

Summary

In *Bacillus licheniformis* 7491, BlaP β -lactamase is induced by the presence of β -lactam antibiotics outside the cell. The first step in the induction mechanism is the detection of the antibiotic by BlaR1, a membrane-bound penicillin receptor. This protein is composed of two functional domains: a carboxy-terminal domain exposed outside the cell, BlaR-CTD, which acts as a penicillin sensor and an amino-terminal domain anchored into the cytoplasmic membrane, BlaR-NTD, which works as a transducer-transmitter. The acylation of BlaR-CTD by the antibiotic generates an intramolecular signal that leads to the activation of the L3 cytoplasmic loop of the transmitter. The BlaI repressor, which represses β -lactamase production, is then inactivated by a protein relay including the activated L3 loop and the BlaR2 protein, yet to be identified.

The main objectives of our work were to contribute to the understanding of the BlaR1 L3 loop activation mechanism, the identification of the cytoplasmic signal launched by this activated loop and the highlighting of the blaR2 locus.

To highlight the residues that are important for the BlaR1 L3 loop activity, we have generated a multiple sequence alignment of *B. licheniformis* BlaR1 and of the BlaR1 and MecR proteins of *Staphylococcus aureus*. A consensus sequence containing strictly conserved residues was then deduced. The presence of HExxH conserved motif suggests that L3 loop could be a metalloprotease. Site-directed mutagenesis of selected conserved residues postulated to be involved in catalysis or zinc chelation, combined with zinc-blot, Western blot and β -lactamase induction analyses confirmed that the cytoplasmic BlaR1 L3 loop belongs to gluzincin metallopeptidase superfamily and that its cleavage during BlaR1 activation occurs by self-proteolysis. The cleavage site should be included in the K³⁰³R[↓]R conserved motif, in which « ↓ » indicates the cleaved peptide bond. The RR/AA cleavage site mutant also sheds new light on the BlaR1 intramolecular transduction mechanism. Indeed, in presence of β -lactam antibiotics, the acylation of the mutated BlaR1 receptor results in a slight induction of BlaP β -lactamase production (about 20% of the wild type) but without L3 cleavage. In fact, in this mutant, we have decoupled the L3 activation caused by BlaR-CTD acylation from the one due to L3 selfproteolysis. In this case, the uncleaved L3 loop exhibits a residual activity which seems unable to perform a successful autocleavage but is able to produce a sufficient amount of coactivator to partially inactivate BlaI. This finding is in agreement with a selfproteolysis mechanism in which it is necessary that BlaR-CTD acylation slightly activates L3 loop to allow its own proteolysis and its full activation.

During this work, the production of the membrane-bound protein BlaR1 has been achieved by producing the E²¹³A mutant (E²¹³ is the catalytic residue of the L3 loop) with a StrepTag at its C-terminal extremity. This tag allows a single step purification protocole on a StrepTactin column. A protocol for the reinsertion of the purified BlaR1 protein was also determined.

Production tests of the isolated L3 loop were made. Several protein partners were used to obtain a soluble protein. Best results were obtained with the NusA-L3 fusion protein. Purified NusA-L3 and *B. subtilis* membranes containing BlaR1 were used for studying the L3 loop activity. Any protease activity of the L3 loop could be detected in our experimental conditions.

In the frame of *blaR2* locus study, two different approaches were used. The first one consists of the prediction of genes that could be regulated by BlaI with the PREDEtector software. This prediction allowed to highlight the *BLO1303* gene which codes for a protein named Yoch. EMSA and RT-PRC experiments confirmed that the production of Yoch could be regulated by BlaI. This protein

was produced, purified and its activity was studied. We showed that it has an peptidoglycan autolytic activity.

The second approach consisted in the search for proteins involved with peptidoglycan degradation and able to generate the BlaI pro-coactivator. The BlaI coactivator is γ -D-Glu-*m*-DAP dipeptide, as postulated by Amoroso *et al.* (2012). This coactivator could be the result of the clivage of a pro-coactivator by the activated L3 loop. The *ykfABCD* operon encodes four proteins: YkfA, similar to the *E. coli* LdcA L,D-carboxypeptidase; Ykfb, a L-Ala-D/L-Glu isomerase; Ykfc, an endopeptidase and Ykfd, that contains characteristic motifs of ATPase part of ABC transporter. The effect of the inactivation of those genes on the β -lactamase induction was determined. The results showed that inactivation of *ykfA* strongly influences BlaP β -lactamase induction.

By sequence comparison, we were able to exclude the *BL01303* gene and the *ykfABCD* operon as blaR2 candidates. Despite the fact that this locus was not found, our study permitted to propose a completed induction model which includes new implicated proteins.