

The penicillin sensory transducer, BlaR, involved in the inducibility of β -lactamase synthesis in *Bacillus licheniformis* is embedded in the plasma membrane via a four- α -helix bundle

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Summary

Prediction studies, conformational analyses and membrane-topology mapping lead to the conclusion that the penicillin sensory transducer, BlaR, involved in the inducibility of β -lactamase synthesis in *Bacillus licheniformis*, is embedded in the plasma membrane bilayer via four transmembrane segments TM1-TM4 that form a four- α -helix bundle. The extracellular 262-amino-acid-residue polypeptide, S340-R601, that is fused at the carboxy end of TM4, possesses the amino acid sequence signature of a penicilloyl serine transferase. It probably functions as penicillin sensor. As an independent entity, this polypeptide behaves as a high-affinity penicillin-binding protein. As a component of the full-size BlaR, it adopts a different conformation presumably because of interactions with the extracellular 63-amino-acid-residue P53-S115 loop that connects TM2 and TM3. Reception of the penicillin-induced signal requires a precise conformation of the sensor but it does not involve penicilloylation of the serine residue S402 of motif STYK. Signal transmission through the plasma membrane by the four- α -helix bundle may proceed in a way comparable to that of the aspartate receptor, Tar. Signal emission in the cytosol by the intracellular 189-amino-acid-residue Y134-K322 loop that connects TM3 and TM4, may proceed via the activation of a putative metallopeptidase.

Introduction

Beta-lactam antibiotics inactivate a set of membrane-bound proteins, known as penicillin-binding proteins (PBPs), that are essential components of the bacterial cell-wall peptidoglycan-synthesizing machinery (Ghuysen, 1994; Ghuysen *et al.*, 1996). Resistance to these antibiotics is β -lactamase mediated and/or PBP mediated. Resistance may be constitutive or inducible and, in the latter case, to detect the presence of β -lactam antibiotics in their environment and switch on the transcription of resistance genes, bacteria have developed transduction pathways that have unique features.

Gram-negative bacteria recycle their wall peptidoglycan (Mengin-Lecreux *et al.*, 1996). The induction of β -lactamase synthesis is the result of the β -lactam-induced, peptidoglycan hydrolase-mediated deregulation of the peptidoglycan recycling process (Jacobs *et al.*, 1994; 1995; Park, 1996). Gram-positive bacteria do not recycle their wall peptides (De Boer *et al.*, 1981). The induction of β -lactamase synthesis in *Bacillus licheniformis* and *Staphylococcus aureus*, and the induction of the low-affinity PBP2' synthesis in *S. aureus* rely on the penicillin-sensory transducers BlaR and MecR, respectively (Joris *et al.*, 1994). BlaR and MecR are bipartite proteins. An amino-terminal module having multiple membrane-spanning segments and possessing the signature of a zinc-binding motif (presumably located on an intracellular loop) is fused, at its carboxy-terminal end, to a penicilloyl serine transferase module (presumably located at extracellular sites), which has the amino acid sequence signature of the class D β -lactamases.

The regulation of transcription of the β -lactamase-encoding *blaP* in *B. licheniformis* involves three chromosomal regulatory genes (Kobayashi *et al.*, 1987). *blal* encodes a repressor which, in the absence of β -lactam antibiotics, maintains β -lactamase production at a low basal level. *blaR1* encodes the 601-amino-acid-residue BlaR which, it is believed, upon reaction with penicillin allows derepression of β -lactamase synthesis to occur. *blaR2* is

unlinked to *blaP*, *blaI* and *blaR1*, and its product is of unknown function. Mutation in *blaR2* generates a low-level constitutive β -lactamase phenotype. While *blaP* is transcribed from its own promoter, *blaI* and *blaR1* are transcribed as a polycistronic mRNA from the *blaI* promoter in the opposite orientation to *blaP*, and the two promoters lie in the intergenic region between *blaP* and *blaI-blaR1*. The *blaI-blaR1* promoter possesses a single repressor-binding sequence; the *blaP* promoter possesses two dyad repeated binding sequences. As a consequence, transcription of *blaI-blaR1* is autoregulated and repression of *blaP* is tighter than that of *blaI-blaR1*.

When overproduced in *Escherichia coli* independently of the rest of the protein, the penicilloyl serine transferase module of the *B. licheniformis* BlaR folds by itself in the periplasmic space of the host and acquires a high-affinity penicillin-binding conformation (Joris *et al.*, 1990). This property suggests that, in the full-size BlaR, the penicilloyl serine transferase module functions as a penicillin sensor, and that the associated transmembrane module functions as a transducer, allowing the signal to be transmitted to the cytosol. Signal transmission depends on the number, directionality and assembly of the membrane-spanning segments of the transducer. In the studies reported here, prediction methods were used to identify the most-probable membrane topology of BlaR, and the predicted model was investigated experimentally.

Results

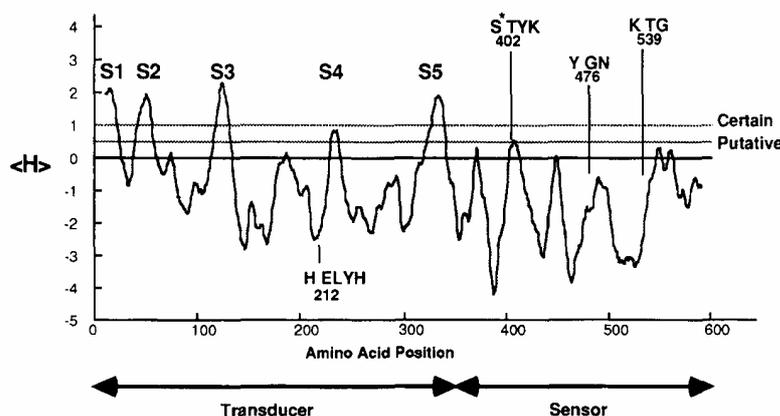
Prediction studies

Figure 1 identifies the H212ELYH zinc-binding motif (Joris *et al.*, 1994) and the S*402TYK (where S* denotes the active-site serine), Y476GN and K539TG penicilloyl serine transferase motifs along the amino acid sequence of BlaR. Because the active-site serine residues of β -lactamases and low-molecular-mass PBPs occur at approximately position 60 in the polypeptide chains, it is assumed that the transducer of BlaR extends from M1 to P339 and that the fused sensor extends from S340 to R601.

Figure 1 also shows the hydrophobicity profile of BlaR. Segments S1, S2, S3 and S5 of the transducer module fell above the upper cut-off value. They were regarded as definite transmembrane segments. Segment S4 fell below the upper cut-off value. As segment S4 is found downstream from the H212ELYH zinc-binding motif, it might be a secondary-structure element of a zinc peptidase borne by the BlaR transducer module.

The hydrophobic property of a helix can be represented on a plot, the vertical axis of which is the hydrophobic moment and the horizontal axis is the mean hydrophobicity (Eisenberg, 1984). In such a plot, transmembrane helices usually fall in a region of high hydrophobicity and small hydrophobic moment. Segments S2, S3 and S5 of the transducer module each fell within the transmembrane region (data not shown). Segment S1 overlapped the transmembrane region and the surface-seeking region. This feature is characteristic of peptide sequences with affinity for the interface between an aqueous and a hydrophobic phase. Segment S4 fell at the edge of the surface-seeking and globular regions. This feature is characteristic of helices that are in contact with the solvent.

Fig. 1. Zinc-binding and penicilloyl serine transferase motifs, hydropathy profile and putative transmembrane segments (S) of the penicillin sensory transducer, BlaR. $\langle H \rangle$ = hydrophobicity. The figure was constructed by moving a window of 20 amino acid residues along the sequence and using the TOPPED u software of Claros and von Heijne (1994).



Conformational analyses

The above prediction studies suggested that segments S2, S3, S5 and possibly S1 of the transducer module of BlaR are membrane-spanning helices. Conformational methods developed for biological membranes and peptides inserted into a lipid matrix (Brasseur, 1991) were used to investigate if and how these segments could be assembled into a multi-helix structure.

Energy minimization studies revealed that segments S2 (V36-A52) and S3 (A116-L133) are likely first to assemble as a stable two- α -helix structure (Fig. 2A) characterized by an antiparallel orientation of the peptides, a steric complementarity and a negative free energy of interaction (which is the sum of the Van derWaals, electrostatic and free-solvation energies of the interacting atoms). The axes of the two helices would be expected to form an 11° angle, with the shortest and longest distances between the two helices being 8.3 Å and 13.6 Å, respectively. The side-chains of Y40 and L47 of segment S2 would be expected to interact with the side-chains of M128 and W121 of segment S3, respectively.

Energy-minimization analysis also showed that segments S1 (F9-I26) and S5 (S323-P339) each would be expected to combine with the previously formed two-helix structure to generate a four- α -helix bundle (Fig. 2B) in which the bulky amino acid side-chains are at the interface between the α -helices, allowing maximal interaction. Figure 3 is a stereo view of the putative four- α -helix bundle.

Fig. 2. Modelling by energy minimization of the four- α -helix bundle formed by segments S1, S2, S3 and S5 of the BlaR transducer.

- A. Assembly of segments S2 and S3 as a stable two-helix structure.
B. Top view of the four- α -helix bundle.

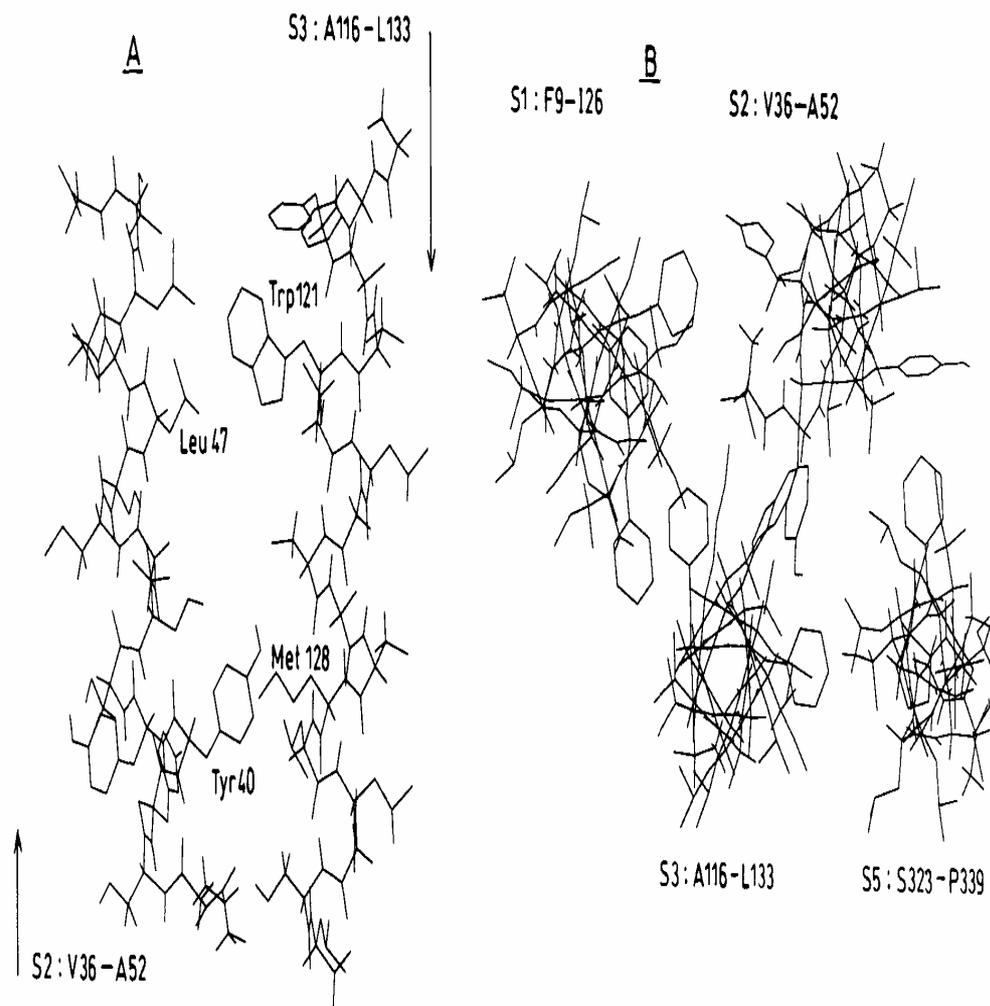
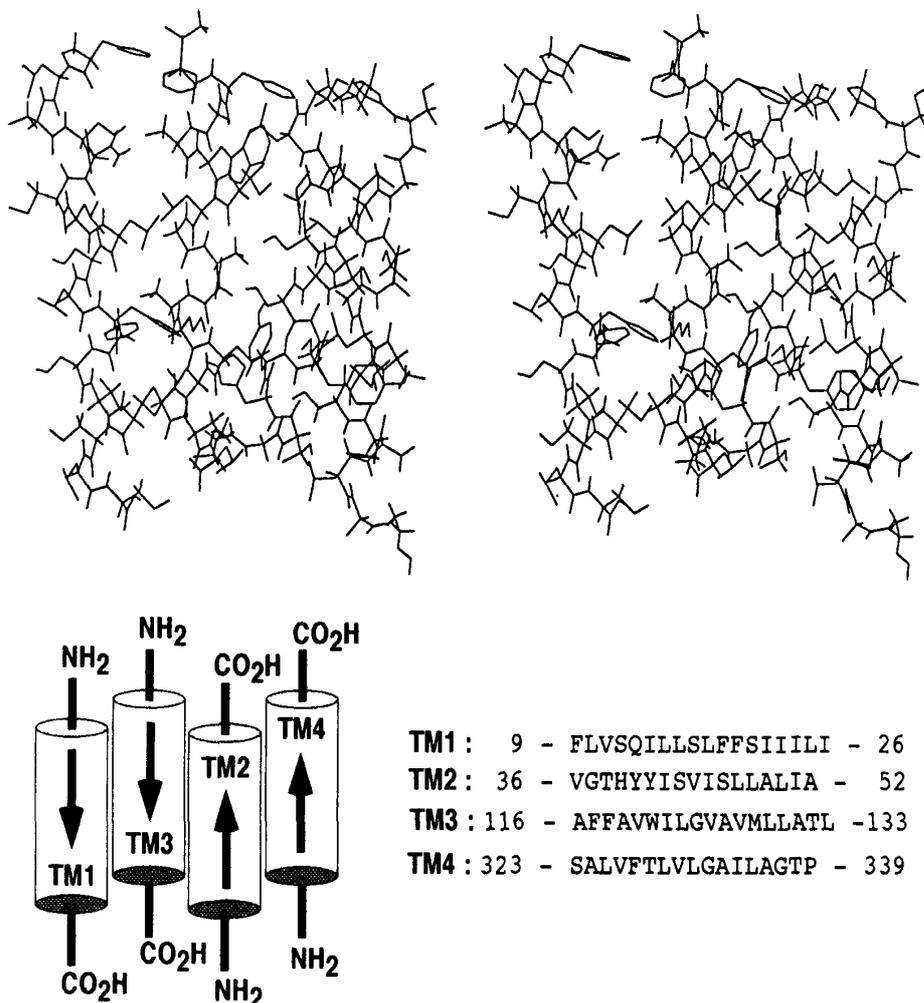


Fig. 3. Stereo view of the four- α -helix bundle of the BlaR transducer. The disposition and the polarity of the transmembrane segments are shown in the lower part of the figure. TM1, TM2, TM3 and TM4 are the hydrophobic segments S1, S2, S3 and S5 of Figs 1 and 2, respectively.



Predicted membrane topology of BlaR

Figure 4 shows the predicted membrane topology of BlaR as derived from the prediction studies and conformational analyses. The K27-T35 loop that connects TM1 and TM2, and the Y134-K322 loop that connects TM3 and TM4 and contains segment S4, are intracellular. The M1-P8 amino-terminal stretch, the P53-S115 loop, which connects TM2 and TM3, and the S340-R601 sensor are extracellular. This membrane topology obeys the positive-inside rule of von Heijne (1992), according to which positively charged amino acid residues of prokaryotic integral-membrane proteins (i.e. K + R + the amino-terminal amino acid residue) are more abundant in cytoplasmic loops than in extracellular loops. The sum 'K + R' for the two intracellular loops is 32. The sum 'M1 + K + R' for the extracellular amino terminal M1-T8 segment and the extracellular loop is 8.

Mapping experiments

The predicted membrane topology of BlaR was probed experimentally using the *E. coli* TEM- β -lactamase reporter method (Broome-Smith *et al.*, 1990).

pDML753 encodes the information for the synthesis of wild-type BlaR. *E. coli* JM105(pDML753) was grown in liquid medium, and BlaR production was induced with IPTG. Analysis of the cytoplasmic, membrane and periplasmic fractions by high-resolution SDS-PAGE showed that the *E. coli* transformants produced, bound to the membrane, one single protein (Fig. 5c) that reacted with the anti-BlaR M346-R601 sensor antibodies and had the same molecular mass as that of the full-size BlaR produced in penicillin-induced *B. licheniformis* (Fig. 5b).

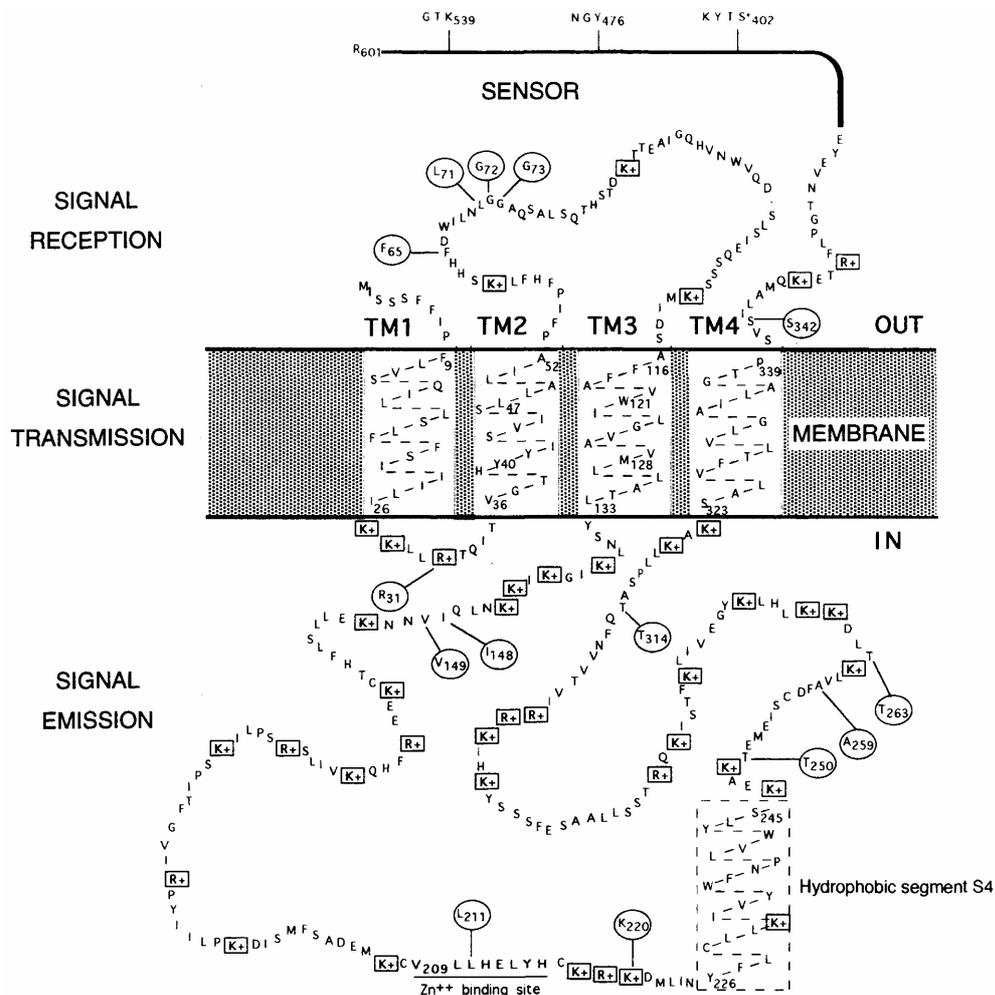
BlaR was the only protein that reacted with the anti-BlaR sensor antibodies in freshly prepared membranes of *E. coli* BlaR in *E. coli*, however, was susceptible to proteolytic degradation (see below).

pDML754(s) encode the information for the synthesis of BlaR-X-TEM hybrids in which BlaR sequences of varying lengths have been fused to the β -lactamase reporter enzyme. The fusion sites (X) are circled in Fig. 4. *E. coli* JM105(pDML754) transformants expressing β -lactamase activity were first identified by 'patch screening' of clones on agar containing $5 \mu\text{g ml}^{-1}$ ampicillin and $100 \mu\text{M}$ IPTG. Under these conditions, colony growth was independent of the location, in the cytosol or in the periplasm, of the β -lactamase moiety of the hybrid.

The β -lactamase-producing *E. coli* transformants fell into two groups. Those of the first group produced BlaR-X-TEM hybrids where X = R31, I148, V149, L211, K220, T250, A259, T263 or T314. All had minimum inhibitory concentration (MIC) values for ampicillin that were lower than $100 \mu\text{g ml}^{-1}$ (in IPTG-containing liquid medium), and they failed to grow as single colonies on agar containing IPTG and $5 \mu\text{g ml}^{-1}$ ampicillin, showing that the fused β -lactamase had no protective effect, consistent with the fusion sites (X) each being in the cytosol.

The transformants of the second group produced BlaR-X-TEM hybrids with X = F65, L71, G72, G73 and S342. Each had an MIC value of greater than $200 \mu\text{g ml}^{-1}$ ampicillin, and each grew as single-cell colonies on IPTG- and ampicillin-containing agar, showing that the fused β -lactamase exerted a protective effect, consistent with the fusion sites (X) each being in the periplasm.

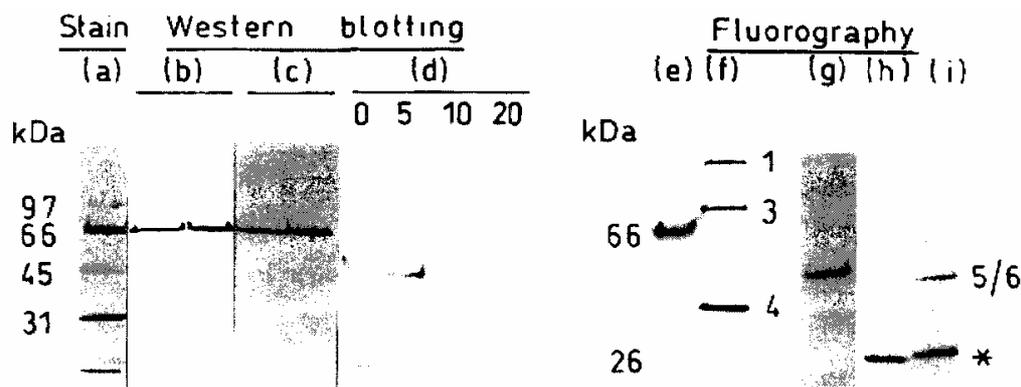
Fig. 4. Membrane topology of the penicillin sensory transducer, BlaR. The amino acid residues to which the TEM β -lactamase was fused are circled. Positively charged amino acid residues (K and R) are boxed. TM1, TM2, TM3 and TM4 are the hydrophobic segments S1, S2, S3 and S5 of Figs 1 and 2, respectively. The metallopeptidase motif (zinc-binding site) and the Y226-S245 hydrophobic segment S4 borne by the intracellular TM3-TM4-connecting loop are indicated.



Spontaneous proteolysis of the BlaR-X-TEM hybrids resulted in the release of the β -lactamase in an active and soluble form. Susceptibilities to proteolysis varied depending on the locations of the fusion sites. Where X = I148, T250 and T314 (in the intracellular TM3-TM4-connecting loop), the β -lactamase was almost exclusively associated with the membrane. Where X = R31 (in the intracellular TM1-TM2-connecting loop), there was more β -lactamase in the cytosol than in the membrane. Where X = G72 (in the extracellular TM2-TM3-connecting loop), there was as much β -lactamase in the periplasm as in the membrane, suggesting that cleavage near the fusion site occurred after translocation to the periplasm. Where X = S340 (at the extracellular end of TM4), there was much more β -lactamase in the periplasm than in the membrane. Proteolytic cleavage was also seen when *E. coli* SF100 (lacking the outer-membrane OmpT protease) and *E. coli* SF103 (lacking the periplasmic protease III) were used as hosts instead of *E. coli* JM105.

The results of these mapping experiments were in agreement with the membrane topology shown in Fig. 4, except that they did not establish whether segment S1 spans the membrane or is located in the cytosol. As shown previously (Zhu *et al.*, 1990), the truncated Δ T38-L353 BlaR (in which the M1-G37 sequence was fused to the P354-R601 sequence) was produced in *Bacillus subtilis* as a membrane-bound protein, suggesting that the truncated BlaR was anchored in the membrane via the membrane-spanning segment TM1.

Fig. 5. Analysis of the full-size BlaR and BlaR-degradation products by SDS-PAGE, Western blotting (b, c and d), and fluorography (f, g and i) of the gels. Western blotting was carried out using the anti-BlaR sensor antibodies. Fluorography was carried out on samples labelled with [14 C]-benzylpenicillin prior to SDS-PAGE. a. Molecular mass markers stained with Coomassie brilliant blue. b. Membranes from penicillin-induced *B. licheniformis* (in duplicate). c. Membranes from IPTG-induced *E. coli* (pDML753) (in duplicate). d. Trypsin-treated protoplasts of penicillin-induced *B. licheniformis*. The incubation times with trypsin (in min) are indicated above the lanes. e. Radioactive serum albumin. f. Membranes from penicillin-induced *B. licheniformis*. The β . *licheniformis* PBPs 1, 3 and 4 are identified. No 66k Da radioactive band (i.e. no radioactive full-size BlaR) is detectable. g. Freshly prepared membranes from IPTG-induced *E. coli* (pDML753). The *E. coli* PBPs 5/6 are identified. No 66 kDa radioactive band (i.e. no full-size BlaR) is detectable. h. Radioactive M346-R661 BlaR penicillin sensor. i. Membranes from IPTG-induced *E. coli* (pDML753) after prolonged storage. The asterisk indicates the BlaR-degradation product also detected by Western blotting.



Extracellular location of the penicilloyl serine transferase module of BlaR

Samples of penicillin-induced cells of *B. licheniformis* were converted into protoplasts and the protoplasts were treated with Sigma Trypsin type XI for increasing times at 37°C. The intact protoplasts and the trypsin-treated protoplasts were analysed by SDS-PAGE and peptides were identified by Western blotting of the gels with the anti-BlaR, M346-R601 sensor antibodies. As shown in Fig. 5d, the molecular mass of the BlaR present in the non-trypsin-treated protoplasts (50 kDa) was smaller than that of the full-size BlaR (66 kDa). However, the anti-BlaR sensor antibodies reacted with the 50 kDa BlaR form, and the 50 kDa BlaR could be totally degraded by

trypsin in intact protoplasts.

As shown previously (Zhu *et al.*, 1992), the amount of full-sized membrane-bound BlaR produced in penicillin-induced *B. licheniformis* cells is increased considerably at 1 and 2h after penicillin induction and then undergoes degradation. Hence, it appears that cell protoplasting somehow causes rapid proteolytic degradation of BlaR. Presumably, the cleavage occurs within the intracellular TM3-TM4-connecting loop, so that the penicillin sensor remains bound at the surface of the protoplasts and susceptible to trypsin degradation.

The penicilloyl serine transferase module of BlaR as penicillin sensor

Knowing that the M346-R601 sensor of BlaR is a high-affinity PBP when produced as an independent entity in the periplasm of *E. coli* (Joris *et al.*, 1990), one could presume that the full-size BlaR of *B. licheniformis* also behaves as a PBP. Experimental data seemed to confirm this conclusion (Zhu *et al.*, 1992). Others, however, have raised the possibility that the induction of β -lactamase synthesis in *B. licheniformis* does not require covalent binding of penicillin to the sensor. As shown by Takagi *et al.* (1993), the mutations S*402→T and K404→A (in motif 1 of the BlaR sensor), and the mutations G538→D and K539→R (in motif 3) were not tolerated, showing that the *in vivo* activity of BlaR requires a precise fold topology of the 'active site' of the sensor. However, the mutation S*402→A was tolerated, showing that the *in vivo* activity of BlaR does not require the presence of a nucleophile at position 402 and does not involve covalent penicilloylation of the sensor.

To resolve the issue, membranes of penicillin-induced cells of *B. licheniformis* were labelled with [¹⁴C]-benzylpenicillin and analysed by 12% high-resolution SDS-PAGE, Western blotting (using the anti-BlaR sensor antibodies), and fluorography. The 66 kDa full-size BlaR of *B. licheniformis* was detected by Western blotting (Fig. 5b), but could not be detected as a PBP (Fig. 5f). It is possible that the low-resolution SDS-PAGE used in previous studies (Zhu *et al.*, 1992) did not allow BlaR to be separated from the *B. licheniformis* PBP3. Similarly, the 66 kDa full-size BlaR of *E. coli* JM105(pDML753) was detected by Western blotting (Fig. 5c) but could not be detected as a PBP (Fig. 5g). However, upon storage of the membranes, BlaR-degradation products of molecular weights smaller than 30000 were identified by penicillin-binding (Fig. 5i) and Western blotting analyses (data not shown), indicating that the transducer module influences the mode of interaction between the sensor module and penicillin.

Discussion

Gram-negative bacteria (see the *Introduction*) and Gram-positive bacteria (this article) have developed different signal mechanisms to serve the same purpose, i.e. the derepression of β -lactamase-encoding genes, in response to the exposure to β -lactam antibiotics. The results of the studies reported here on the *B. licheniformis* penicillin sensory transducer, BlaR, lead to plausible conclusions and raise important questions.

Integral membrane proteins have two basic modalities: the anti-parallel β -barrel and the α -helix bundle. BlaR would appear to adopt the second modality. It is predicted that the protein is embedded in the plasma membrane via four transmembrane segments, TM1 to TM4, that form a four- α -helix bundle. Three polypeptide segments are external: the M1-P8 amino terminal octapeptide substitutes TM1; the 63 amino-acid-residue P53-S115 loop connects TM2 and TM3; and the 262-amino-acid-residue S340-R601 penicilloyl serine transferase sensor is fused to the carboxy-terminal end of TM4. Two polypeptide segments are cytosolic: the K27-T35 nonapeptide connects TM1 and TM2 and the 189-amino-acid-residue Y134-K322 loop connects TM3 and TM4. This latter loop possesses the signature of a zinc-binding motif located upstream from a segment (S4) of moderate hydrophobicity.

The mode of translocation and membrane incorporation of BlaR during synthesis remains unknown. However, one may note that: (i) TM1 has the signature of a reverse signal peptide (von Heijne and Manoil, 1990); (ii) the extracellular TM2-TM3-connecting loop has the minimum length and the distribution of positively charged amino acid residues (+++(TM2-TM3)++) compatible with a Sec-independent translocation (Andersson and von Heijne, 1993); and (iii) TM4 with the positively charged residues located immediately upstream from its amino end; represent the classical signature of a signal peptide (Gierash, 1989).

The question of what mechanism(s) allow BlaR to induce the derepression of β -lactamase synthesis in cells exposed to penicillin remains unanswered, but testable hypotheses can be put forward.

Signal reception by BlaR on the outer face of the plasma membrane requires a precise conformation of the penicilloyl serine transferase module but it does not involve penicilloylation of the essential serine residue,

S*402. When produced as an independent entity, this module adopts a folded structure that allows the γ OH of S*402 to perform nucleophilic attack on the bound penicillin molecule with formation of a stable ester-linked penicilloyl derivative (Joris *et al.*, 1990). In the full-size BlaR, the extracellular TM2-TM3-connecting loop borne by the transducer module probably modifies the conformation of the penicillin sensor, and signal reception is presumably the result of non-covalent interactions between the penicillin sensor, the extracellular loop and the β -lactam compound inducer.

The sensor module of BlaR shares 32% identity with the Oxa-2 β -lactamase of class D, indicating a common polypeptide scaffolding. The sensor module, however, lacks β -lactamase activity (Zhu *et al.*, 1992) although, as stated, it does display a masked penicillin-binding activity. BlaR illustrates the principle according to which modification to an existing protein may result in the creation of a new function. A class D β -lactamase acquires a new property, penicillin binding, via local structural changes; fusion of this PBP to another polypeptide then results in a hybrid that performs another function, i.e. gene regulation.

Signal transmission through the plasma membrane presumably proceeds via the four- α -helix bundle. It is known that the insertion of the tetrapeptide PGGT between T38 and H39 in TM2, and the change of G124 to D in TM3, each give rise to *B. licheniformis* BlaR mutants that are not functional *in vivo* (Zhu *et al.*, 1992), consistent with the above-stated view. The mechanism of signal transmission may be similar to that of the aspartate receptor, Tar, of *Salmonella typhimurium* (Lynch and Koshland, 1991; Milburn *et al.*, 1991). Tar is a homodimer with two 60 kDa subunits forming a four- α -helix bundle, with two additional short helices located at the exterior of the membrane. Upon binding aspartate, the binding-pocket residues are brought closer to each other, the two subunits undergo a 4° rotation, and the four transmembrane helices realign, somehow allowing the information to be transmitted to the interior of the cell.

BlaR signal emission in the cytosol is probably mediated by the intracellular TM3-TM4-connecting loop. Dominant forms of signal emission in bacteria in response to environmental changes generally proceed via phosphoryl transfers or are associated with sites of methylation and demethylation (Surette and Stock, 1994). In particular, realignment of the four transmembrane helices of Tar occurs concomitantly with methylation of four glutamic acid residues in the cytosol. The intracellular Y134-K322 loop of BlaR has no recognizable site of methylation/demethylation and has no recognizable site that could be associated with a histidine kinase (and to which generation of an intracellular signal via phosphorylation-dephosphorylation could be attributed). The loop does, however, possess the H212ELYH consensus sequence of a neutral zinc metallo-peptidase (Vallee and Auld, 1992). The isology is probably not coincidental because this amino acid sequence signature is conserved in the *S. aureus* proteins BlaR and MecR (Joris *et al.*, 1994). One may hypothesize that this putative peptidase is inactive when BlaR is in the resting state, and that it adopts an active conformation in response to the signal transmitted via the four- α -helix bundle. Following this hypothesis, BlaR would be another example of control of gene activity via proteolytic degradation of key regulatory proteins, in this case, presumably, the Blal repressor. Degradation of the transcription factor σ^{32} in *E. coli* is mediated by the membrane-bound zinc protease FtsH, the intracellular domain of which possesses an ATPase motif of the AAA-protein family and a HExxH zinc-binding motif (Tomoyasu *et al.*, 1995).

Experimental procedures

Conformational analysis of transmembrane peptides

The multi-step procedure of Brasseur (1991) was used for the conformational analysis of transmembrane peptides. The conformational energy of each of the peptides under consideration was calculated as the sum of the London-Van der Waals interaction energies between all pairs of non-bonded atoms, the Coulomb's electrostatic energies between atomic charges, the potential energies of rotation of torsional angles, and the free-solvation transfer energy of each atom. The lowest energy conformer obtained by Simplex minimization, with resolution of less than 5° for each torsional angle, defined the most-stable α -helix. Starting with single helical peptides, each in their most stable conformation, the pair of peptides that formed the most-stable two-helix structure was identified by translation and rotation, and this two- α -helix structure was then used to build, step-wise, the most-stable multi-helix structure. The interactions between all the atoms were optimized by energy minimization of the first stable pair of helices at the lipid-water interface, followed by a third helix, a fourth helix, and so on. The interaction energy was the sum of the Van der Waals, electrostatic and free-solvation energies of interaction between atoms. The calculations were performed on an Olivetti cp486 microcomputer equipped with an Intel 80486 arithmetic co-processor, using the PC-PROT+ (Proteins Analysis) and PC-TAMMO+ (Theoretical Analysis of Molecular Membrane Organization) programs (Centre de Biophysique Moléculaire Numérique, Faculté des Sciences Agronomiques, Gembloux, Belgium). Graphs were drawn using the pc-MGM (Molecular Graphic

Manipulation) program.

Plasmids, oligonucleotides and E. coli transformants

E. coli JM105 (Sambrook *et al.*, 1989), SF100 and SF103 (Baneyx and Georgiou, 1990; 1991) were used as hosts. pRWT8 carried *blaR1* (Kobayashi *et al.*, 1987). pJBS633 (carrying the mature TEM β -lactamase-encoding *blaM*) and pYZ4 (Broome-Smith *et al.*, 1990) were provided by B. G. Spratt. The oligonucleotides 01-07 (Fig. 6) used to construct the plasmids described below were from Eurogentec.

Fig. 6. Oligonucleotides used in this work. The sequence in bold corresponds to *blaR1*. The asterisk indicates the mutation T→G introduced to create the *HaeI* site.

- 01 5' AACTGCAGT**CATGAGCAGTTCTTTCTTTATTC** 3' (sense)
Met1
PstI BspHI
- 02 5' **CGATTGAGCTCCTCCTAAATTC** 3' (antisense)
SacI
- 03 5' CAGGACTGTGCTGGTCATTAA 3' (sense)
- 04 5' **GTGATTTGAGGCCTGAGCAGT** 3' (antisense)
*Arg31
HaeI
- 05 5' CTGCGTCACCCAGAAAC 3' (sense)
- 06 5' **AAGCAAAACACATTTTCATTTTC** 3' (antisense)
Leu211
- 07 5' **TTTTTCGTTTGCAATGGT** 3' (antisense)
Lys220

pDML753 (harbouring *blaR1*). *lacZ'* in pYZ4 was substituted by *blaR1* in such a way that *blaR1* was under the control of the *lacUV5* promoter. For this purpose, a *BspHI* site was created in the start codon of *blaR1* of pRWT8 by the polymerase chain reaction (PCR) using oligonucleotides 01 and 02 (Fig. 6). The reaction product (i.e. the first 217 bp of *blaR1* with an internal *SstI* site) was cloned between *NcoI* and *SstI* of pYZ4, giving rise to pDML751. An *SstI*, *HindIII*, *Clal*, *PstI*, *BamHI* and *EcoRI* polylinker was generated by annealing two synthetic oligonucleotides, and this was inserted between *SstI* and *EcoRI* of pDML751, giving rise to pDML752. The *blaR1*-containing *SstI*-*HindIII* fragment of pRWT8 was cloned into the corresponding sites of pDML752, yielding pDML753.

pDML754(s) (harbouring truncated *blaR1*-*X*-*blaM* fused genes, where *X* denotes the amino acid residue of *BlaR* involved in the fusion with the TEM β -lactamase). *blaR1* in pDML753 was randomly shortened by the combined action of exonuclease III and S1 nuclease. Nuclease-sensitive ends were created with restriction enzymes that cut *blaR1* once and generated overhanging 5' ends. The rate of degradation (as determined by analytical digestion of 0.1 μ g of linearized pDML753) was 10 bp min⁻¹ at 30°C. The reaction was carried out on 0.5 μ g of pDML753. The samples were then restricted with *Mlu*I and the resulting DNA fragments were ligated into pJBS633 that had

been digested with *PvuII* and *MluI*.

To obtain *blaRI-X-blaM* where X = R31, a *HaeI* site was created in the R31 codon of *blaRI* (Fig. 4), and a 565 bp segment covering the *lacUV5* promoter region and the first 104 bp of *blaRI* was amplified using pDML753 as template and oligonucleotides 03 and 04 (Fig. 6) as primers. The PCR product was digested with *HaeI* and *MluI* and the fragment was cloned between *MblI* and *PvuII* of pJBS633.

The *blaRI-X-blaM* fusions where X = L211 and X = K220 (Fig. 4) were obtained using pDML753 as template and oligonucleotides 05 and 06, and oligonucleotides 05 and 07 (Fig. 6) as primers, respectively.

All the polymerase chain reactions were carried out in a Trio-thermoblock thermocycler (denaturation, 1 min at 94°C; annealing, 2 min at $T = T_m$ of the oligonucleotides -2°C; elongation, 1 min at 72°C; number of cycles, 30).

E. coli cells were transformed with the appropriate plasmids, kanamycin-resistant transformants were selected, and clones containing in-frame-fused *blaRI-X-blaM* genes were selected using the method of Broome-Smith *et al.* (1990). The nucleotide sequences across the junction of the fused *blaRI-X-blaM* genes were checked by dideoxy sequencing (T7 Sequencing kit; Pharmacia) using the plasmids as templates and a polynucleotide complementary to codons 19-24 of the mature β -lactamase-encoding *blaM* as primer. The lengths of the PCR products were sequenced completely.

Growth, cell fractionation and β -lactamase activity of E. coli transformants

E. coli transformants were grown at 37°C in Luria-Bertani (LB) medium containing 25 $\mu\text{g ml}^{-1}$ kanamycin. When the OD₆₀₀ was 0.7, IPTG was added to a final concentration of 1 mM and incubation was continued for a further 2 h. The periplasmic, cytoplasmic and membrane fractions of the cells were obtained by lysozyme treatment, as described by Lindstrom *et al.* (1970). Beta-lactamase activity was determined using nitrocefin as the substrate (O'Callaghan *et al.*, 1972).

Growth of B. licheniformis 749 and penicillin-induced β -lactamase synthesis. Preparation and trypsin treatment of protoplasts

Overnight cultures of *B. licheniformis* in LB medium were diluted 10 times in LB medium containing 100 nM benzylpenicillin and the cells were allowed to grow at 37°C until the cultures reached an OD₆₀₀ of 0.6 to 1.0. Protoplasts were prepared as described by Quax (1990), except that the washed protoplasts were resuspended in 10 ml, instead of 2 ml, of sucrose buffer (0.5 M sucrose, 20 mM sodium maleate pH6.5, 20 mM MgCl₂). Samples (500 μl) were stored at -70°C. For trypsin treatment, the protoplasts were centrifuged (2000 $\times g$ for 5 min at 20°C) and resuspended in 200 μl of sucrose buffer. Samples (20 μl) were incubated at 37°C with 1 μg Sigma Trypsin type XI for time intervals of 5-60 min. The samples were supplemented with 5 μl of 5x denaturing buffer (0.3 M Tris-HCl pH 6.8; 50% glycerol (v/v), 5% SDS, 2.5% β -mercaptoethanol, and 0.01% bromophenol blue), heated at 95°C for 10 min, then analysed by SDS-PAGE and Western blotting of the gels.

Antibodies, Western blotting, and [¹⁴C]-benzylpenicillin binding

Antibodies used against the BlaR sensor were those described by Zhu *et al.* (1992). Rabbit anti-TEM β -lactamase antisera were collected and purified by (NH₄)₂SO₄ precipitation and anti-*E. coli* antibodies were removed by affinity chromatography, as described by Sambrook *et al.* (1989). Western blotting using the anti-BlaR sensor and anti- β -lactamase antibodies was performed according to the Bio-Rad protocol (Bio-Rad Laboratories), using the goat anti-rabbit IgG-alkaline phosphatase conjugate-detection system.

Samples previously labelled with [¹⁴C]-benzylpenicillin (54 mCi mmole⁻¹; Amersham) were subjected to SDS-PAGE and the gels analysed by fluorography.

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