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Review

Bacterial oligopeptide-binding proteins

V. Monnet

Unité de Biochimie et Structure des Protéines, Institut National de la Recherche Agronomique, 78352 Jouy en Josas cedex (France), Fax: +33 1 34 65 21 63, e-mail: monnet@jouy.inra.fr

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Abstract. This review focuses on bacterial oligopeptide-binding proteins, which form part of the oligopeptide transport system belonging to the ATP-binding cassette family of transporters. Depending on the bacterial species, these binding proteins (OppA) capture peptides ranging in size from 2 to 18 amino acids from the environment and pass them on to the other components of the oligopeptide transport system for internalisation. Bacteria have developed several strategies to produce these binding proteins, which are periplasmic in Gram$^-$ bacteria and membrane-anchored in Gram$^+$, with a higher stoichiometry (probably necessary for efficient transport) than the other components in the transport system. The expression of OppA-encoding genes is clearly modulated by external factors, especially nitrogen compounds, but the mechanisms of regulation are not always clear. The best-understood roles played by OppAs are internalisation of peptides for nutrition and recycling of muropeptides. It has, however, recently become clear that OppAs are also involved in sensing the external medium via specific or non-specific peptides.

Key words. Oligopeptide; transport; bacteria; nutrition; binding; sensing; stoichiometry.

Oligopeptide-binding protein as part of the oligopeptide transport system

Oligopeptide transport systems from bacteria belong to the ATP-binding cassette (ABC) family of transporters. As indicated by their name, these systems draw their energy from ATP hydrolysis, which is coupled to peptide transport. They are composed of five subunits: an extracellular oligopeptide-binding protein which specifically captures the substrates, two transmembrane proteins forming the pore and two proteins in charge of ATP hydrolysis. These subunits are generally named OppA, OppB, OppC, OppD and OppF. However, in addition to OppA, several other names have been given to oligopeptide-binding proteins, as shown in table 1. In Gram$^-$ bacteria, oligopeptide-binding proteins are periplasmic, whereas in Gram$^+$ bacteria, they are anchored to the membrane via a NH_2-terminal lipomodification. These two forms of oligopeptide-binding proteins are probably derived from a common ancestor [1]. In both cases, the mobility of oligopeptide-binding proteins is relatively limited: in Gram$^-$ bacteria because the periplasm is thin and has a gel-like consistency which limits the diffusion of proteins [2], and in Gram$^+$ bacteria because oligopeptide-binding proteins are linked to the membrane. Restricted diffusion of the substrate to two dimensions through binding to the oligopeptide-binding protein facilitates its interaction with the limited number of transport systems [3].

Oligopeptide-binding proteins from Gram$^+$ bacteria are lipoproteins, synthesized as precursors in the cytoplasm and translocated across the membrane via the Sec secretion machinery. Three subsequent modifications take place outside the membrane: (i) formation of a thioether linkage between the N-terminal cysteine and diacylglycerol, (ii) cleavage of the signal peptide by a lipoprotein-specific signal peptidase (signal peptidase II) and (iii) N-acylation of the N-terminal cysteine with phospholipids

Table 1. Different names given to functional and characterized bacterial oligopeptide binding proteins.

Species	Oligopeptide-binding protein	Specific substrate	Accession number when known	Reference
Bacillus subtilis	Spo0KA	Phr peptides	M57689	[66]
	AppA		U20909	[10]
Borrelia burgdorferi	OppA-I, II, III (o) OppA-IV,V (i)		AF043071	[7]
Enterococcus faecalis	OppA			
	PrgZ	sex pheromone	L14285	[77]
	TraC	sex pheromone	D28859	[18]
Escherichia coli	OppA		J05433	[33]
	MppA	muropeptides	U88242	[16]
Lactococcus lactis	OppA		L18760	[23]
	OptA		AE005176	[12]
Lactobacillus delbrueckii	OppA1 (o) OppA2		AY040221	[14]
Listeria monocytogenes	OppA		AF103793	[20]
Mycoplasma hominis	P100		X99740	[21]
Salmonella typhimurium	OppA		X05491	[29]
Streptococcus gordonii	HppA (o) HppG, HppH		L41358	[6]
Streptococcus pneumoniae	AmiA (o)		Z15136	[5]
	AliA or PlpA, AliB (i)		L20556	[64]
Streptococcus thermophilus	AmiA1 (o) AmiA2, AmiA3		AF316884 to 6	[15]
Treponoma denticola	OppA	plasminogen, fibronectin	AF042861	[56]

(o) indicates a location of the binding protein encoding gene in the operon with genes coding for other components of the transport system; (i) indicates a location independent from the genes coding for other components of the transport system.

[4]. In most cases, the lipoprotein nature of oligopeptide-binding proteins has been assessed by metabolic labelling with [^{3}H] palmitate (as is the case for other lipoproteins) [5–7].

Alignment of binding proteins has led to their classification into eight clusters, amongst which cluster 5 combines peptide- and nickel-binding proteins. Proteins in this group are the largest solute-binding proteins, ranging from 493 to 543 residues, and share the following common signature: (LIVM)AXX(WI)$X_{1\ or\ 2}$(SN)(KE)DX_4T(FY)X(LIV)RX_3K [8].

At a genetic level, the five genes encoding the five proteins which form the oligopeptide transport system are always organised in an operon, except for additional copies of the *oppA* gene when they are present. In some bacteria, two copies of the *opp* operon have been identified. In *Bacillus subtilis*, a second Opp system, called App, has been described. It is composed of proteins homologous to OppA, B, C, D and F but whose encoding genes are linked in the order *oppD*, *F*, *A*, *B* and *C*. The App system is inactive in the laboratory strain BS168 because of a frame-shift mutation in the oligopeptide-binding protein encoding gene *appA*. This mutation is absent from other *B. subtilis* strains. The two operons are transcribed at different growth phases: exponential for *opp* and stationary for *app* [9]. The repaired App system of strain BS168 can transport tetra- and pentapeptides but not tripeptides, as can the Opp system. It is capable of completely replacing the Opp system in both sporulation and competence processes, which are detailed below. The reason for this second oligopeptide transport system in *B. subtilis* remains unknown [10]. In *Lactococcus lactis*, the *opp* locus is located in a genome area subject to rearrangements which give rise to strains with no *opp* or with one, two or more copies on the chromosome or on a plasmid [11]. In the available genome sequence, i.e. that of strain IL-1403, two homologous operons have been found: the *opp* one (*oppABCDF*) and the similarly organised *opt* one [12]. In addition, another gene which may encode a third oligopeptide-binding protein, *optS*, has been found just upstream of *optA*. The expression of at least the *optA* and *oppA* genes has been demonstrated through the detection of two corresponding proteins on two-dimensional electrophoresis gels [M.-Y. Mistou, personal communication]. However, the role and importance of these two operons remains unclear.

OPPA stoichiometry

It is striking to observe that to achieve efficient oligopeptide-binding, it is essential that OppA be produced with a higher stoichiometry than other components in the transport system. Consequently, OppA from Gram⁻ bacteria has been described as one of the most abundant periplasmic proteins in *Salmonella typhimurium* and, particularly, in *Escherichia coli,* where its concentration can reach 1 mM [13]. To increase the expression of *oppA*-encoding genes and to render it more independent, bacteria have developed three strategies which can be applied simultaneously in the same species.

The first is to carry from two to five copies of *oppA* genes in their genomes (fig. 1). The lactic acid bacteria *Lactobacillus delbrueckii* subsp. *bulgaricus* possesses two copies of *oppA* (*oppA1* and *oppA2*) in the same operon with other transport system genes [14]. Streptococci typically possess three copies of OppA-encoding genes: one or two in the operon with other transport system genes, and one or two others independently on the chromosome [5, 6, 15]. In *Borrelia burgdorferi*, the phenomenon is further amplified because the bacteria possess five copies of *oppA* genes: three in the operon with other transport system genes and a further two on an independent plasmid. The *oppA* copies are generally very similar but not identical (see table 2), thus allowing the bacteria both to increase their OppA concentration and broaden the variety of transported peptides. In a few cases, the specialization of OppA homologues has been demonstrated. *E. coli* synthesizes a protein, MppA, which is similar in size and 46% identical to OppA, but which is in a minority in the periplasm. MppA works with the other components of the Opp system and exhibits high affinity for muropeptides [16]. In *Enterococcus faecalis*, PrgZ and TraC, homologous to OppA, bind sex pheromones with high affinity and also recruit the OppBCDF system to transport them into cells [17–19].

The second solution adopted by some bacteria is to render the transcription of *oppA* independent. This is the case in *Listeria monocytogenes*, where a stem-loop structure has been found between *oppA* and the other genes of the operon, leading to a predominant expression of *oppA* alone during growth in BHI medium at 37 °C [20]. A similar stem-loop active structure allowing independent transcription of the *oppA* gene has been observed in *Mycoplasma hominis* [21]. In other bacteria, such as *Streptococcus gordonii*, a potentially active terminator downstream of the *hppA* gene has also been identified [6].

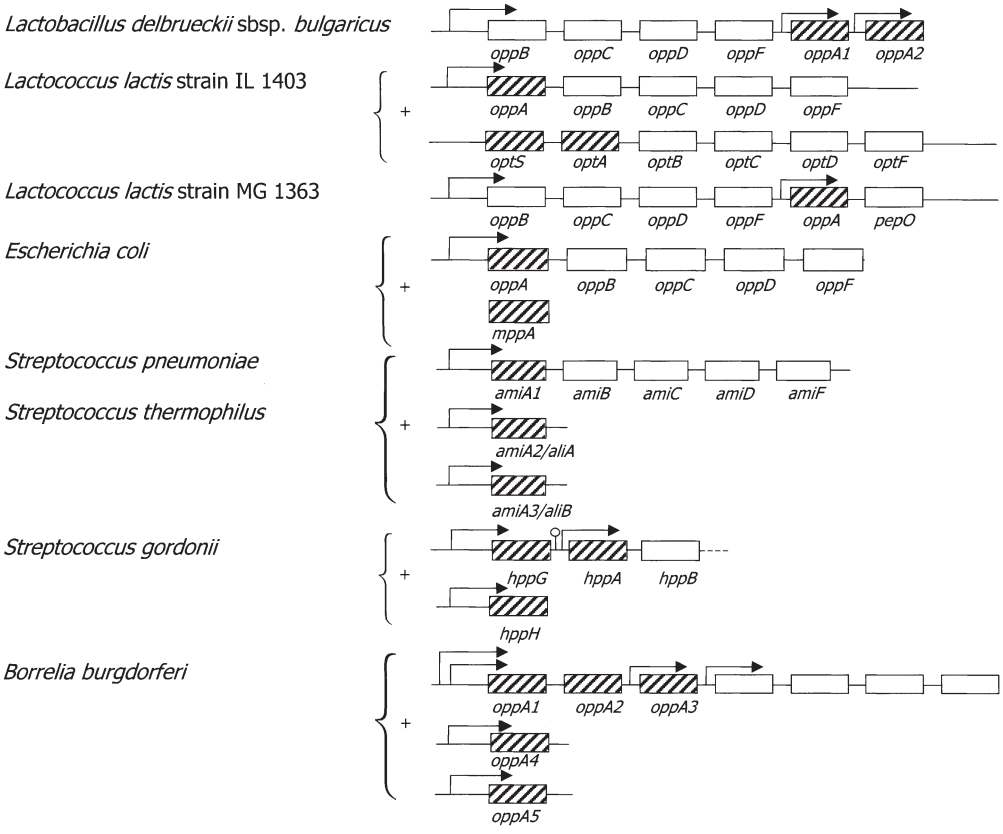

Figure 1. Examples of genetic organisation of *opp* systems, especially those including several oligopeptide-binding proteins. Rectangles representing oligopeptide-binding proteins are hatched. Promoters whose functionality has been demonstrated are represented by arrows.

Table 2. Identity between amino acid sequences of different copies of oligopeptide-binding proteins found in the same bacterial species.

Streptococcus thermophilus		*Streptococcus pneumoniae*		*Streptococcus gordonii*		*Escherichia coli*	
	AmiA1		AmiA		HppA		OppA
AmiA2	97,6%	AliA	67%	HppG	68%	Mppa	46%
AmiA3	87,1%	AliB	65%	HppH	65%		
Borrelia burgdorferi		*Lactobacillus delbrueckii*		*Lactococcus lactis*		*Enterococcus faecalis*	
	OppA1		OppA1		OppA		PrgZ
OppA2	67%	OppA2	66%	OptA	34%	TraC	70%
OppA3	65%			OptS	37%		

The third solution, which is seen, for example, in *Lactococcus lactis* strain MG1363 and in *L. delbrueckii* subsp. *bulgaricus,* is to have a specific promoter for *oppA* sited exceptionally at the end of the *opp* operon. This promoter allows transcription of the two genes (*oppA* and *pepO*) in *L. lactis* [22, 23]. In *L. delbrueckii* subsp. *bulgaricus,* in addition to the operon structure, the *oppA1* gene is also expressed as a monocistronic transcript, while under experimental growth conditions (in whey broth) *oppA2* was expressed at low levels as a separate transcript [14]. The expression level of *oppA1* was about 14-fold that of the whole operon because of the majority expression of *oppA1* under its own promoter under these growth conditions [14]. Similarly, *B. burgdorferi* most frequently initiates transcription of its *oppA* genes from individual promoters located upstream of each of the five *oppA* genes [24, 25]. It is also interesting to note that in most cases, the *oppA* gene is located at one extremity of the *opp* operon, which may be of significance in terms of messenger RNA (mRNA) stability. Examples of mRNA degraded to shorter and more stable ones can be found in the literature [26, 27]. Differences in stability of polycistronic mRNA segments can contribute to the stoichiometry of protein complexes [28]. We cannot exclude the possibility that this conserved position of *oppA* in the operon provides a further means for bacteria to favour OppA production over that of other components in the Opp system.

Regulation of *oppA* expression

Early reports on the regulation of *oppA* suggested non-regulated expression of the oligopeptide-binding protein-encoding gene. Variations in the carbon, nitrogen or amino acid source induced variations to the periplasmic protein composition but not to OppA in *E. coli* [13]. The constitutive expression of *opp* genes in *Salmonella typhimurium* was demonstrated with gene reporter expression and was explained by its possible role in muropeptide transport and peptidoglycan turnover [13, 29, 30]. Since then, in contrast to these examples, so many articles reporting the effects of environmental conditions on *oppA* expression have been published that it is now difficult to believe that *oppA* is constitutively expressed in bacteria. Modifications to the external environment of bacteria are generally associated with changes in *oppA* expression. In *E. coli*, *oppA* expression is clearly repressed in rich medium (= LB) compared with minimum medium (GM + glycine) [31]. In the latter case, *oppA* expression was studied using translational gene fusion, while transcriptional gene fusion was used by Jamieson and Higgins, who did not detect any regulation of *oppA* by the medium [30]. This finding suggests that LB-mediated repression of *oppA* either involves translational control or differs from one strain to another. In *Streptococcus gordonii*, random, arbitrarily priming polymerase chain reaction (PCR) made it possible to identify *hppB* as a downregulated gene when bacteria grown in BHI (brain heart infusion) medium were put in the presence of saliva [32]. *B. burgdorferi* also differentially regulates the expression of its *oppA* genes in response to different hosts which, in the case of one study, were fed/unfed ticks or mice [25].

More precisely, several external factors and molecules influencing *oppA* transcription and translation have been identified, and the list given below continues to grow with the development of global approaches in microbiology. A physical parameter such as temperature is capable of modulating *oppA* expression. In *Listeria monocytogenes,* a second promoter upstream of *oppA* is indeed activated at low temperatures (5 °C) and independently of the cold-shock response [20]. The expression of some OppA-encoding genes from *B. burgdorferi* is also modulated by temperature. The results, obtained using two methods set up in parallel [quantification of mRNA by reverse transcriptase (RT-PCR) and measurement of β-galactosidase activity resulting from fusion with *oppA* promoter regions], were comparable. They indicated that temperature is an important signal for *oppA*-V, whose mRNA transcripts increased markedly more at 37 °C (32-fold) than at

25 °C. Still, this factor is not very significant (less than 5-fold) in the other four *oppAs* [25]. These authors also studied the effect of pH variations, but no change in promoter activity was observed.

Among the molecules affecting *oppA* expression, nitrogen sources including peptides, amino acids, polyamines and ammonium chloride are, as expected, the most frequently reported. As an example, the promoters located upstream of *oppA-I* and *oppA-IV* in *B. burgdorferi* cloned in promoter-less β-galactosidase reporter plasmids in *E. coli* are activated by increasing concentrations of NH_4Cl [83]. The mechanisms governing regulation by nitrogen compounds, however, have been studied in detail in only a few cases. The most detailed example concerns the regulation of *oppA* translation by polyamines. In 1990, in *E. coli*, Kashiwagi et al. [33] observed an increase in OppA synthesis (up to five-fold stimulation) which occurred following the addition of polyamine to the growth medium. This increased OppA synthesis was followed by *E. coli* cell growth. The regulation mechanism was identified at the translation level and involved both the position and secondary structure of the Shine-Delgarno sequence. Polyamines induce structural changes to *oppA* mRNA, especially at the Shine-Delgarno sequence and start codon, by binding the G-C-rich stem loop close to the former. This binding exposes these sequences and facilitates formation of the initial translation complex [34]. The same authors [35] also demonstrated that the weaker the match between the Shine-Delgarno sequence and other regions of the mRNA, the stronger stimulation by polyamines.

The influence of amino acids on *oppA* activation or repression takes place via intracellular pools of amino acids and global regulators in both $Gram^+$ and $Gram^-$ bacteria. In *E. coli* K-12, exogenous leucine increases the transport of peptides via the Opp system. More precisely, leucine induces an increase in OppA synthesis. The transport of leucine or alanine, or di-, tri- peptides containing leucine or alanine, as shown to activate *opp* expression, while no effect was observed with peptides lacking leucine and alanine [36]. The leucine/alanine effect occurred at the transcriptional level, was demonstrated by measuring reporter gene expression in strains carrying *opp-lacZ* fusions [37]. Subsequently, the observation that a mutation in the global regulator Lrp (leucine-responsive regulatory protein) induced a constitutive expression of *opp* operon led to the conclusion that the regulation of *opp* expression by leucine was Lrp mediated [38, 39]. The situation is therefore complex, as leucine uptake activates Lrp, which, in turn, induces *oppA* expression and the uptake of peptides by Opp, which, again, modulates Lrp. In $Gram^+$ bacteria, the regulation of *oppA* by CodY has been reported in detail with respect to *Lactococcus lactis*. In the strain used, *oppA* is cotranscribed either with other genes from the transport system and the oligopeptidase *pepO*-encoding gene, or with the *pepO* gene alone from two different promoters: P_{oppD} localised upstream of the whole operon and P_{oppA} upstream of the last two genes *oppA* and *pepO* (fig. 1). The two promoters are negatively regulated by peptides (up to 150-fold repression of translation) or more precisely by a set of dipeptides, all of which contain at least one branched-chain amino acid [22]. Random mutagenesis has made it possible to obtain mutants derepressed for this nitrogen control and to identify the CodY regulator as a key element in *opp* regulation. These authors showed that repression of the *opp* operon by regulatory dipeptides was indeed completely removed in the *codY* mutant, and that regulatory dipeptides needed to enter the cell and to be cleaved into amino acids in order to repress *opp-pepO* transcription. Their observations suggest that CodY repression depends on branched-chain amino acids or derivatives. The fact that *opp-pepO* transcription is still repressed in a mutant unable to catabolise branched-chain amino acids (i.e. aminotransferase negative) indicates that the signal for CodY repression is the branched-chain amino acid pool itself [40]. Another regulator, the ScoC transcriptional factor, also affects *opp*/*app* expression in *B. subtilis*. The transcription of both operons was completely curtailed by overproduction of the ScoC regulator and enhanced in a *scoC*-negative mutant. However, in this case, the hypothetical effector molecule for ScoC is still unidentified [9].

More surprising are two examples of the modulation of *oppA* expression by non-nitrogen sources in *E. coli,* suggesting the existence of complex regulation of *oppA* expression. Indeed, glycerol represses *oppA,* although no cyclic AMP catabolite activator protein (cAMP-CAP) binding site has been identified in the *oppA* promoter region. The hypothesis retained to explain this catabolite repression is possibly indirect regulation by a cAMP-CAP-dependent regulator [41]. The deletion of *gcvB*, a gene encoding a small-size untranslated mRNA, also derepresses the *oppA* gene in rich medium. In minimum medium the effect of *gcvB* deletion on *oppA* expression is no longer visible, suggesting that another level of regulation occurs in this situation and overrides *gcvB*-mediated regulation.

To conclude, we can say that *oppA* expression is certainly not constitutive and that it is affected by numerous factors. Furthermore, although the effects of many external factors have clearly been demonstrated, the mechanisms through which they act on *oppA* expression are mostly still unknown.

OppA release/shedding

Several observations have revealed that lipoproteins from $Gram^+$ bacteria may be released from the membrane. This phenomenon may constitute a further means of regulating

the level of OppA in bacteria. OppA from *Bacillus cereus* and *B. subtilis* are clearly visible in two-dimensional electrophoresis gels of extracellular proteomes, i.e. bacterial proteins released or secreted in the medium during growth when they are expected to be found in the membrane fraction [42, 43]. The release of OppA has been more closely monitored by electrophoretic analysis of both the supernatant and cell-associated proteins during the growth of *B. subtilis*. OppA release clearly increased as the cells progressed towards the stationary phase. The proportion of OppA in the growth medium at the stationary phase was shown to represent more than 90% of the entire OppA content. Because this release took place very early during growth, it could be dissociated from the lysis phenomenon, which occurs at sporulation [3]. The reversibility and mechanism governing OppA release are poorly understood. However, the two mechanisms proposed so far with respect to lipoprotein release, i.e. release of the lipoprotein with its lipid moiety or release with neither its lipid moiety nor its N-terminal cysteine, would hardly seem compatible with a recapture of OppA by bacteria [42]. A fact which would tend to reinforce the idea that proteolytic cleavage releases OppA into the medium is that the N-terminal cysteine was no longer detectable in the OppA sequence of *B. cereus* released in the supernatant [M. Gohar, personnal communication].

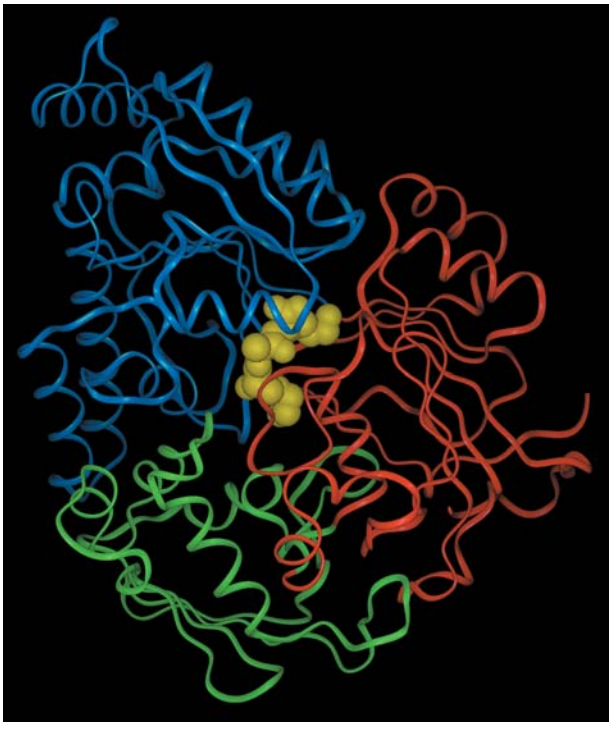

Figure 2. Diagram showing the overall topology of the OppA molecule from *S. typhimurium* binding the tripeptide KAK. This figure has been prepared with InsightII (Accelerys package from the tridimensional structure of OppA deposited in the Brookhaven Protein Database at RCSB with the accession number 1jet [44, 45]. Domain I is in red, domain II in green, domain III in blue, and the tripeptide is represented in the CPK format.

OppA structure, mechanism of binding and specificity

Structural studies have been performed mainly on OppA from Gram$^-$ bacteria in which OppA is one of the larger periplasmic proteins [13]. The three-dimensional structure of OppA from *S. typhimurium*, crystallised with various di-, tri- and tetrapeptides, has been established with high resolution up to 1.2 Å (fig. 2) [44, 45]. The 517-residue protein comprises three domains: domains I and III enclosing the ligand, and domain II, which is not involved in ligand binding (thus accounting for the large size of OppA) and whose role remains unclear. Domains I and III are structurally related to their counterparts in a dozen other binding proteins, even though they only share certain similarities with them in terms of size and amino acid sequences [46]. The OppA binding site is formed by a cleft between domains I and III, connected by a flexible hinge. When ligand binding occurs, OppA markedly changes its conformation, the cleft closes and the ligands are captured. This mechanism is referred as the 'Venus flytrap', at the end of which the ligands are completely buried within the OppA protein. The mechanism by which substrates are transported via ABC transporters has been most deeply described for maltose transport in *E. coli*. This mechanism couples transport and ATP hydrolysis and can most probably be extended in broad outline to other bacterial transport systems of the same family. It involves conformational changes of both the binding protein and the membrane complex (fig. 3).

Three steps can be distinguished. In a first one, the binding protein closes on the substrate, generating a high-affinity binding site and binding to the membrane complex to initiate transport and ATP hydrolysis. Upon binding, conformational changes of the binding protein and membrane complex occur, leading to the second step, called transition state. During this step, the binding protein opens and transfers the substrate to the membrane complex, modifying its conformation and bringing the two ATP hydrolysis subunits into the close proximity necessary for ATP hydrolysis. Then, in the third step, the binding protein is released from the membrane complex, and the substrate is liberated into the cytoplasm [47, 48].

Observations of liganded OppA structures justify the following two conclusions: (i) a protonated α-amino group and an unmodified α-peptide bond are essential for peptide binding, and (ii) little direct interaction exists between OppA and peptide side chains; they mainly occur via salt bridges between termini of the peptides and charged OppA residues. OppA is consequently capable of binding a wide variety of peptides in large hydrated cavities [45].

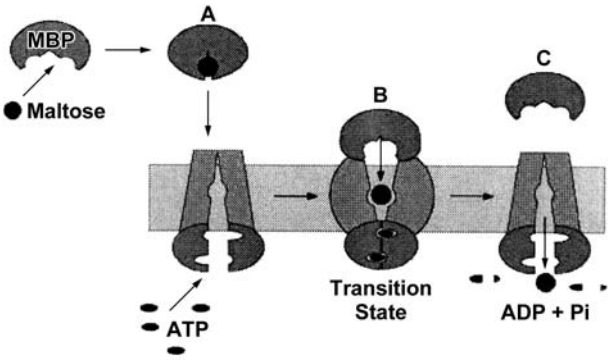

Figure 3. Model for maltose transport in *E. coli*. (*A*) The maltose binding protein binds maltose, adopts a close conformation, generating a high affinity for maltose and binds to the membrane complex. (*B*) In the transition state, MPB and the membrane complex are tightly bound and have opened, exposing internal binding sites to each other. The opening of MBP weakens the interaction with maltose, facilitating the transfer of maltose to the low-affinity binding site of the membrane complex. Conformational modification of the membrane complex allows ATP-induced dimerisation and the activation step that completes both nucleotide-binding sites with residues donated from the opposite subunit. Copyright: National Academy of Sciences, USA [47].

Using isothermal titration calorimetry, Sleigh et al. studied the binding of 20 Lys-X-Lys peptides to OppA. They confirmed that OppA uses water to extend its capability to bind peptides in a way that differs from other proteins which use water to bind their ligands more selectively [49]. These observations are in agreement with previous competition studies performed to characterise the oligopeptide transport system of *E. coli*. These studies showed that two principal peptide features are necessary for efficient peptide binding to OppA: first, unmodified N- and C-termini and, second, a peptide length of two to five amino acids with a preference for tripeptides. Oligopeptide permease exhibits broad tolerance of the amino acid acyl chain, but side-chain composition affects binding to OppA [50]. Although no OppA structure has been reported in Gram$^+$ bacteria, detailed studies to determine OppA specificity have been performed, mainly in *L. lactis*. As a possible common and distinctive feature, Opp from Gram$^+$ bacteria are capable of binding and transporting larger peptides than the Opp from Gram$^-$ bacteria. Indeed, OppA from *L. lactis* binds peptides with a length of up to 35 amino acids and can transport 18 amino acid peptides [51, 52]. Poolman et al. demonstrated that the transfer of oligopeptides from OppA to the membrane complex determines in large part the rate of transport [53]. Based on the OppA structure of *S. typhimurium*, site-directed mutagenesis has been performed on the amino acids which may be involved in the binding site of *L. lactis* OppA [53]. All the mutants obtained exhibited modified peptide transport kinetics compared with the wild type strain (mainly modifications to V_{max} values), and the majority of them exhibited highly defective bradykinin transport. A model for the ligand-binding mechanism of peptides to lactococcal OppA was proposed during study of the transport of a combinatorial peptide library in liposomes carrying anchored OppA. OppA-bound peptides within the range of 5–35 amino acids, with optimum binding for nonapeptides. Positions 4, 5 and 6 were more selective, and the differences in affinity for given side chains were small, although a hypothesis of overall hydrophobicity was favoured. Glycine, proline and negatively charged amino acids reduced binding affinity. Residues 7, 8 and 9 did not find a site in the cleft, but associated in an unspecific manner with the outer surface of OppA. Additional residues led to a steric hindrance which decreased affinity (fig. 4) [52]. The biodiversity of lactococci in oligopeptide transport capacity was recently demonstrated. Both the transport rate of peptides and the type of peptides transported vary from one strain to another. For example, peptide DRVYIHPFHL is efficiently transported or not transported at all, and each strain tested exhibits some specific preferences for peptide utilisation. The differences observed in peptide consumption cannot be attributed solely to differences in OppA sequences, as it has been shown that the cloning of two different *oppAs* in the same *ΔoppA* strain produces the same peptide consumption profiles for the two modified strains [54]. This result indicates that despite the essential role of OppA in

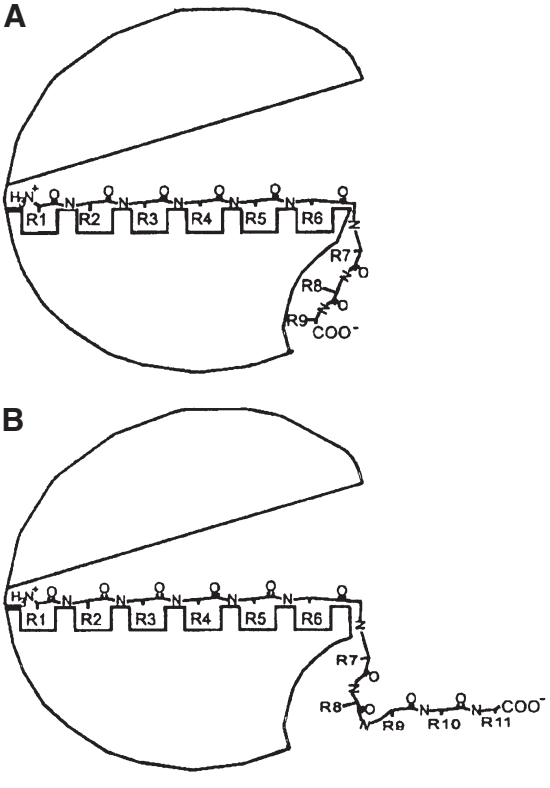

Figure 4. Schematic representation of oligopeptide-binding mechanism to OppA in *L. lactis*. (*A*): Nonapeptide; (*B*): undecapeptide. From Copyright National Academy of Sciences.

peptide transport, it is not the sole determinant of peptide transport specificity in *L. lactis*. In the same bacteria, it was recently demonstrated that all the peptides binding to OppA were not necessarily transported by Opp. More specifically, charged casein-derived peptides may obstruct the route for the transport of other peptides, thus limiting the growth rate of *L. lactis* in milk [55].

In *S. thermophilus,* where the situation is complex because of the presence of three functional and homologous AmiAs, it has been observed that cell-wall proteinase negative ($PrtS^-$) strains consume peptides of up to 4000 Da in mass, exhibiting a preference for neutral and cationic peptides during their growth in a chemically defined medium containing αs_2-casein hydrolysate as the amino acid source [V. Juillard, personal communication]. The transport of such large intact peptides, suggested by these experiments, remains to be confirmed.

The question of the possible or necessary association of several oligopeptide-binding proteins to ensure efficient peptide transport has been raised by several observations, particularly in streptococci, which possess three homologous binding proteins [5, 6, 15]. The three oligopeptide-binding proteins were able to work either as three independent transporters or as a unique transport system with three binding and interacting proteins. In *S. gordonii*, the different types of *hppG* mutations indeed gave rise to different phenotypes. When the mutation caused an absence of HppG, protein peptides were transported, but when the mutation led to a truncated HppG protein, peptide transport no longer occurred. It was as if the truncated protein was inhibiting peptide binding by disturbing the possible association of several oligopeptide-binding proteins (fig. 5) [6]. The putative association of several identical or homologous oligopeptide-binding proteins with only one OppBCDF system is consistent with the higher stoichiometry of OppA.

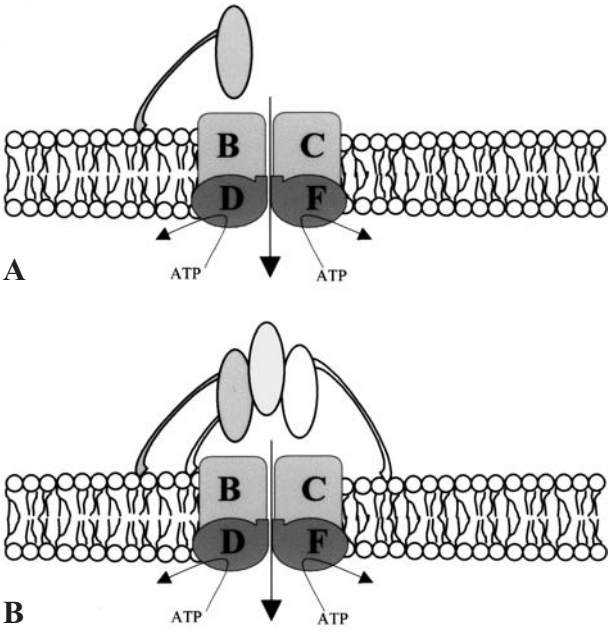

Figure 5. Possible oligopeptide-binding associations with the oligopeptide transport system in streptococci. (*A*): One oligopeptide-binding protein/one transport system ratio; (*B*): three oligopeptide-binding proteins/one transport system ratio.

Substrates and roles for OppA

Because it is responsible for peptide uptake, the oligopeptide transport system plays an essential role in both nutrition and sensing by providing bacteria with nutrients and information on the state of the environment. On the basis of this information, the bacteria can adapt to any changes. In addition, oligopeptide-binding proteins may also be involved in adhesion and chaperoning.

Nutrition and recycling

In terms of nutrition, the best-documented roles of OppA are, first, capture of peptides as a source of the amino acids necessary for growth, and, second, binding of muropeptides for recycling. The internalisation of peptides for nutrition is essential, especially when bacteria are auxotroph for several amino acids. In this context, OppA is responsible for the binding of a variety of α-linked peptides. The nutritional role of OppA has been demonstrated through observation of the growth of mutants in media with different nitrogen sources. In *L. lactis*, an $OppA^-$ mutant grows similarly to the wild-type strain, with amino acids as its nitrogen source. It has been, however, shown that its growth is completely abolished when an essential amino acid is supplied in a tetrapeptide [23]. In bacteria carrying several copies of *oppA* genes, the multiplicity of oligopeptide-binding proteins renders the effects of mutation less drastic. In *S. gordonii*, oligopeptides binding protein-negative mutants grow similarly to the wild-type strain when amino acids are supplied as the nitrogen source, while in more complex media, such as BHY or TY, the doubling time of an *hppA* mutant is 1.8-fold higher than that of the wild type. Conversely, mutations in *hppG* and *hppH*, alone or in combination, do not affect the growth of cells in a complex medium [6]. Mutations in *amiA* genes from *S. thermophilus* have a different effect on growth in milk, a medium which contains insufficient free amino acids to sustain streptococcal growth. The growth rates of AmiA1 and AmiA2 mutants are not significantly different from that of the wild-type strain, while those of AmiA3 and AmiA1AmiA2AmiA3 mutants are significantly reduced (fig. 6) [15]. These two examples demonstrate the lack of equivalence of different copies of oligopeptide-binding proteins in terms of their specificity or levels of expression, even when they are almost identical. Similarly, the

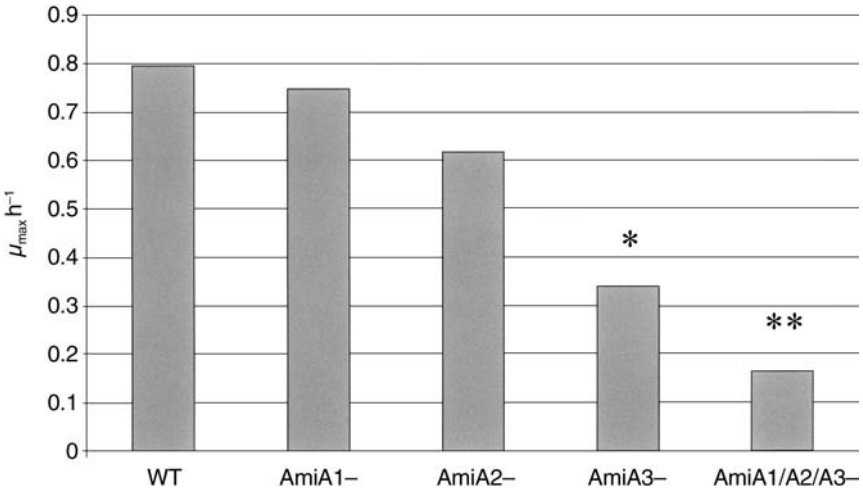

Figure 6. Growth rates (μ_{max}) of *S. thermophilus* wild-type strain and *amiA* derivative mutants in milk. From [15].

authors suggested that in *Treponoma denticola*, where the *oppA* mutant still grows like the wild-type strain in a medium containing yeast extract, OppA forms part of a redundant system [56].

The second type of ligand known for oligopeptide-binding proteins are muropeptides. Two independent systems involved in muropeptide recycling have been reported in *E. coli*. In the first, the single-component permease AmpG specifically imports muropeptides containing the disaccharide *N*-acetylglucosaminyl-β-1,4 anhydro-*N*-acetylmuramic acid. This system works independent of the peptide side chain and peptide chain length [57, 58]. The second system involves the oligopeptide transport system and works with MppA as binding protein. Tri- and tetra-muropeptides do, indeed, bind to the oligopeptide-binding protein MppA in *E.* and *S. typhimurium*. In the latter, up to 50% of the muropeptides are recycled per generation. The OppMppA system has been identified as the only means by which *E. coli* efficiently internalises cell-wall peptides such as L-Ala-γ-D-Glu-meso-diaminopimelic acid, even though it transports α-linked tripeptides poorly. An earlier paper reported that an OppA mutant could no longer transport muropeptides [59], but a different group has since demonstrated that another protein, MppA, a paralog of OppA which has evolved towards affinity for muro-tripeptides, is responsible for efficient cell-wall peptide internalisation [16]. The first observation may have been due to the polar character of the *oppA* mutation affecting the entire *opp* operon.

Sensing

Sensing and signalling via oligopeptides seem to be specific to Gram$^+$ bacteria, while Gram$^-$ bacteria prefer to communicate via *N*-acylhomoserine lactones, even if, in some cases, they also use dipeptide transport to induce chemotaxis [60]. However, because Gram$^-$ bacteria possess an oligopeptide transport system similar to that of Gram$^+$ bacteria, their ability to sense the environment using oligopeptides cannot be completely excluded. In Gram$^+$ bacteria, secreted signal oligopeptides can act either from the outer part of the membrane, generally by activating two-component systems, or in the cytoplasm after internalisation by the oligopeptide transport system. In the context of this review, we will focus on this latter group of transported peptides. The role of oligopeptide-binding proteins, and more generally of the whole oligopeptide transport system, in sensing is complex. Two different, but probably not exclusive pathways have been shown to explain how Gram$^+$ bacteria use internalised oligopeptides to react to environmental changes.

The first pathway works via nonspecific peptides and intracellular amino acid pools. This strong hypothesis agrees with many observations of *opp* mutations often being linked with highly pleitropic phenotypes. Opps internalise peptides, which, in the cell, are hydrolysed into amino acids by a set of peptidases. Opp activity increases the pool of amino acids, which in turn activates global regulators such as Lrp or CodY, as mentioned above in the section on regulation. These global regulators act on the group of genes belonging to their regulons, leading to extended modification of bacterial metabolism [61] (fig. 7 A). This hypothesis provides an explanation for indirect modification of adhesion properties observed following mutation in genes encoding oligopeptide-binding proteins and which are caused by the modulation of adhesion gene expression. In *S. gordonii*, expression of the *cshA* gene, encoding an adhesin, is indeed repressed in an *hppa* mutant [62]. Similarly, mutations in *amiA* and *plpA* (= *aliA*) from *Streptococcus pneumoniae* modulate adhe-

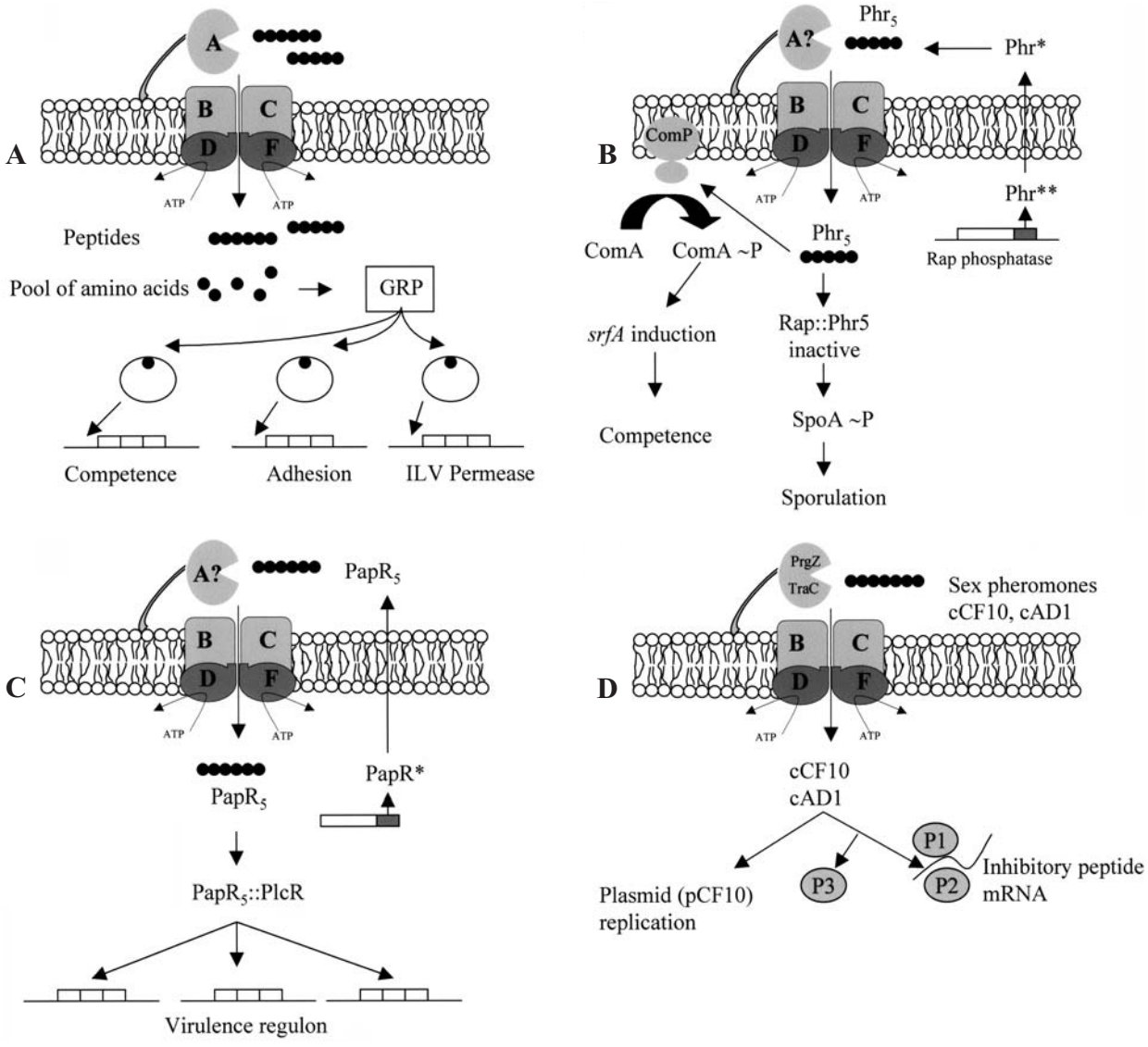

Figure 7. Examples of peptide-mediated signalling involving the oligopeptide transport system. *A, B, C, D, F* represent the different components of the Opp system. (*A*): Signalling via nonspecific peptides and inducing pleiotropic effects. GRP, global regulatory protein; ILV, isoleucine, leucine, valine. From [61]. (*B*): Competence and sporulation signalling via Phr peptides in *B. subtilis*. Phr**, native Phr; Phr*, processed and exported Phr; Phr_5, Phr pentapeptide form. Rap::Phr_5, Rap phosphatase-Phr_5 peptide complex. From [84]. (*C*): Virulence regulation via PapR peptide in *Bacillus*. PapR*, native peptide; $PapR_5$, PapR pentapeptide; PapR5::PlcR, PapR pentapeptide-PlcR virulence regulator complex. From [72, 73]. (*D*): Conjugation regulation via cCF10/cAD1 peptides in *E. faecalis*. PrgZ, TraC, specific pheromone-binding proteins; P1, P2, P3, proteins interacting intracellularly with the pheromones. From [17].

sion of bacteria to eukaryotic cells via a mechanism which is unknown [63, 64]. The likely link between the transport of oligopeptides and global regulators may also explain the lower intracellular survival of $OppA^-$ *Listeria monocytogenes* in macrophages after phagocytosis when compared with an $OppA^+$ strain [20]. The induction of competence is probably also partially explained by modulation of the amino acid pool. Competence is a physiological status which always occurs at the same cellular density and allows bacteria to import DNA. More precisely, in *B. subtilis*, the link between oligopeptide transport and the general metabolic regulator, CodY, has also been established, since in an *opp* mutant, the CodY-dependent repression of two competence genes (*srfA* and *comK*) was lost [65]. The link between oligopeptide transport and competence has been clearly established for several naturally competent bacteria and more specifically in *B. subtilis,* where a *spo0KE* (= *oppF*) mutant has exhibited a 1000-fold reduction in competence [66]. Involvement of the different components of the oligopeptide transport system, Spo0KA, B, C, D, has been demonstrated, since a nonpolar deletion in each of their encoding genes led to mutants which were affected in terms of competence and sporulation [67]. The regulation of com-

Table 3. Signal peptides transported via oligopeptide transport systems.

Name	Sequence	Bacterial species	Oligopeptide-binding protein	Reference
Virulence factors				
PapR	LPFEF LPFEY MPFEY VPFEY VPYEY VPFEF	*Bacillus cereus* *Bacillus thuringiensis* *Bacillus anthracis* *Bacillus mycoïdes*	OppA ?	[72, 73]
Competence and sporulation factors				
PhrA	ARNQT	*Bacillus subtilis*	OppA	[70, 71]
PhrC (or CSF) and other Phr homologues	ERGMT	*Bacillus subtilis*	OppA	
Conjugation factor				
cCF10	LVTLVFV	*Enterococcus faecalis*	PrgZ	[17, 76, 78]
cAD1	LFSLVAG	*Enterococcus faecalis*	TraC	

petence has been precisely described in *S. pneumoniae*. It depends on a quorum-sensing mechanism induced by a 17-amino acid competence-stimulating peptide (CSP) which is exported. In this bacterium, the $AmiA^{-}AliA^{-}AliB^{-}$ triple mutant does not, like the wild-type strain, display a sharp peak of competence but, rather, lower and constant competence throughout the whole exponential growth phase. In addition, an *aliB* plasmid insertion mutant no longer exhibits any competence. However, even though the link between competence and oligopeptide transport has been demonstrated, competence can be still induced in these mutants by incubating cells with synthetic competent-stimulating factor (CSP), thus suggesting that oligopeptide-binding proteins do not bind CSP [68, 69].

The second possible pathway for oligopeptides in sensing is via specific peptides. So far, only three groups of such peptides have been described, and they are linked to competence, sporulation, virulence and conjugation processes (table 3). These peptides, secreted as precursors, processed and secreted by bacteria, are then taken back by the Opp system in bacteria, where they interact with specific targets. In most cases, the essential role of the Opp system in the transport of these specific peptides has been demonstrated. However, the oligopeptide-binding proteins involved in the binding of specific peptides have not always been unambiguously identified.

In *B. subtilis*, two parallel cell-cell signalling systems involving secreted peptides have been described, one involving the ComX pheromone, which interacts at the surface of the membrane and induces a phosphorelay cascade in the cell, and a second involving Phr peptides, which are competence- and sporulation-activating factors and which need to be transported into the cell via Opp to reach their targets. These peptides (PhrA and PhrC, also called CSF for competence and sporulation factor) act similarly and probably have similar functions. Their encoding genes are linked to Rap phosphatase-encoding genes. The peptides are produced from 40- and 44-amino acid precursors which are processed and secreted in the medium. Their mature forms are pentapeptides which are taken up in the cell by the Spo0K system. Different effects on the induction of competence by PhrC have been observed by measuring induction of the *srfA* gene and they are dependent on PhrC concentration. At low levels, i. e. within the range 2 – 10 nM, PhrC stimulates *srfA* expression, while at higher levels, i. e. 20 nM, it inhibits this expression. Alanine substitution at each position of the tetrapeptide demonstrates that each position is important to the stimulation of *srfA* expression, while only the second and fourth positions are essential for inhibition. For the sporulation process to occur, all tetrapeptide positions (except the third) are of importance. These observations strongly suggest that the PhrC peptide has different targets in the cell which affect gene expression. Two of these have been identified: (i) in the case of sporulation, Phr peptides probably inhibit Rap phosphatases, thus affecting the level of Spo0A~P, the key transcription factor required for the initiation of sporulation, and (ii) in the case of competence, they may activate the ComP histidine protein kinase, which is required for the phosphorylation of ComA and subsequent activation of *srfA*, an early gene of competence [70] (fig. 7 B).

Differences in the ranges of concentration of signalling peptides (nM range) and nutritional peptides (mM) have led to the question about the transport and receptor role of the Opp system. In the case of PhrC, its ATP-dependant transport by Opp has been clearly demonstrated, thus answering this question. Several other putative genes encoding Phr homologues have been identified in the genome of *B. subtilis*, suggesting that this bacterium possesses multiple Phr peptides. But the significance of this excess is still unknown [71]. The production of a PhrA peptide inside the cell does not totally suppress the sporu-

lation defect in a *spo0K* null mutant, thus suggesting another role for the Spo0K oligopeptide transport system in addition to Phr peptide transport, that of initiating sporulation [70].

Peptides transported by the Opp transport system may also constitute virulence activators. In the *Bacillus cereus* group, PlcR regulates a large regulon composed of genes encoding at least 15 extracellular virulence factors, including phospholipases C, enterotoxins and proteases. Activation of this regulon is partly responsible for the opportunistic pathogenicity of *B. cereus* and *Bacillus thuringiensis*. A mutation in the *opp* operon abolishes the expression of the PlcR regulon and consequently reduces the virulence of these bacteria, suggesting that Opp transports a signal involved in PlcR regulon activation. The disruption of a short open reading frame downstream of *plcR* prevents expression of the PlcR regulon. The product of this gene, PapR, has been characterised in greater detail: it appears to be secreted, processed and transported via Opp back into the cell where it is active as a pentapeptide (LPFEF), the sequence of which varies slightly within the *B. cereus* group. PapR is necessary for PlcR to bind to its DNA recognition sites and probably increases the affinity of PlcR for its DNA target. It may, thus, induce a conformation change [72, 73] (fig. 7 C). Complementation experiments between a *B. thuringiensis ΔpapR* and other *Bacillus* strains have shown that the activation of PlcR by PapR is strain dependent [73]. Furthermore, PapR is completely different from another peptide, AgrD, which acts at the outer part of the membrane and induces virulence in *Staphylococcus aureus* [74].

Conjugation in *Enterococus faecalis* is the third example of a peptide-mediated process via Opp which allows the acquisition of new properties, including antibiotic resistance, by these bacteria. *E. faecalis* is endowed with several conjugative plasmids, the transfers of which are made possible by peptide pheromones secreted by recipient cells lacking these plasmids. Two plasmid/pheromone couples have been studied in detail: pCF10/cCF10 and pcAD1/cAD1. Pheromones are small hydrophobic peptides with no acidic or basic residues whose amino acid sequence is a critical determinant in biological activity. *E. faecalis* is extremely sensitive to the induction of pheromones which are very active at concentrations as low as the picomolar range [75]. The sequences of cCF10, cAD1 and their related peptides are always found in the carboxy-terminal end of prolipoprotein signal peptides and are processed by several proteolysis steps before being secreted [76]. The essential role of the Opp system in transporting these pheromones has been demonstrated. Disruption of the *opp* operon has a deleterious effect on cCF10-mediated signalling [17]. However, specific oligopeptide-binding proteins, whose encoding genes are located on the conjugative plasmids, are responsible for the binding of pheromones and recruit OppBCDF to transport the peptides. PrgZ is dedicated to the binding of cCF10, while TraC is responsible for that of cAD1. In the absence of PrgZ, OppA can only replace it if the pheromone concentration is high [17, 77]. In this bacterium, pheromones activate conjugation via a set of *prg* genes (pheromone-responsive genes), including a gene encoding an adhesin which facilitates donor/recipient mating [78]. They probably act by interacting with several effectors (including ribonucleoprotein complexes) and by displacing an inhibitory peptide encoded by *prgQ* on plasmid pCF10 and *iad* on plasmid pAD1 [17] (fig. 7 D). More precisely, response to the cAD1 peptide involves two key regulatory proteins: TraE1, which positively regulates the genes necessary for conjugation, and TraA, which binds DNA and negatively regulates the expression of *traE1*. Direct interaction between TraA and the cAD1 peptide causes TraA release from its DNA binding site. The C-terminal part of TraA is more especially involved in pheromone recognition and differentiation between cAD1, its competitive inhibitor peptide iAD1 and other pheromones [78].

In conclusion, sensing via specific peptides appears to be a highly specific process, recognizing very low levels of peptides. Bacteria are capable of discriminating between peptides and using them as species or strain-specific systems.

Transport of toxic peptides

OppA also binds toxic peptide analogues. This phenomenon occurs naturally and has been exploited for screening and characterisation purposes. Most of the documentation in this field concerns *E. coli* and *S. typhimurium*. Opp from these species are capable of transporting the phytotoxic tripeptide, phaseolotoxin [(*N*γ-phosphosulfamyl) ornithylalanyl homoarginine] produced by *Pseudomonas syringae*. The toxic peptide inhibits the ornithine carbamoyltransferase and is toxic to both bacteria and plants [79]. OppA is also a good target for *E. coli* in its fight against aminoglycoside antibiotics (kanamycin, streptomycin, isepamycin, neomycin). Two independent studies have analysed the OppA content of *E. coli* strains (including clinical strains) resistant to aminoglycosides, and they revealed that the majority of these resistant strains displayed reduced or undetectable levels of OppA [80, 81]. Mutations were due to nonsense mutations or a reduction in OppA synthesis at the translational level. These results support the suggestion that aminoglycoside antibiotics are transported into bacteria via the *opp* system and that transport plays an important role in resistance to such antibiotics. It is interesting to note that mutants expressing low levels of OppAs have also frequently been found to be defective in ornithine decarboxylase and arginine decarboxylase. They are, consequently, weak producers of polyamines which stimulate OppA expres-

sion. Aminopterin (4-aminopimelic acid) has been used successfully as an oligopeptide binding protein substrate in (amongst others) *S. pneumoniae* and *S. thermophilus*. The AmiA-negative mutants available in these two species have been used to demonstrate that AmiAs are responsible for the binding of this peptide analogue [5, 15].

Transport of other molecules

Oligopeptide-binding proteins probably bind other, as yet unidentified, substrates. As an example, their possible role in the transport of cryoprotectants has been suggested by the observation that a specific promoter for *oppA* from *L. monocytogenes* is activated at low temperatures [20].

Other roles: adhesion and chaperoning

Finally, in addition to the peptide transport function of oligopeptide-binding lipoproteins described above, another direct role in adhesion to host cells has been demonstrated for the OppA of the cell-wall free bacteria *Mycoplasma hominis* [13]. This observation confirms that lipoproteins may, as cell-surface molecules, mediate the adhesion of bacteria to different substrates, tissues or environments [82]. In the pathogen *Treponoma denticola*, OppA is also capable of binding soluble fibronectin and plasminogen, thus suggesting a role for OppA in interactions with host tissues [56].

Several binding proteins, including OppA, are capable of acting in *E. coli* as chaperones involved in protein renaturation after stress. In vitro, OppA promotes the folding of citrate synthase and α-glucosidase after urea denaturation and prevents aggregation of citrate synthase under heat shock conditions. Both liganded and free forms of OppA display this chaperone-like function, suggesting that in addition to its function in transport, OppA from *E. coli* is probably implicated in protein folding and protection from stress in the periplasm [83].

Conclusions and perspectives

Unquestionably, a wealth of knowledge has been gained over the past 15 years on how bacteria sense their environment and capture elements from it. During these sensing/capture processes, both receptors and binding proteins located at the outer surface of the bacterial membrane constitute key elements because they are the first to bind extracellular signals. Oligopeptide-binding proteins are known to bind several classes of peptides necessary to bacterial life: nutritional and signalling peptides. The nutritional role of oligopeptide-binding proteins is not clearly understood and is all the more important since bacteria are auxotroph for amino acids. The signalling role of oligopeptide-binding proteins has been demonstrated more recently, and further revelations in this field are expected in the near future. We still need to understand how oligopeptide-binding proteins achieve such efficient binding of signal peptides which are present at very low concentrations, how specialised they are, what the level of their specialisation is, what the significance of their high stoichiometry is and how important they are to the specificity of cell-cell signalling. Ultimately, we will also need to determine whether nonvirulent, nonsporulating and noncompetent bacteria or Gram$^-$ bacteria use their oligopeptide-binding proteins to communicate and if so, what messages they emit.

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