The EMBO Journal vol.7 no.12 pp.3971 - 3974, 1988

Evidence for high affinity binding-protein dependent transport systems in Gram-positive bacteria and in *Mycoplasma*

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Communicated by M.Hofnung

Gram-negative bacteria are surrounded by two membranes. In these bacteria, a class of high affinity transport systems for concentrating substrates from the medium into the cell, involves a binding protein located between the outer and inner membranes, in the periplasmic region. These 'periplasmic binding-proteins' are thought to bind the substrate in the vicinity of the inner membrane, and to transfer it to a complex of inner membrane proteins for concentration into the cytoplasm. We report evidence leading us to propose that a Gram-positive bacterium, *Streptococcus pneumoniae*, and a mycoplasma, *Mycoplasma hyorhinis*, which are surrounded by a single membrane and have therefore no periplasmic region, possess an equivalent to the high affinity periplasmic binding-protein dependent transport systems, i.e. extra-cytoplasmic binding lipoprotein dependent transport systems. The 'binding lipoproteins' would be maintained at proximity of the inner membrane by insertion of their N-terminal glyceride-cysteine into this membrane.

Key words: periplasmic protein/binding-protein/lipoprotein/transport/Gram-negative/Gram-positive/*Mycoplasma*

Introduction

In Gram-negative bacteria, a number of substrates (such as maltose and maltodextrins, histidine, oligopeptides, phosphate, etc.) are concentrated into the cell by high affinity transport systems which include several proteins, and a common organization (reviewed in Ames, 1986; Shuman, 1987). A central component of these systems is a binding protein (e.g. MalE for maltose and maltodextrins in *Escherichia coli*, HisP for histidine in *Salmonella typhimurium*, OppA for oligopeptides in *S.typhimurium*) which is located in the periplasm between the outer and inner membranes. These water soluble 'periplasmic binding-proteins' or 'PBP' present high affinity for their specific substrates ($K_d \sim 1$ μM). Their concentration in the periplasm can reach very high values (1 mM) so that they increase the availability of (bound) substrate in the vicinity of the inner membrane. Upon specific interactions of the liganded PBP with a complex of inner membrane proteins, the substrate is transferred to the complex and further transported into the cytoplasm. Two proteins of the complex are hydrophobic and are intrinsic inner membrane proteins (MalF−MalG for maltose, HisQ−HisM for histidine and OppB−OppC for oligopeptides). Depending on the system there are also one (MalK, HisP), or two (OppD, OppF), hydrophilic proteins in the complex; they are believed to be bound to the inner face of the cytoplasmic membrane. These hydrophilic proteins belong to a family of homologous proteins associated with various systems for trans-membrane transport of very different substrates in a variety of organisms (haemolysin secretion in *E.coli*, pigment export in *Drosophila*, drug expulsion from mammalian cells, etc.) (Gilson *et al.*, 1984; Higgins *et al.*, 1986; and reviewed in Henderson and Maiden, 1987). These proteins bind ATP and are believed to provide energy for transport through the membrane.

Here we report evidence that Gram-positive bacteria and mycoplasma possess high affinity transport systems with an analogous organization. We suggest that these organisms, which have no outer membrane, maintain the extra-cytoplasmic protein in the vicinity of the cytoplasmic membrane by means of an NH_2 terminal lipo-amino acid anchor.

Results and discussion

Homologies between PBP and proteins from Gram-positive bacteria

We have found extensive homology between two PBP involved in transport in Gram-negative bacteria (MalE for maltose and maltodextrins and OppA for oligopeptides) and two proteins from the Gram-positive bacterium *Streptococcus pneumoniae*, respectively MalX and AmiA (Figure 1).

MalX is a maltose inducible membrane bound protein (Weinrauch and Lacks, 1981; Lacks *et al.*, 1982). The homology with MalE corresponds to 27% identity over nearly the entire length of the two proteins (in an overlap of 388 residues) (Figure 1). If homologous residues are not distinguished, the homology increases to 67%. Only a few small gaps are necessary for optimal alignment. MalX is induced by maltose and its structural gene belongs to a cluster involved in maltose and maltodextrins utilization (Lacks, 1968). It is assumed to play a role in the uptake of maltodextrins such as maltotetraose, but is not needed for transport of maltose itself.

The AmiA protein is encoded by a gene of the *ami* locus (Alloing *et al.*, 1986). Mutations at the *ami* locus have pleiotropic effects. They confer: (i) increased resistance to aminopterin (Sicard and Ephrussi-Taylor, 1965) and methotrexate (Trombe *et al.*, 1986), to Celiptium (2-*N*-methyl-9-hydroxy-ellipticium) (Sautereau and Trombe, 1986); (ii) sensitivity to an inbalance in concentrations in the medium between the three branched amino acids, leucine, isoleucine and valine (Sicard, 1964); (iii) decrease in the trans-membranous electric potential (Trombe *et al.*, 1984). AmiA (493 residues) (G.Alloing *et al.*, in preparation) and

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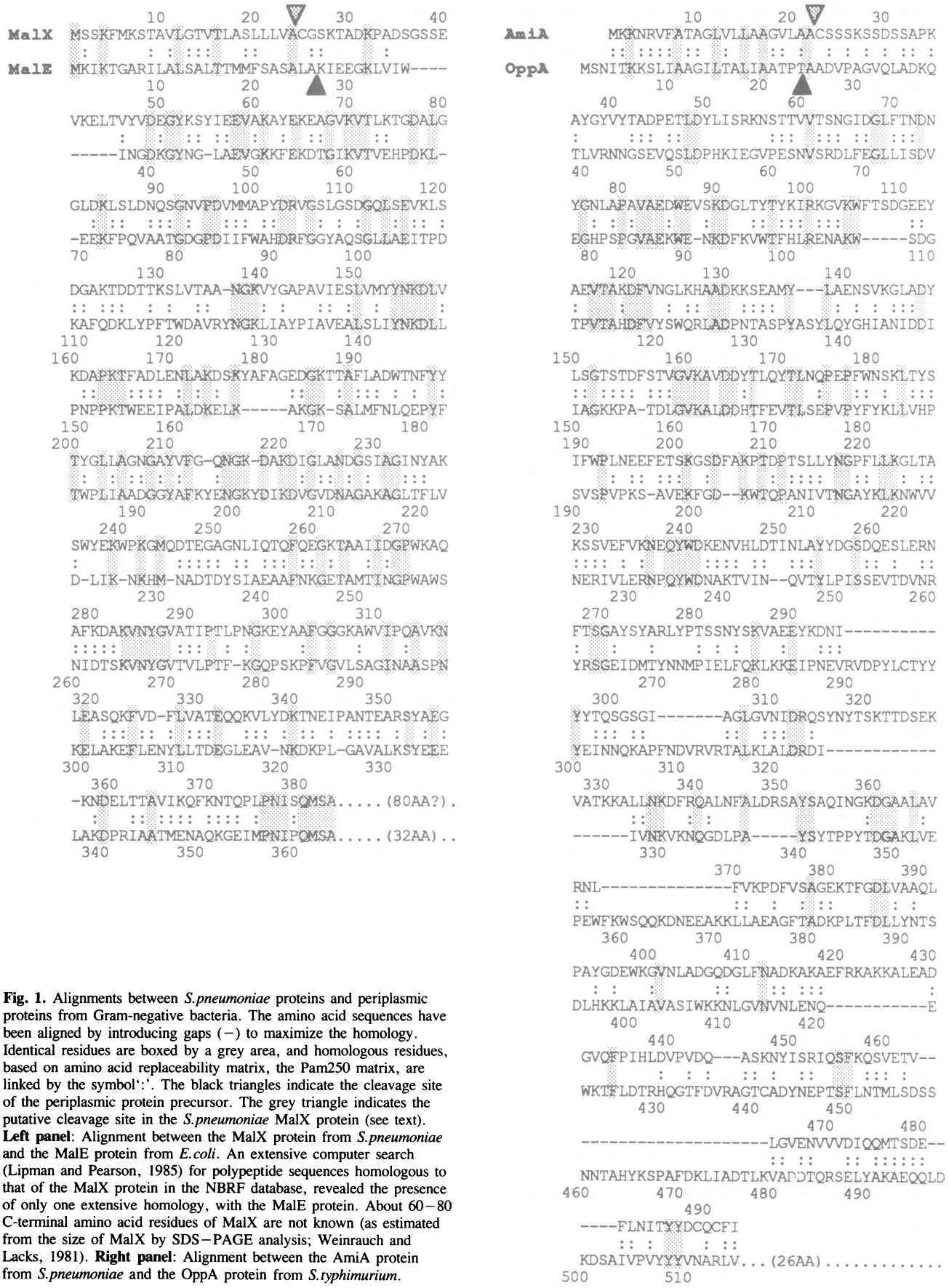

Fig. 1. Alignments between *S.pneumoniae* proteins and periplasmic proteins from Gram-negative bacteria. The amino acid sequences have been aligned by introducing gaps (−) to maximize the homology. Identical residues are boxed by a grey area, and homologous residues, based on amino acid replaceability matrix, the Pam250 matrix, are linked by the symbol':'. The black triangles indicate the cleavage site of the periplasmic protein precursor. The grey triangle indicates the putative cleavage site in the *S.pneumoniae* MalX protein (see text). **Left panel**: Alignment between the MalX protein from *S.pneumoniae* and the MalE protein from *E.coli*. An extensive computer search (Lipman and Pearson, 1985) for polypeptide sequences homologous to that of the MalX protein in the NBRF database, revealed the presence of only one extensive homology, with the MalE protein. About 60−80 C-terminal amino acid residues of MalX are not known (as estimated from the size of MalX by SDS−PAGE analysis; Weinrauch and Lacks, 1981). **Right panel**: Alignment between the AmiA protein from *S.pneumoniae* and the OppA protein from *S.typhimurium*.

OppA (540 residues) (Hiles *et al.*, 1987) have 24% of identical residues in an overlap of 299 residues. If homologous residues are not distinguished, the homology increases to 40%.

At this point, it is worth recalling that PBP involved in the transport of different substrates in the same bacterial species present very little, or no detectable, sequence homology (Duplay *et al.*, 1984) In contrast, PBP from different Gram-negative bacteria with identical substrate specificity appear very homologous in sequence (Dahl *et al.*, in preparation). Thus, the strong homologies between proteins MalE and MalX suggest that they may perform similar functions. The same holds true for OppA and AmiA. This idea is reinforced by the fact that similar levels of homology have been found between isofunctional proteins of *E.coli* and Gram-positive bacteria (Henner *et al.*, 1984; Mannarelli *et al.*, 1985; Parsot, 1986; Priebe *et al.*, 1987).

The complete nucleotide sequence of the *ami* locus revealed that it consists of an operon encoding six proteins, Ami ABCDEF (G.Alloing *et al.*, in preparation). In addition to the AmiA−OppA homology, the other proteins encoded by the *ami* locus exhibit homology with other components of the oligopeptide permease cluster (*opp*). AmiE and AmiF are homologous to the peripheral 'energizing' components OppD−OppF. The AmiC (over its last 230 residues) and AmiD proteins are strongly hydrophobic and are >30% homologous to OppB and OppC, respectively. Such homologies were never found between inner-membrane components of PBP dependent systems with different substrates (Dassa and Hofnung, 1985), so that it appears very likely that the *ami* and *opp* encoded transport systems have at least overlapping, and possibly identical, specificities.

Binding lipoproteins, Gram-positive bacteria and mycoplasma

Proximity of the PBP to the cytoplasmic membrane is believed to be critical for the functioning of these high affinity transport systems. How could a similar system work in Gram-positive bacteria where no outer membrane exists? Examination of the NH_2 terminal sequences of the *malX* and *amiA* gene products gives a possible clue to this question.

Let us first examine the case of *malX* and *malE*. The NH_2 terminal parts of the *malE* and *malX* gene products represent 'signal peptides' characteristic for proteins which are exported through the cytoplasmic membrane. In the case of MalE, it was directly shown that the 26 first residues are cleaved upon export (Duplay *et al.*, 1984). In the case of MalX the region of the potential cleavage site belongs to a well defined category; it carries a sequence L-V-A−C-G-S corresponding to the consensus (L-*Y*-*Z*-cleavage site-C-*y*-*z*; where *Y* is A, S, V, Q, T; *Z* is G or A; *y* is S, G, A, N, Q, D; *z* is S, A, N, Q) of the precursors of lipoproteins (reviewed in Wu, 1987; Yamaguchi *et al.*, 1988). It appears thus very likely that the MalX protein is a lipoprotein. Lipoproteins are also exported through the cytoplasmic membrane, but the NH_2 terminal cysteine is transformed into a lipo-amino acid. This lipophilic modification is thought to be responsible for the membrane anchorage of a number of exported proteins (Nielsen and Lampen, 1982). The membrane attachment of MalX is thus likely to occur through the same mechanism. The protein itself would be exposed to the outside face of the membrane in a water soluble form.

The situation is similar for the *amiA* and *oppA* gene product. In the case of OppA, it was shown that a peptide of 23 residues is cleaved upon export (Hiles and Higgins, 1986). For AmiA, the region of the potential cleavage site carries the sequence L-A-A−C-S-S, which corresponds also exactly to the consensus of the lipoprotein precursors.

Finally, a gene cluster with the same organization as PBP dependent system from Gram-negative bacteria has been described in *Mycoplasma hyorhinis* (Dudler *et al.*, 1988). The mature extracytoplasmic component, the p37 protein, also presents the characteristics of a lipoprotein: the NH_2 terminal sequence of the mature proteins starts with C-S-N-, which corresponds to the consensus, and the protein is bound to the membrane (R.Dudler, unpublished results). The sequence before the potential cleavage site is less typical (A-I-S-cleavage site), but it should be noted that the putative signal sequence is rather peculiar, since it contains four phenylalanine residues (Dudler *et al.*, 1988). As very little is known about signal sequences in mycoplasma, it is possible that they differ slightly from those in other microorganisms.

Binding lipoprotein dependent transport systems in Gram-positive bacteria and mycoplasma

The evidence presented leads us to propose that MalX and AmiA, in Gram-positive bacteria, and p37 in mycoplasma, are the functional equivalent of periplasmic binding proteins involved in transport in Gram-negative bacteria. In the case of AmiA, the other components of the transport system are encoded by the rest of the *ami* locus (G.Alloing *et al.*, in preparation) and the substrate is likely to be oligopeptide(s). For MalX, the other components have yet to be identified, and the substrate is likely to be a maltodextrin. For p37, the other components are known, but not the substrate.

Our proposal contradicts the commonly admitted idea that transport systems depending on a component located outside the cytoplasm are confined to Gram-negative bacteria. However, the anchoring of this extra-cytoplasmic component by its NH_2 terminal lipoaminoacid anchor would allow such systems to operate in Gram-positive bacteria and in mycoplasma. In that respect, it is interesting that a mutant MalE protein which is anchored, by its uncleaved NH_2-terminal signal peptide, to the external face of the cytoplasmic membrane can still operate in transport in *E.coli* (Fykes and Bassford, 1987). Sequestration of the protein by the outer membrane may thus not be required for transport, even in Gram-negative bacteria.

A systematic search for lipoproteins in *Bacillus licheniformis* and *B.cereus* revealed sets of lipoproteins which were released from protoplasts by mild trypsin treatment, suggesting an orientation to the outside of the membrane (Nielsen and Lampen, 1982). In addition, the β-lactamases involved in resistance to penicillins are periplasmic proteins in Gram-negative bacteria and exist in substantial amounts under a lipoprotein membrane bound form in Gram-positive bacteria such as *B.licheniformis* and *Staphylococcus aureus* (Nielsen *et al.*, 1981). This led to the early suggestion that lipoproteins in Gram-positive bacteria could play roles equivalent to some of the free periplasmic proteins from

E.coli (Nielsen and Lampen, 1982). However, until now, no physiological role had been identified for these Gram-positive lipoproteins, (considering that penicillinase activity is not a physiological function). It was also suggested that the "lipoprotein modification offers the Gram-positive bacterium an efficient way of retaining a variable portion of its secreted proteins in an active releasable form" (Nielsen and Lampen, 1982). In that perspective, it would be interesting to see if any of the proteins MalX, AmiA and P37, gives rise to a processed secreted form.

The common organization of these high affinity transport systems for the transport of very different substrates and their existence in different bacterial species could reveal a common evolutionary origin. In this hypothesis, the ancestral system would have existed before the divergence between these bacterial species ($\sim 1.5 \times 10^9$ years ago) (Ochman and Wilson, 1987) . Many questions may be raised. For example, did this ancestral system include a lipoprotein which would have evolved to a regular protein, or did the converse happen? It is also interesting to recall that the *mdr* locus, involved in multidrug resistance by efflux in mammalian cells, encodes a protein with the properties of the inner membrane components of PBP dependent transport systems (review in Ferro-Luzzi Ames, 1986). However, in this case, no extra-cytoplasmic component has been identified. The question may be raised whether such systems may also function for influx of certain molecules and whether an extra-cytoplasmic component may also exist under the form of a membrane anchored protein (see, e.g. Caras *et al.*, 1987).

Acknowledgements

We thank Jean Marie Clément, David Perrin, William Saurin and Philippe Marlière for discussion. This work was supported by grants from the Fondation pour la Recherche Médicale, the Ligue Nationale contre le Cancer, and the Association pour la Recherche sur le Cancer.

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Received on August 25, 1988