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Expression in *Escherichia coli* of the carboxy terminal domain of the BLAR sensory-transducer protein of *Bacillus licheniformis* as a water-soluble M_r 26 000 penicillin-binding protein

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1. SUMMARY

A cloning vector has been constructed which allows production and export by *Escherichia coli* of the Met346-Arg601 carboxy terminal domain of the 601 amino acid BLAR sensory-transducer involved in β-lactamase inducibility in *Bacillus licheniformis*. The polypeptide, referred to as BLAR-CTD, accumulates in the periplasm of *E. coli* in the form of a water-soluble, M_r 26 000 penicillin-binding protein. These data and homology searches suggest that BLAR has a membrane topology similar to that of other sensory-transducers involved in chemotaxis.

2. INTRODUCTION

As previously reported [1,2], BLAR appears to be a two-domain protein with an amino terminal domain that consists of several transmembrane segments and a carboxy terminal domain that protrudes on the outer face of the membrane and serves as penicillin receptor. On the basis of this presumed membrane topology and given that the penicillin receptor per se, i.e. BLAR-CTD, has high similarity, in the primary structure, with the class D Oxa-2 β-lactamase of *Salmonella typhimurium* [2], a cloning vector was constructed in which that portion of the *bla*R gene encoding BLAR-CTD was placed under the control of the promoter, ribosome-binding site and signal peptide-encoding sequence of the Oxa-2 β-lactamase. The expectation was that *Escherichia coli* harbouring the resulting combinant plasmid pDML307 would export BLAR-CTD in the form of a novel, water-soluble penicillin-binding protein.

3. MATERIALS AND METHODS

Plasmid pRTW8 [1] was the source of the *bla*R gene, plasmid R46 [3] was the source of the gene encoding the Oxa-2 β-lactamase and plasmid

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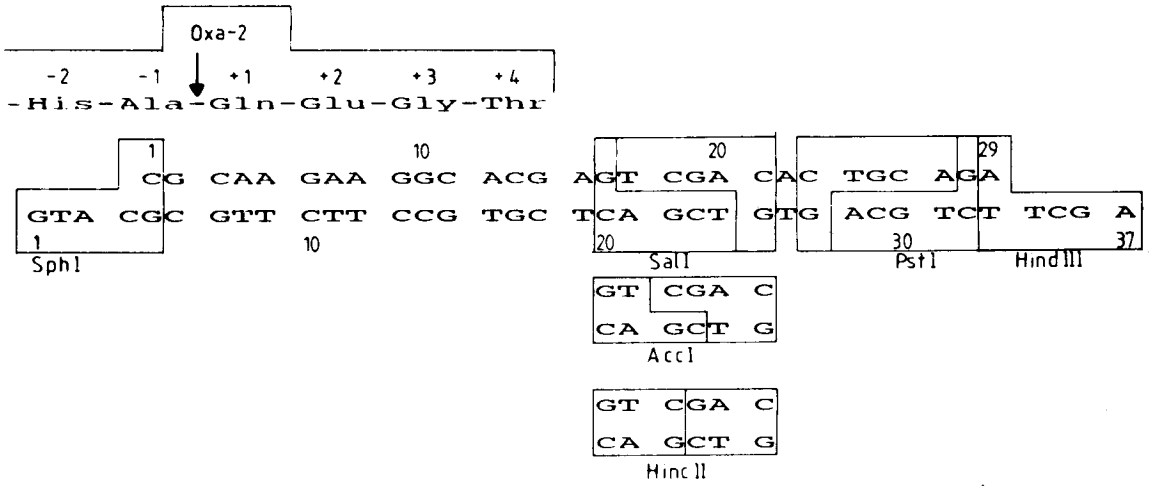

Fig. 1. Nucleotide sequence of the polylinker. The nucleotides 1 to 14 of the 29-mer were designed to reconstitute the −1 to +4 amino acid sequence of the Oxa-2 β-lactamase. The arrow indicates the site of cleavage of the β-lactamase precursor by the leader peptidase. The 29-mer–37-mer hybrid provides cohesive ends specific of *Sph*I and *Hin*dIII.

pBGS18$^+$ [4] was used as vector. pBGS18$^+$ contains the multiple cloning region of M13mp18 inserted in the 5′ end of the *lac*Z′ gene and has the *Km*R gene conferring resistance to kanamycin. *E. coli* JM105 (*lac*$^-$, *proAB, thi, rps*L, *end*A, *sbc*B15, *hspR*4 [F′, *pro*$^+$, B$^+$, *lac*I^{q}Z M15, *tra*D]) [5] was used as host. Standard DNA recombinant [6] and nucleotide sequencing [7] techniques were used. The polylinker shown in Fig. 1 was synthesized with a DNA synthesizer Biosearch Cyclone (New Brunswick Scientific Co., San Raphael, CA, USA). Bacterial growth was carried out at 37 °C in liquid YT medium [8] and on 2 × YT agar plates. Selection of kanamycin- and ampicillin-resistant strains was made on agar plates containing 25 μg antibiotic ml^{-1}. Detection of *lac*Z′ α-complementation was made on agar plates containing per ml 100 μg 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and 40 μg isopropyl-β-D-thiogalactopyranoside.

Cell fractionation was carried as described in [9]; β-galactosidase and β-lactamase activities were assayed on *o*-nitrophenyl-β-galactopyranoside [10] and nitrocefin [11], respectively; DD-carboxypeptidase (reaction catalysed: Ac_2-L-Lys-D-Ala-D-Ala + H_2O → Ac_2-L-Lys-D-Ala + D-Ala) and DD-transpeptidase (Ac_2-L-Lys-D-Ala-D-Ala + [^{14}C]D-Ala → Ac_2-L-Lys-D-Ala-[^{14}C]D-Ala + D-Ala) activities were measured and penicillin binding (using [^{35}S]benzylpenicillin) was carried as described in [12,13].

pDML307 (see INTRODUCTION) was constructed as indicated in Fig. 2 Subcloning of the Oxa-2 β-lactamase was made by inserting the 2.5 kb *Pst*I 50860-*Bam*HI 1160 DNA fragment of R46, in the *Bam*HI-*Pst*I site of the multiple-cloning region of pBGS18$^+$ (arrow). The ligation mixture was used to transform *E. coli* JM105. Ampicillin-resistant and kanamycin-resistant transformants were selected. The isolated and purified recombinant plasmid pDML303 contained: (i) from *Bam*HI 170 to *Sph*I 1670 (with an additional internal *Sph*I site 1030) and downstream of *Ava*I 1500, the promotor, the ribosome-binding site and the sequence coding for the signal peptide of the Oxa-2 β-lactamase; and (ii) from *Sph*I 1670 to *Hin*dIII 2670 the rest of the Oxa-2 β-lactamase gene. The next step was therefore to replace this latter piece of DNA by the *bla*R-CTD gene which, in pRTW8, extends from *Bal*I 4360 to *Hin*dIII 5590. This was achieved as follows. (i) The *Sph*I 1030 - *Hin*dIII 2670 DNA fragment was excised from pDML303 and replaced by the *Sph*I*Hin*dIII polylinker of Fig. 1, yielding plasmid pDML304. Note that the nucleotides 1 to 14 of the 29-mer strand of the polylinker were designed to recon-

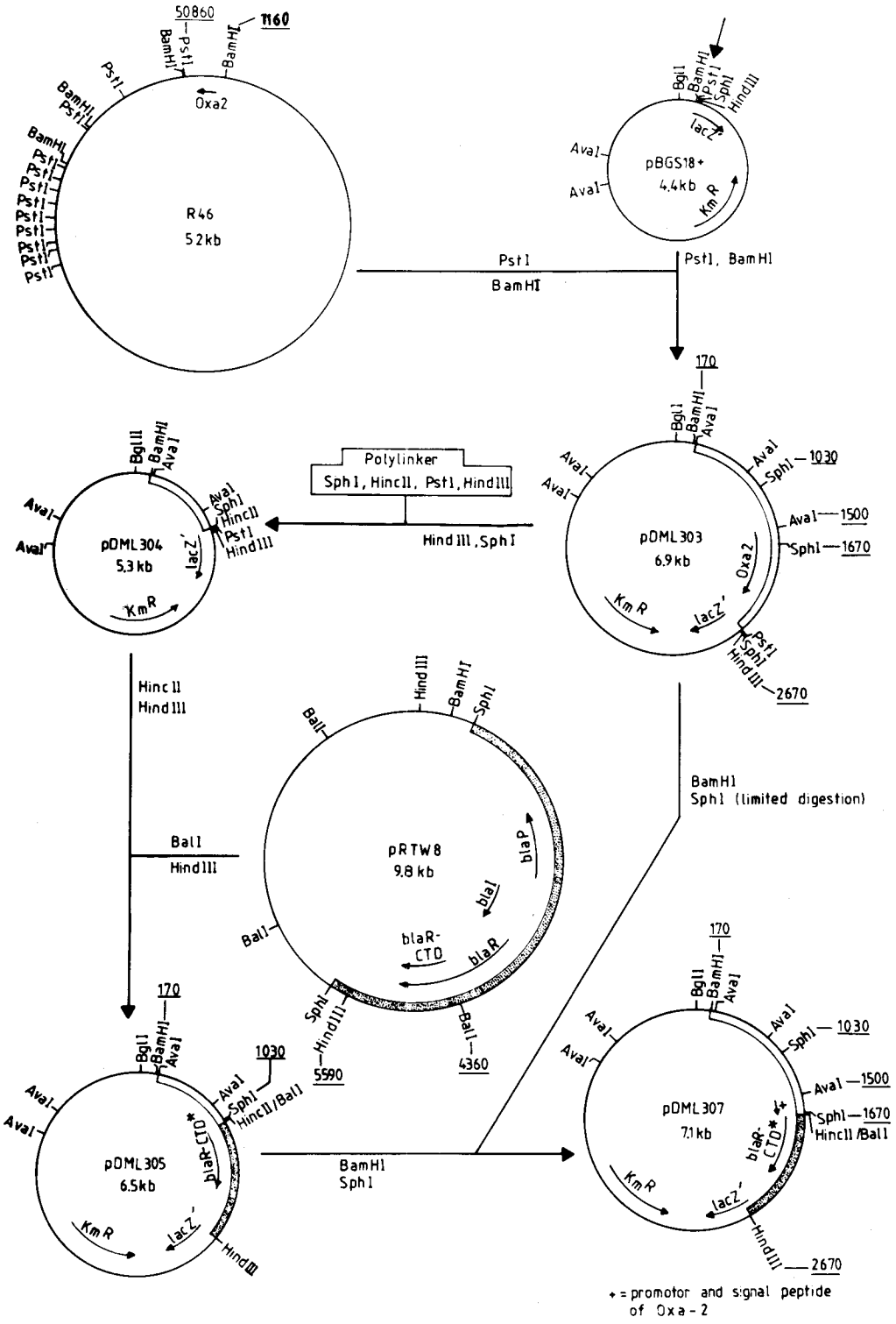

Fig. 2. Construction of plasmid pDML307.

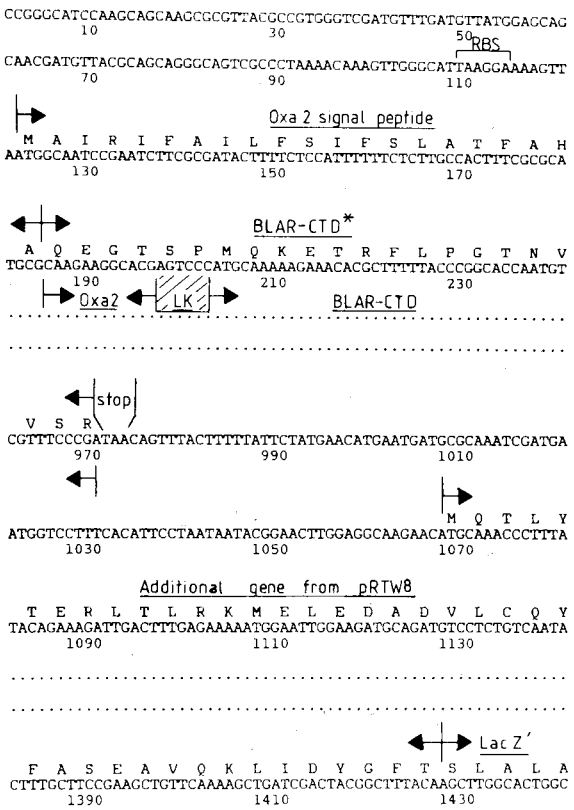

Fig. 3. Organization of the *bla*R-CTD* gene in pDML307. The promoter, the ribosome-binding site (RBS) and the 75 base-pair sequence coding for the signal peptide and the tetrapeptide QEGT of the amino terminal region of the Oxa-2 β-lactamase were linked to the *bla*R-CTD gene via a 6-base-pair sequence (originating from the linker and coding for the dipeptide SerPro). The *bla*R-CTD gene was followed by another *B. licheniformis* gene (from pRTW8) itself in frame with the *lacZ'* gene.

stitute the −1 to +4 sequence of the Oxa-2 β-lactamase, i.e. the sequence AQEGT containing the AQ site of cleavage by the leader peptidase. (ii) The polylinker in pDML304 was digested with *Hin*cII and *Hin*dIII thus providing the sites for the insertion of the *Bal*I 4360–*Hin*dIII 5590 DNA fragment of pRTW8 that contained the *bla*R-CTD gene. The resulting plasmid was called pDML305. iii) The *Bam*HI 170–*Sph*I 1030 DNA fragment of pDML305 was excised and replaced by the *Bam*HI 170–*Sph*I 1670 DNA fragment of pDML303. The recombinant plasmid was called pDML307.

4. RESULTS

The organization of the insert in pDML307 is shown in Fig. 3. pDML307 was used to transform *E. coli* JM105 and the resulting strain was called *E. coli* JM105/pDML307. Given the site of cleavage by the leader peptidase (vertical arrow in Fig. 3), *E. coli* JM105/pDML307 was expected to produce and export into the periplasm, a 262 amino acid polypeptide referred to as BLAR-CTD*—i.e. BLAR-CTD with a QEGTSP hexapeptide amino terminal extension. Since this small extension should not interfere with the folding of the polypeptide, the exported BLAR-CTD* was

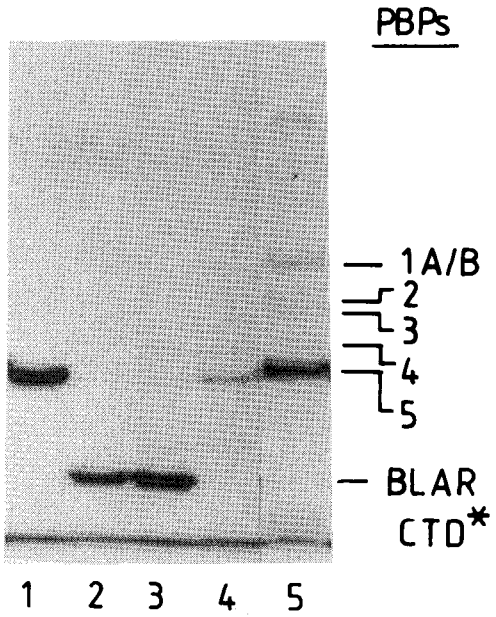

Fig. 4. Periplasmic location of BLAR-CTD* expressed by *E. coli* JM105/pDML307. The periplasmic fraction (8 and 60 μg protein; lanes 2 and 3, respectively), the cytoplasmic fraction (175 μg protein; lane 4) and the plasma membrane fraction (20 μg protein; lane 5) were labelled with [^{35}S]benzylpenicillin, submitted to SDS–polyacrylamide gel electrophoresis and the radioactively labelled penicillin-binding proteins were visualized by fluorography. The 38000 M_r DD-peptidase/penicillin binding proteins of *Streptomyces* R61 (5 pmol; lane 1) used as standard was similarly treated. The cytoplasmic fraction (lane 4; 175 μg protein) is slightly contaminated by membrane components. Lane 5 shows the membrane-bound penicillin-binding proteins (PBPs), from PBPs 1A/1B (molecular weight: 93636 and 94266, respectively) to PBP5 (molecular weight: 44444), of the host *E. coli* JM105.

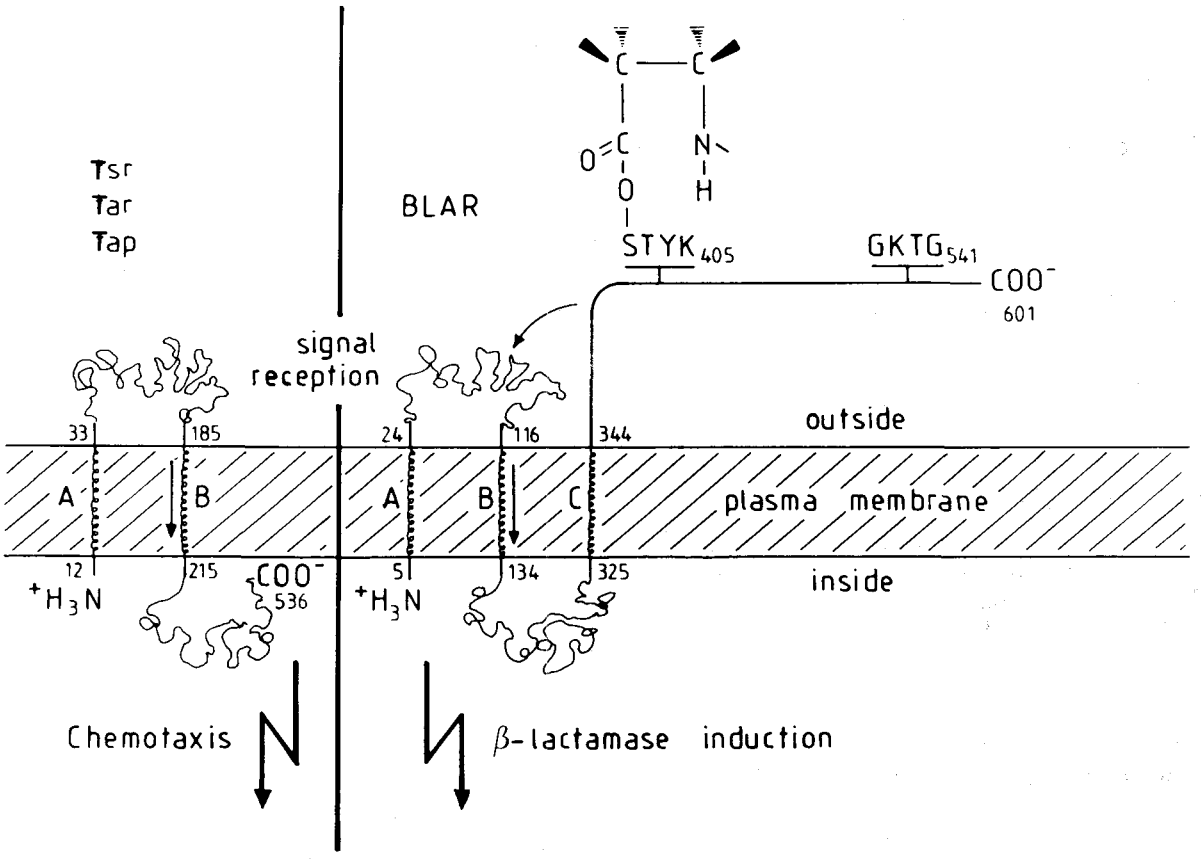

Fig. 5. Membrane topology of the chemotactic transducers Tsr, Tar and Tap. Proposed membrane topology of BLAR and site of penicilloylation.

also expected to behave as a 26 000-M_r penicillin-binding protein. Finally, *E. coli* JM105/pDML307 was expected to be a producer of intracellular β-galactosidase (as a result of an in-frame fusion between an additional gene from pRTW8 and *lacZ'*) and of extracellular β-lactamase (given that the host *E. coli* JM105 had the chromosome-encoded *amp*C gene).

Analysis of the penicillin-binding proteins in isolated cell fractions revealed that *E. coli* JM105/pDML307 produced during growth, a water-soluble, 26 000-M_r penicillin-binding protein that accumulated exclusively in the periplasm (Fig. 4). Using the *Streptomyces* R61 DD-peptidase/penicillin-binding protein as reference [14], one overnight culture of *E. coli* JM105/pDML307 contained about 1 mg BLAR-CTD* per litre of culture. The periplasmic fraction lacked detectable DD-carboxypeptidase of DD-transpeptidase activity, suggesting that the only property of BLAR-CTD* was to bind penicillin. Analysis of the *E. coli* JM105/pDML307 cell fractions also showed that 80% of the expressed β-galactosidase was cytoplasmic and 80% of the expressed β-lactamase was periplasmic.

Note that the *E. coli* JM105 strains harbouring pBGS18$^+$, pDML303 or pDML304 did not produce any periplasmic penicillin-binding protein and that *E. coli* JM105/pDML305 produced trace amounts of BLAR-CTD* (presumably intracellularly). Given that immediately downstream of the *Bal*I site, there occurs and initiation codon ATG,

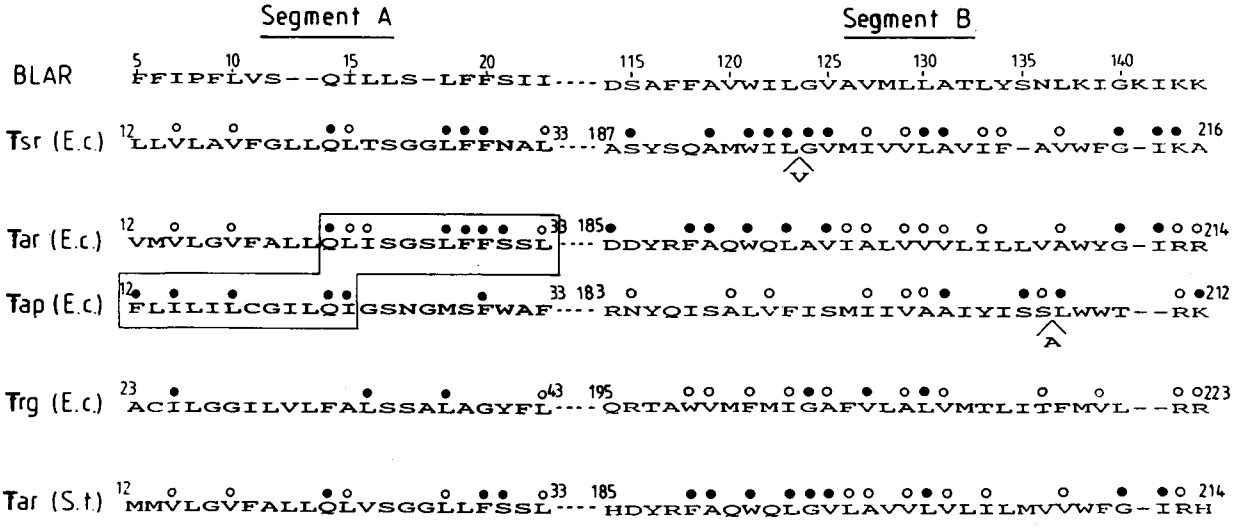

Fig. 6. Alignment of the 5-23 amino acid segment and the 114-144 amino acid segment of BLAR with the transmembrane segments and neighbouring amino acids, of the Tsr, Tar, Tap and Trg sensory transducer proteins of Gram-negative bacteria. E.c = *E coli*; S.t = *Salmonella typhimurium*; ● = identities; ○ = conservative replacements; ∧ = insertions. The transducers are involved in chemotactic responses to serine (Tsr), aspartate and maltose (Tar), galactose and ribose (Trg) and dipeptides (Tap). A and B are transmembrane segments (Fig. 5).

this low expression indicated the presence of a promoter in the *Bam*HI 170-*Sph*I 1030 DNA segment.

5. DISCUSSION

Transducers are transmembrane proteins specialized in the transmission of a chemical signal from the environment to the interior of the bacterial cell. They contain an extracellular domain which is located on the outer face of the membrane and is responsible for signal reception, and a cytosolic domain which is responsible for the generation of an intracellular signal (Fig. 5). In chemotaxis, the Tsr, Tar, Trg and Tap transducers are sensitive to amino acids (serine, aspartate), carbohydrates (maltose, galactose, ribose) and dipeptides [15]. Attractants either bind directly to the transducer or, alternatively, they bind first to soluble binding proteins (located in the periplasm of Gram-negative bacteria) and the attractant: attractant-binding protein then binds to the transducer.

Amino acid alignments (Fig. 6) revealed that the polypeptide segment D115-K143 of BLAR is very similar, in its primary structure, to the transmembrane segment B which links the receptor domain to the cytoplasmic domain of Tsr in *E. coli* (12 strict identities out of 20 amino acids) or Tar in *Salmonella typhimurium* (9 strict identities). Also , the polypeptide segment F5-S21 of BLAR exhibits similarity with the transmembrane segment A of Tar and Tap of *E. coli*. On the basis of these homologies and the fact that, as shown above, BLAR-CTD is the penicillin-binding domain of BLAR, a likely molecular organization of BLAR is that shown in Fig. 5. The 325 amino acid amino terminal region, i.e. the transducer per se, would have the same general membrane topology as that found in the chemotactic transducers Tsr, Tar and Tap. As observed with some transducers, the attractant of BLAR, i.e. penicillin, does not bind directly to the transducer but binds to the 257 amino acid penicillin-binding BLAR-CTD. The peculiarity of BLAR is that the BLAR-CTD is fused to the carboxy terminus of the transducer by means of an additional transmembrane segment C, which fusion is a direct consequence of the absence of periplasm in *B. licheniformis*.

One may hypothesize that penicilloylation of BLAR-CTD induces conformational changes and that this signal is transmitted to the extracellular domain of the transducer, and from this, to the intracellular domain via the transmembrane segment B. In agreement with this view, the Gly124 → Asp and Gly538 → Asp BLAR mutants are desensitized to penicillin and fail to induce β-lactamase synthesis in *B. licheniformis* (in preparation). The mutation Gly124 → Asp affects the transmembrane segment B and the mutation Gly538 → Asp occurs upstream of the KTG box which, in the active-site serine, penicillin-interactive proteins of known three-dimensional structure, occurs on the innermost strand of the five-stranded β-sheet and form part of the active-site [19–22]. The intracellular domain of BLAR has no multiple sites of methylation/demethylation as found in the chemotactic transducers [16] (though a dyad Glu-Glu occurs at positions 163 and 164); it lacks sequences that would suggest the occurrence of ATP binding sites [23]; it does not contain the sequence consensus characteristic of transmitters [24], but it contains five cysteine residues at positions 161, 208, 217, 229 and 256.

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REFERENCES

[1] Kobayashi, T., Zhu, Y.F., Nicholls, N.J., and Lampen, J.O. (1987) J. Bacteriol. 169, 3873–3878.
[2] Zhu, Y.F., Curran, I.H.A., Joris, B., Ghuysen, J.M., and Lampen, J.O. (1990) J. Bacteriol. 72, 1137–1141.
[3] Brown, A.M.C., Coupland, G.M., and Willets, N.S. (1984) J. Bacteriol. 159, 472–481.
[4] Spratt, B.G. Hedge, P.J., te Heesen, S., Edelman, A., and Broome-Smith, J.K. (1986) Gene 41, 337–342.
[5] Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Gene 33, 103–109.
[6] Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory. Cold Spring Harbor, New York.
[7] Sanger, F., Nicklen, S., and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5463–5467.
[8] Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
[9] Linquist, S., Galleni, M., Lindberg, F. and Normark, S. (1989) Mol. Microbiol. 3, 1091–1102.
[10] Craven, G.R., Steers, E., and Anfinsen, C.B. (1965) J. Biol. Chem. 240, 2468–2478.
[11] O'Callaghan, C.H., Morris, A., Kerby, S.M., and Shingler, A.H. (1972) Antimicrob. Agents Chemother. 1, 283–288.
[12] Nguyen-Distèche, M., Leyh-Bouille, M., Pirlot, S., Frère, J.M. and Ghuysen, J.M. (1986) Biochem. J. 235, 167–176.
[13] Leyh-Bouille, M., Nguyen-Distèche, M., Pirlot, S., Veithen, A., Bourguignon, C. and Ghuysen, J.M. (1986) Biochem. J. 235, 177–182.
[14] Duez, C., Piron-Fraipont, C., Joris, B., Dusart, J., Urdea, M.S., Martial, J.A., Frère, J.M., and Ghuysen, J.M. (1987) Eur. J. Biochem. 162, 509–518.
[15] MacNab, R.M. (1987) in *Escherichia coli* and *Salmonella typhimurium* cellular and molecular biology, Vol. 1 (F.C. Neidhardt, J.L. Ingraham, K. Brooks Low, B. Magasanik, M. Schaechter and H.E. Umbarger, eds.), pp. 732–759, American Society for Microbiology, Washington, D.C.
[16] Bollinger, J., Park, C., Harayama, S., and Hazelbauer, G.L. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 3287–3291.
[17] Krikos, A., Mutoh, N., Boyd, A., and Simon, M.I. (1983) Cell 33, 615–622.
[18] Russo, A.F., and Koshland Jr, D.E. (1983) Science 220, 1016–1020.
[19] Herzberg, O., and Moult, J. (1987) Science 236, 694–701.
[20] Dideberg, O., Charlier, P., Wéry, J.P., Dehottay, P., Dusart, J., Erpicum, T., Frère, J.M. and Ghuysen, J.M. (1987) Biochem. J. 245, 911–913.
[21] Joris, B., Ghuysen, J.M., Dive, G., Renard, A., Dideberg, O., Charlier, P., Frère, J.M., Kelly, J.A., Boyington, J.C., Moews, P.C., and Knox, J.R. (1988) Biochem. J. 88, 313–324.
[22] Kelly, J.A., Knox, J.R., Zhao, H., Frère, J.M., and Ghuysen, J.M. (1989) J. Mol. Biol., 209, 281–295.
[23] Walker, J.E., Saraste, M., Runswick, M.J., and Gay, N.J. (1982) EMBO J. 1, 945–951.
[24] Kofoid, E.C., and Parkinson, J.S. (1988) Proc. Natl. Acad. Sci. USA 85, 4981–4985.