S. aureus*, it cannot be excluded that expression of the *blaR2* gene depends on a cellular stress due to the inducing antibiotic.

In the induction mechanism of the *S. cacaoi* BlaU and BlaL β -lactamases, only the cytoplasmic proteins BlaA and BlaB are known, but it is reasonable to suggest that a cellular relay exists, signalling the presence of the antibiotic outside the cell. This cellular relay must be common to some *Streptomyces* species, since the BlaL β -lactamase is inducible in *Streptomyces lividans*, after the plasmid-borne *blaL-blaA-blaB* divergeon has been transformed into this organism [31]. As in *Bacillus*, it is quite possible that this cellular relay depends on a cellular stress due to the presence of the inducing antibiotic in the external medium. This hypothesis is reinforced by the fact that BlaA and AmpR belong to the same family of regulators (LysR family) and that those are generally regulated by a coactivator

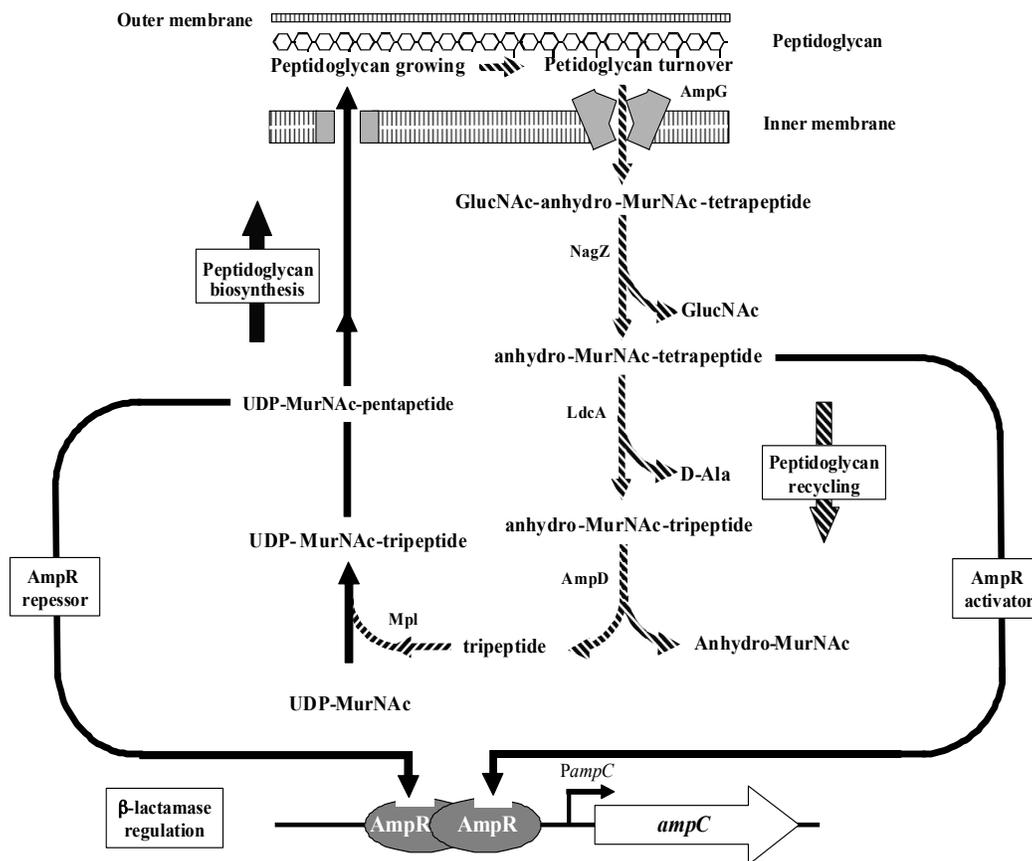


Figure 5. Control of β -lactamase biosynthesis in *C. freundii*: peptidoglycan synthesis (left) and recycling pathways (right).

In the absence of inducing β -lactam and with active AmpG and AmpD proteins, the concentrations of anhydro-MurNAc-tetra- and -tripeptides are very low. In *ampD* mutants, the latter accumulates and binds to AmpR transforming it into an activator. In the presence of β -lactams, the GNac-anhydro-MurNAc-pentapeptide is the main product which reenters the cytoplasm (not shown). It is hydrolysed by NagZ to yield the anhydro-MurNAc-pentapeptide which is a substrate of AmpD (for details, see text) but the activity of the latter is not sufficient to keep the concentration of anhydro-MurNAc-pentapeptide at a low level. In turn, this compound transforms AmpR into an activator. It is easy to understand why *ampG ampD* double mutants exhibit the phenotype of *ampG* mutants.

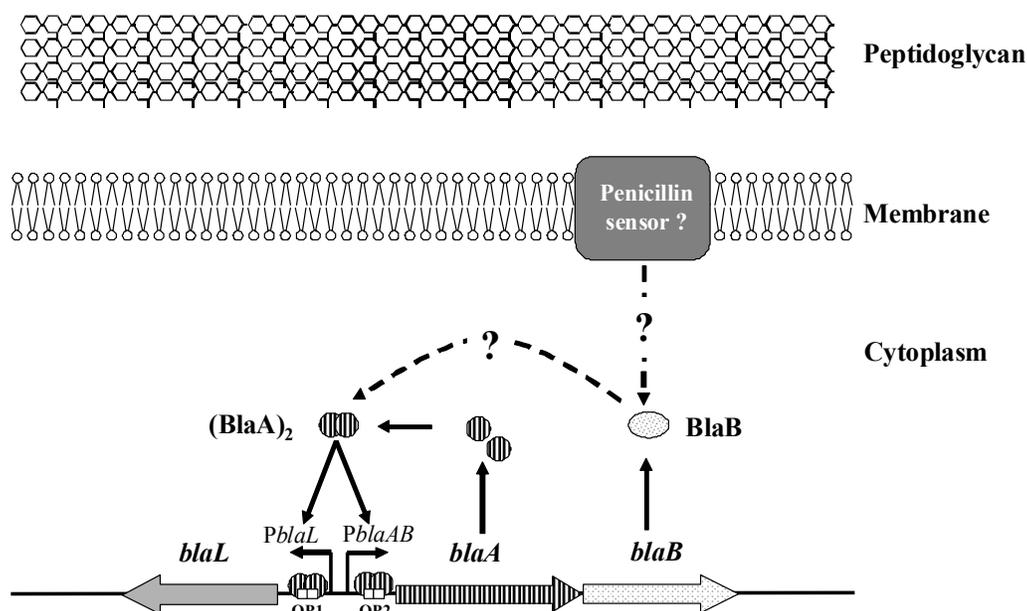


Figure 6. Induction mechanism of the BlaL β -lactamase in *S. cacaoi*.

The *blaL*, *blaA* and *blaB* genes code for the β -lactamase BlaL, the activator-repressor BlaA and the cytoplasmic protein BlaB (of unknown functions), respectively. The mechanism by which the presence of penicillin is detected outside the cell is unknown, as is the nature of the signal transmitted to BlaA. See text for more details.

In *A. hydrophilia*, the nature of the signal detected by BlrB when the β -lactam inducer is added in the medium remains unknown. However, it is possible that it is linked to the effect of the antibiotic on the growing cell. So, in addition to the BlrB activation, a cellular stress would be necessary for β -lactamase induction in *A. hydrophilia*.

On the basis of our knowledge on the four induction mechanisms of β -lactamases, it would seem that in each of them, a stress due to the inducing antibiotic is necessary. If this hypothesis is correct, it would be noteworthy that during the evolution, four different mechanisms have been selected to link this cellular stress to the induction of a resistance protein, allowing the survival of the bacterium in the presence of the antibiotic. It might be difficult to verify if this hypothesis but it represents an excellent starting point for the elucidation of the still unknown mechanisms.

REFERENCES

- [1] Philippon, A., Dusart, J., Joris, B., and Frere, J. M. (1998) The diversity, structure and regulation of beta-lactamases, *Cell Mol Life Sci* 54, 341-346.
- [2] Duval, V., Swinnen, M., Lepage, S., Brans, A., Granier, B., Franssen, C., Frere, J. M., and Joris, B. (2003) 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 beta-lactamase synthesis, *Mol Microbiol* 48, 1553-1564.

-
- [3] Mallorqui-Fernandez, G., Marrero, A., Garcia-Pique, S., Garcia-Castellanos, R., and Gomis-Ruth, F. X. (2004) Staphylococcal methicillin resistance: fine focus on folds and functions, *FEMS Microbiol Lett* 235, 1-8.
- [4] Jacobs, C., Frere, J. M., and Normark, S. (1997) Cytosolic intermediates for cell wall biosynthesis and degradation control inducible beta-lactam resistance in gram-negative bacteria, *Cell* 88, 823-832.
- [5] Avison, M. B., Niumsup, P., Nurmahomed, K., Walsh, T. R., and Bennett, P. M. (2004) Role of the 'cre/blr-tag' DNA sequence in regulation of gene expression by the *Aeromonas hydrophila* beta-lactamase regulator, BlrA, *J Antimicrob Chemother* 53, 197-202.
- [6] Segatore, B., Massidda, O., Satta, G., Setacci, D., and Amicosante, G. (1993) High specificity of cphA-encoded metallo-beta-lactamase from *Aeromonas hydrophila* AE036 for carbapenems and its contribution to beta-lactam resistance, *Antimicrob Agents Chemother* 37, 1324-1328.
- [7] Ross, C. L., Thomason, K. S., and Koehler, T. M. (2009) An extracytoplasmic function sigma factor controls beta-lactamase gene expression in *Bacillus anthracis* and other *Bacillus cereus* group species, *J Bacteriol* 191, 6683-6693.
- [8] Filee, P., Vreuls, C., Herman, R., Thamm, I., Aerts, T., De Deyn, P. P., Frere, J. M., and Joris, B. (2003) Dimerization and DNA binding properties of the *Bacillus licheniformis* 749/I BlaI repressor, *J Biol Chem* 278, 16482-16487.
- [9] Van Melckebeke, H. V., Vreuls, C., Gans, P., Filee, P., Llabres, G., Joris, B., and Simorre, J. P. (2003) Solution structural study of BlaI: implications for the repression of genes involved in beta-lactam antibiotic resistance, *J Mol Biol* 333, 711-720.
- [10] Vreuls, C., Filee, P., Van Melckebeke, H., Aerts, T., De Deyn, P., Llabres, G., Matagne, A., Simorre, J. P., Frere, J. M., and Joris, B. (2004) Guanidinium chloride denaturation of the dimeric *Bacillus licheniformis* BlaI repressor highlights an independent domain unfolding pathway, *Biochem J* 384, 179-190.
- [11] Boudet, J., Duval, V., Van Melckebeke, H., Blackledge, M., Amoroso, A., Joris, B., and Simorre, J. P. (2007) Conformational and thermodynamic changes of the repressor/DNA operator complex upon monomerization shed new light on regulation mechanisms of bacterial resistance against beta-lactam antibiotics, *Nucleic Acids Res* 35, 4384-4395.
- [12] Hardt, K., Joris, B., Lepage, S., Brasseur, R., Lampen, J. O., Frere, J. M., Fink, A. L., and Ghuysen, J. M. (1997) The penicillin sensory transducer, BlaR, involved in the inducibility of beta-lactamase synthesis in *Bacillus licheniformis* is embedded in the plasma membrane via a four-alpha-helix bundle, *Mol Microbiol* 23, 935-944.
- [13] Joris, B., Ledent, P., Kobayashi, T., Lampen, J. O., and Ghuysen, J. M. (1990) Expression in *Escherichia coli* of the carboxy terminal domain of the BLAR sensory-transducer protein of *Bacillus licheniformis* as a water-soluble Mr 26,000 penicillin-binding protein, *FEMS Microbiol Lett* 58, 107-113.
- [14] Hanique, S., Colombo, M. L., Goormaghtigh, E., Soumillon, P., Frere, J. M., and Joris, B. (2004) Evidence of an intramolecular interaction between the two domains of the BlaR1 penicillin receptor during the signal transduction, *J Biol Chem* 279, 14264-14272.

-
- [15] Kerff, F., Charlier, P., Colombo, M. L., Sauvage, E., Brans, A., Frere, J. M., Joris, B., and Fonze, E. (2003) Crystal structure of the sensor domain of the BlaR penicillin receptor from *Bacillus licheniformis*, *Biochemistry* 42, 12835-12843.
- [16] Kobayashi, T., Zhu, Y. F., Nicholls, N. J., and Lampen, J. O. (1987) A second regulatory gene, blaR1, encoding a potential penicillin-binding protein required for induction of beta-lactamase in *Bacillus licheniformis*, *J Bacteriol* 169, 3873-3878.
- [17] Yasukawa, K., Kusano, M., and Inouye, K. (2007) A new method for the extracellular production of recombinant thermolysin by co-expressing the mature sequence and pro-sequence in *Escherichia coli*, *Protein Eng Des Sel* 20, 375-383.
- [18] Filee, P., Benlafya, K., Delmarcelle, M., Moutzourelis, G., Frere, J. M., Brans, A., and Joris, B. (2002) The fate of the BlaI repressor during the induction of the *Bacillus licheniformis* BlaP beta-lactamase, *Mol Microbiol* 44, 685-694.
- [19] Fuda, C. C., Fisher, J. F., and Mobashery, S. (2005) Beta-lactam resistance in *Staphylococcus aureus*: the adaptive resistance of a plastic genome, *Cell Mol Life Sci* 62, 2617-2633.
- [20] Zhang, H. Z., Hackbarth, C. J., Chansky, K. M., and Chambers, H. F. (2001) A proteolytic transmembrane signaling pathway and resistance to beta-lactams in *staphylococci*, *Science* 291, 1962-1965.
- [21] Garcia-Castellanos, R., Marrero, A., Mallorqui-Fernandez, G., Potempa, J., Coll, M., and Gomis-Ruth, F. X. (2003) Three-dimensional structure of MecI. Molecular basis for transcriptional regulation of staphylococcal methicillin resistance, *J Biol Chem* 278, 39897-39905.
- [22] McKinney, T. K., Sharma, V. K., Craig, W. A., and Archer, G. L. (2001) Transcription of the gene mediating methicillin resistance in *Staphylococcus aureus* (mecA) is corepressed but not coinduced by cognate mecA and beta-lactamase regulators, *J Bacteriol* 183, 6862-6868.
- [23] Safo, M. K., Zhao, Q., Ko, T. P., Musayev, F. N., Robinson, H., Scarsdale, N., Wang, A. H., and Archer, G. L. (2005) Crystal structures of the BlaI repressor from *Staphylococcus aureus* and its complex with DNA: insights into transcriptional regulation of the bla and mec operons, *J Bacteriol* 187, 1833-1844.
- [24] Birck, C., Cha, J. Y., Cross, J., Schulze-Briese, C., Meroueh, S. O., Schlegel, H. B., Mobashery, S., and Samama, J. P. (2004) X-ray crystal structure of the acylated beta-lactam sensor domain of BlaR1 from *Staphylococcus aureus* and the mechanism of receptor activation for signal transduction, *J Am Chem Soc* 126, 13945-13947.
- [25] Wilke, M. S., Hills, T. L., Zhang, H. Z., Chambers, H. F., and Strynadka, N. C. (2004) Crystal structures of the Apo and penicillin-acylated forms of the BlaR1 beta-lactam sensor of *Staphylococcus aureus*, *J Biol Chem* 279, 47278-47287.
- [26] Marrero, A., Mallorqui-Fernandez, G., Guevara, T., Garcia-Castellanos, R., and Gomis-Ruth, F. X. (2006) Unbound and acylated structures of the MecR1 extracellular antibiotic-sensor domain provide insights into the signal-transduction system that triggers methicillin resistance, *J Mol Biol* 361, 506-521.
- [27] Li, J., Cross, J. B., Vreven, T., Meroueh, S. O., Mobashery, S., and Schlegel, H. B. (2005) Lysine carboxylation in proteins: OXA-10 beta-lactamase, *Proteins* 61, 246-257.
- [28] Golemi-Kotra, D., Cha, J. Y., Meroueh, S. O., Vakulenko, S. B., and Mobashery, S. (2003) Resistance to beta-lactam antibiotics and its mediation by the sensor domain of

- the transmembrane BlaR signaling pathway in *Staphylococcus aureus*, *J Biol Chem* 278, 18419-18425.
- [29] Park, J. T., and Uehara, T. (2008) How bacteria consume their own exoskeletons (turnover and recycling of cell wall peptidoglycan), *Microbiol Mol Biol Rev* 72, 211-227).
- [30] Wiedemann, B., Pfeifle, D., Wiegand, I., and Janas, E. (1998) beta-lactamase induction and cell wall recycling in gram-negative bacteria, *Drug Resist Updat* 1, 223-226.
- [31] Hervé M, Boniface A, Gobec S, Blanot D, Mengin-Lecreulx D.(2007) Biochemical characterization and physiological properties of *Escherichia coli* UDP-N-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase, *J Bacteriol* 189, 3987-95.
- [32] Lenzini, V. M., Magdalena, J., Fraipont, C., Joris, B., Matagne, A., and Dusart, J. (1992) Induction of a *Streptomyces cacaoi* beta-lactamase gene cloned in *S. lividans*, *Mol Gen Genet* 235, 41-48.
- [33] Raskin, C., Gerard, C., Donfut, S., Giannotta, E., Van Driessche, G., Van Beeumen, J., and Dusart, J. (2003) BlaB, a protein involved in the regulation of *Streptomyces cacaoi* beta-lactamases, is a penicillin-binding protein, *Cell Mol Life Sci* 60, 1460-1469.
- [34] Avison, M. B., Niumsup, P., Walsh, T. R., and Bennett, P. M. (2000) *Aeromonas hydrophila* AmpH and CepH beta-lactamases: derepressed expression in mutants of *Escherichia coli* lacking creB, *J Antimicrob Chemother* 46, 695-702.