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ENGINEERING SIGNAL TRANSDUCTION  
PATHWAYS IN BACTERIA

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*Εἰς μνημὴν τοῦ πατέρα μου Ματθαίου Μιχαλοδημητρακῆ*

*In memory of my father Matthaios Michalodimitrakis*

## ***Acknowledgements***

My father was a physicist with remarkable teaching abilities; although we were very young children, he managed to present to us in a very simple and intriguing way basic concepts of science. Thus science appeared to my eyes like a magic key, revealing the true nature of the world, which I had to have. Due to my father's influence, I could never imagine myself away from science. Although advised by my father not to take everything for granted and to question all theories that I came across, I started my journey in the world of science having a very romantic and idealistic concept of how the world of science is. Unfortunately I started this journey alone, having lost my father in a car accident when I was only 13 years-old.

Keeping my father's advice in mind helped me during my first steps in the scientific world, as an undergraduate chemistry student, to keep an open mind and have a lot of constructive discussions, sometimes even arguments, with my professors and get even more involved in science. However the deeper I get into science the more the myth around it, that I had created in my mind, falls apart. Science is far from the ideal icon that I had in my mind, something that I realised the "hard way" during the last year of my undergraduate studies and during my post-graduate studies. During my stay at EMBL I have had a plethora of stimuli and experiences, in all aspects of my life, which helped me mature as a person and start seeing things in a more realistic manner. I never regret following my dreams or for any of the choices I have made, although now that I have a more clear picture of the scientific life, I feel the need to re-consider my priorities and re-define the path I will follow.

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## *Abbreviations*

PCR-ATW	Polymerase Chain Reaction Around-The-World
aTc	Anhydro-tetracycline
Lrp	Leucine responsive protein
MBP	Maltose Binding Protein
SA	AT142 cells carrying Taz and pR2
CB	Chemotaxis Buffer
TetO1	TetR repressor operator 1 site
R	Ratio of YFP/CFP fluorescence
TB	Tryptone Broth
wt	Wild-type
aa	Amino acid
RMS	Root-Mean-Square
CRP	cyclic AMP receptor protein

## ***SUMMARY***

Engineered proteins have proven to be a very powerful tool both for basic research and industry. The next step in this direction is engineering biological pathways, such as signal transduction, which can set the foundations for a broad range of applications ranging from gene therapy to introducing novel properties in organisms. However a lot of biological processes are subjected to a lot of noise, which for most applications is not desired. For example, within a population of cells, different cells can express a gene at different levels ranging from zero to high expression. Therefore it is very important to design pathways whose output can be tightly controlled. Such a challenge is not easy to tackle, so before trying to addressing it in complex organisms, it is better to start from “simple” well-studied ones, like signal transduction in *E.coli*.

Signal transduction in *E.coli* takes place mainly through two-component systems, consisting of a sensor-kinase and a response-regulator, which usually is a transcription factor, with few exceptions like CheY from the chemotaxis signalling pathway. In this study two of the best characterised signalling systems of *E.coli* are combined through a chimeric sensor, in order to construct a novel pathway: the ligand-binding domain of the aspartate receptor, Tar, from the chemotaxis pathway is combined with the catalytic domain of the osmosensor EnvZ resulting in the chimeric receptor Taz. Taz can activate gene expression from the porin promoters through phosphorylation of the response regulator OmpR, upon binding of a proper ligand, like aspartate.

The aim of the study was to use this system as a template in order to construct a pathway whose output would be controlled by a gene circuit and to change the input signal through rational design. As an output a destabilised GFP was chosen and different circuitry were designed: competition of OmpR-P with TetR repressor, expressed from a synthetic promoter, for activation of the *pompC* promoter, expression of anti-sense RNA for the reporter gene (GFP) and finally a gene toggle switch, established by TetR and a temperature sensitive CI, which would be activated by OmpR-P and provide the tightest regulation of the output. Despite the different approaches used it was impossible to obtain stable constructs with sufficient promoter strength to have any effect compared to the simple reporter *pompC*-GFP.



Rational design using Perla and Fold-X algorithms and SwissPdbViewer program was implemented on the crystal structure of the periplasmic domain of Tar in complex with aspartate. Although the power of this approach seemed very promising, when it was tested to predict the effect of mutations in the wild-type chemotaxis system, it was not successful in the case of changing the chimera's ligand. This can be attributed to the fact that in the chimera the periplasmic moiety has a different conformation to the one in the wild-type receptor, caused by the fusion to the cytoplasmic domain of EnvZ. The latter is supported by experimental results showing that Tar and Taz do not have exactly the same sensing properties i.e. some signals of the same type for Tar can elicit opposite responses through Taz.

However during the study it was necessary to re-examine in more detail the properties of Taz, leading for the first time to the discovery of ligands (amino acids) which can inhibit the receptor. This inhibition is proven to be stereo-specific and extremely tight, dominating even the effect of activators like aspartate. Furthermore, the intrinsic properties of the Taz-OmpR system found in this study gave a different perspective for the wild-type systems: activation of the EnvZ-OmpR system in *E.coli* during cell growth seems to take place through EnvZ and the catalytic domain of EnvZ is not sufficient to account fully for it. The most important finding was for the chemotaxis system where some of the inhibitory amino acids for Taz gave a novel complex response and revealed the existence of a yet uncharacterised adaptation pathway.

*Part I*

*Introduction*

Signal transduction in bacteria takes place mainly through two-component systems. The classical paradigm of such a system consists of a sensor protein and an effector protein called response regulator (Mizuno, 1998). The sensor is a kinase, usually a dimer, which utilises ATP to undergo upon sensing the proper signal *trans* auto-phosphorylation on a histidine residue which resides in a highly conserved domain, named Hpt (**H**istidine **p**hospho-transfer domain, Aravind and Ponting, 1999) or H box (Parkinson and Kofoid, 1992). The phosphate group is subsequently transferred to an aspartate of the response regulator (RR) which causes a conformational change and/or a change in the oligomerisation state of the response regulator leading to a change in its activity. The RR is usually able to dephosphorylate itself, but the presence of the sensor (or sometimes of another protein) increase the dephosphorylation rate. It is worth noticing that the metal ion required for the phospho-transfer reaction resides in the RR. Therefore the RR seems to be the sensor's phosphatase and the phosphorylated RR is an enzyme intermediate with intrinsic long life-time, compared to other enzyme intermediates (Perraud *et al*, 1999). From this point of view the enhancement of dephosphorylation by the sensor can be due to an allosteric effect or by contribution of some residues in the formation of an active site with better catalytic properties. The latter is supported in cases like the EnvZ-OmpR system, where the reverse reaction (phospho-transfer from phosphorylated OmpR to EnvZ) has been observed (Duta and Inouye, 1996).

The significance of these systems has rendered them a topic of detailed study, generating a lot of qualitative and quantitative information which can be used as basis for engineering new signalling pathways. However, there are still a lot of unknown parameters, even in the best described systems. Combining parts of different pathways can in some cases help to reveal unknown parameters or players, or create novel properties that can be used to engineer new pathways.

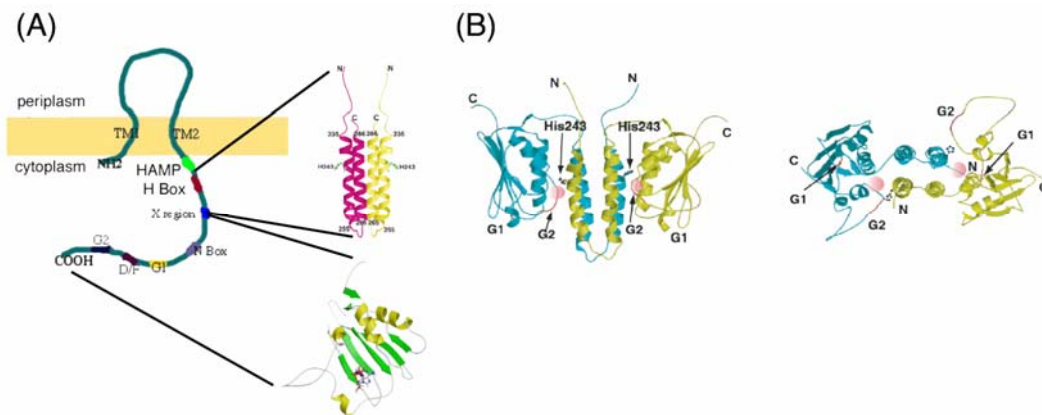
In this study components from the chemotaxis signalling pathway and the EnvZ-OmpR system from *E.coli* are combined in order to by-pass some inherent problems of each system. The new system retains some of the properties of the original systems, but also has novel properties, some of which high-light properties, originating from the

parental systems, which were not described up to now. Before looking into the system used, it is important to look into the two parental systems and their intrinsic properties.

### The EnvZ-OmpR two component system

Although the EnvZ-OmpR system is found only in enterobacteria, its structure is that of the typical two-component system, having extended homology of EnvZ to other histidine kinases and OmpR to response regulators, rendering it a model two-component system. In fact OmpR establishes a family of structurally homologous transcription factors (Itou and Tanaka, 2001).

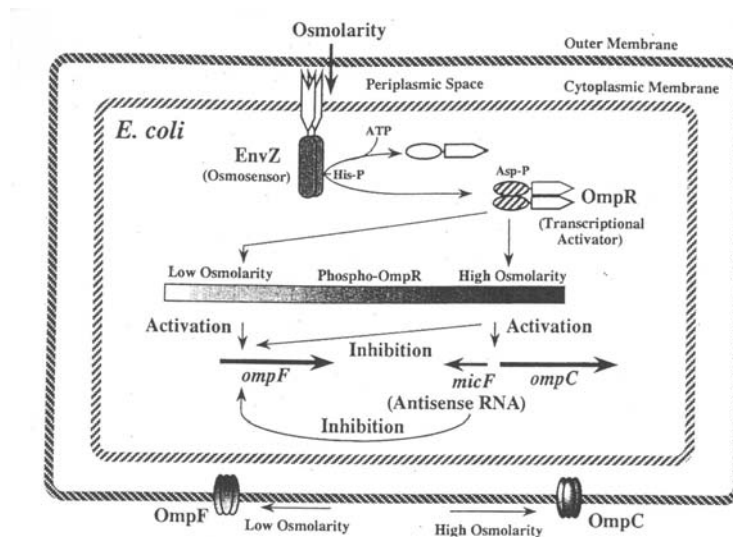
The main stimulus of the EnvZ-OmpR system in *E.coli* is the osmolarity of the environment. This system controls the expression of the porins OmpF and OmpC (Lan, and Igo, 1998). Like all two-component sensors, EnvZ has the ability of trans-autophosphorylation and transfer of the phosphate group to OmpR, yielding OmpR-P, which is the active form of OmpR. Moreover it plays a crucial role in the dephosphorylation of OmpR-P. A schematic drawing of EnvZ depicting the characteristic domains of Histidine kinases and a model for the trans-phosphorylation, based on the available crystal structures, is shown in fig.I-1.



**Fig I-1.** (A) Schematic drawing of the EnvZ monomer depicting the conserved regions in histidine kinases and the available crystal structures for the dimerization and catalytic domain.(adapted from Hsing *et al*, 1998 and Tomomori *et al*, 1999) (B) Model of the transphosphorylation based on the available crystal structures. The pink sphere represents ATP (adapted from Cai *et al* 2003)

Thus EnvZ's conformational state will determine the balance of the two opposing reactions, leading to a certain concentration of OmpR-P. Under low osmotic pressure, this concentration is sufficient to activate transcription mainly of the porin which forms a pore with bigger diameter, OmpF. As the osmolarity increases, the concentration of OmpR-P increases as well, leading to activation of transcription of OmpC (fig.I-2). This occurs through co-operative binding of OmpR-P to three sites upstream the promoter (*pompC*) termed C1, C2 and C3 (Mizuno and Mizushima, 1986). Simultaneously, OmpF is down-regulated by the anti-sense RNA *micF* and by binding of OmpR-P to an inhibitory low-affinity site, so that the sum of the two different porins remains constant (Mizuno, 1998). This is true only until the mid-logarithmic phase of cell growth, after which there seems to be an activation of transcription of both porins through an unknown mechanism (Martinez-Flores *et al*, 1995).

Despite the fact that we know a lot on the system, the actual way that EnvZ senses osmolarity remains elusive, rendering EnvZ inappropriate for engineering, i.e. since the exact signal is unknown it is impossible to try to change the specificity of the receptor towards a chosen small molecule, through rational design.

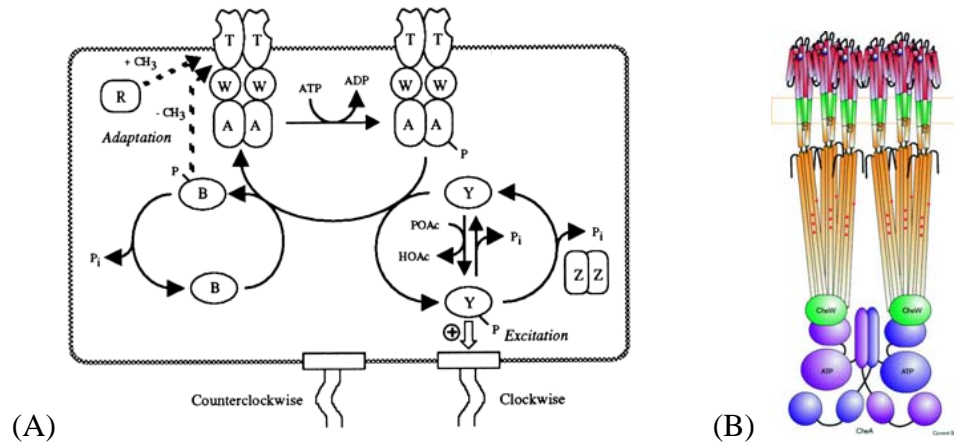


**Fig. I-2.** The EnvZ-OmpR system. Increase in the medium osmolarity increases the level of OmpR-P to levels that activate transcription of the porin OmpC and repress transcription of OmpF. The anti-sense RNA for OmpF, *micF*, ensures a tight regulation of OmpF expression. Adapted from (Mizuno, 1998)

### **The chemotaxis signal transduction pathway in *E.coli*.**

*E.coli* have the ability to sense and swim towards higher concentrations of nutrients (attractants) and away from higher concentrations of noxious chemical compounds (repellents). The way they achieve this is the following (Alberts *et al.*): *E.coli* have multiple flagella. When the flagella rotate counter-clockwise they all form a bundle allowing the cell to swim towards a certain direction, whereas when they rotate clockwise they fly apart, so that the cell tumbles chaotically without moving forward. In the absence of any stimuli the rotation changes direction with a certain frequency so that the cell swims smoothly in a straight line which is interrupted by abrupt, random changes in direction, caused by tumbling. In the presence of an attractant the rotation of the motor which controls the flagella will be biased towards the counter-clockwise direction, resulting in longer periods of smooth swimming which help the cell swim towards higher concentrations of the attractant. Similarly, in the presence of a repellent the rotation is biased towards the clockwise rotation which increases the tumbling and results in a change in the direction of swimming, so that in the end the cell moves away from the higher concentration of the repellent.

The bias of the rotation towards either direction is controlled by the binding of the phosphorylated RR CheY (CheY-P) to the motor complex (fig.I-3A): binding of CheY-P to the motor favours tumbling. Therefore the cell chooses its fate by regulating the levels of CheY-P according to the environmental stimuli. The cognate kinase of CheY is CheA but unlike most two-component systems CheA is not a receptor. CheA is a dimer which binds both directly and through the adaptor protein CheW to multiple dimers of chemotaxis receptors (figI-3B). These receptors can sense a plethora of stimuli ranging from small molecules to temperature and pH and transduce the signal to CheA. Attractants lead to a decrease of CheA activity so that the levels of CheY-P are reduced and smooth swimming is favoured. Repellents have the opposite effect. Regulation of the level of CheY-P is completed by CheZ which increases the dephosphorylation of CheY-P and ensures a rapid turn-over of the signalling species, which is necessary for fast responses to changes in the environment.



**Fig. I-3.** (A) Schematic representation of the chemotaxis pathway in *E. coli*. The signalling complex phosphorylates CheY (Y) which regulates the bias of the motor rotation. Repellents increase CheA (A) activity leading to increase of Y-P, which favours tumbling. The system adapts to the stimuli by the CheB/CheR system which controls the methylation state of the signalling complex (B) Schematic representation of the of the signalling complex. The exact stoichiometry is receptor dimers:CheW:CheA dimer  $7\pm 1:3\pm 1:1$ . The receptor homodimers can belong to different types of chemoreceptors. (Adapted from Abouhamad *et al*, 1998 and Stock and Levit 2000).

However, cells should be able to sense not absolute concentrations of the chemical signals but rather changes in their concentration. Otherwise, for example when they encounter an attractant they would keep swimming and eventually move away from it or when they encounter a repellent they would mainly tumble and not escape from it. This means that when the cells are exposed to a certain concentration of a compound they have to adapt to it so that they can later on sense changes in the concentration and keep moving towards the higher concentration of attractant and away from the higher concentration of repellent. Adaptation takes place, within minutes (Niedhart *et al*), through the methylation and de-methylation of specific glutamate residues on the cytoplasmic tail of the chemotaxis receptors. Methylation is carried out by CheR and leads in a increase of CheA activity. De-methylation occurs through CheB, which can also de-amidate some glutamines to glutamates, which are also methylation targets, leading to a decrease of CheA activity. CheB itself is regulated by CheA through phosphorylation. Thus when a cell encounters a given concentration of an attractant the

level of CheA kinase activity will drop and the level of CheY-P will decrease leading to smooth swimming. However, at the same time the level of phosphorylated CheB will drop, leading to an increase of the methylation state of the receptor, restoring CheA kinase activity to the former level. It should be noted that the methylation level of the receptor influences the ligand binding affinity as well.

In order to cover the whole range of chemical stimuli *E.coli* have 5 chemotaxis receptors: two of them, Tsr and Tar, are the most abundant ones and are responsible mainly for amino acid sensing. Trg and Tap which are less abundant are responsible mainly for sensing of sugars and dipeptides respectively and Aer is responsible for oxygen and generally energy taxis. It should be noted that the actual number of signals sensed by each receptor is very high., e.g. Tar is described as the aspartate receptor, because it can sense aspartate as an attractant, but also has other attractants like glutamate, maltose (which is recognised through its complex with the Maltose-Binding-Protein, MBP), high temperature, low pH and repellents like Ni<sup>+2</sup> and Co<sup>2+</sup> (Mowbray and Sandgren, 1998).

The situation gets even more complicated by the fact that the chemoreceptors not only share some common signals, but localise together at the cell pole, forming a heterogeneous lattice (Maddock and Shapiro, 1993) with intrinsic properties. Within the lattice the stoichiometry of the complex is Receptor Homodimer:CheW:CheA dimer 7±1:3±1:1 (Liu *et al*, 1997), where the homodimers could belong to different types of receptors. It has been shown that formation of the lattice enhances the sensitivity of the receptors (Duke and Bray, 1999; Sourjik and Berg, 2002; Mello and Tu, 2003) but also facilitates extensive cross-talking between the different types of chemoreceptors (Gestwicki and Kiassling, 2002,). Thus the sensing properties of each type of receptor within the lattice depend not only on its own expression level, but on the presence and relative amount of the rest of the chemoreceptors (Sourjik and Berg, 2004). Formation of the lattice requires CheA and CheW (Maddock and Shapiro, 1993), indicating that the part of the chemoreceptors involved in the lattice formation is the cytosolic domain. Moreover there are specific residues in this domain which have been shown to participate in the formation of trimers of receptor dimers (Ames *et al*, 2002).



It is worth noticing that, up to now, the assignment of recognition of a compound to a certain chemoreceptor has been done with single or double deletion mutants of some of the receptors. Taking into account the cross-talking of the receptors within the lattice, one could realise that past observations were actually reflecting the properties of a mixture of receptors under the specific experimental conditions and possibly the call of whether a given compound is recognised or not by a certain receptor could sometimes be wrong. As more data are compiled on the interactions of the receptors within the lattice, it becomes more and more clear that chemotaxis studies could give unambiguous results only in strains that express a single chemoreceptor.

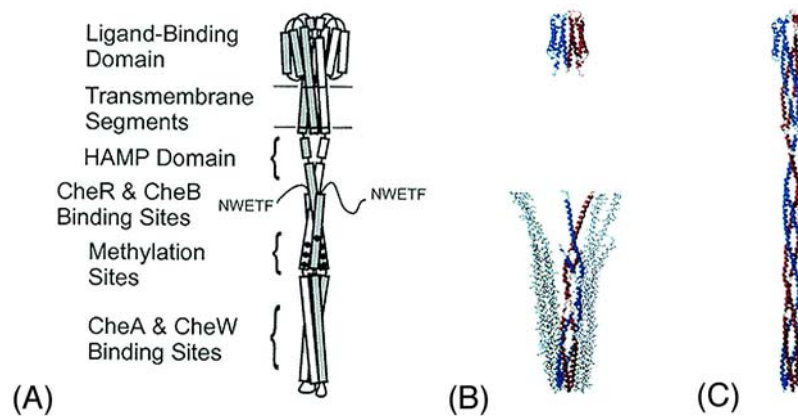
The *E.coli* chemotaxis signalling pathway seems a very promising system for engineering, since there are a lot of experimental data on it. However one of its major draw-backs is that it is not coupled to gene expression, which limits its applications. Furthermore, its complexity, the extensive cross-talking among receptors and adaptation are additional problems for engineering.

The methyl accepting transducers are conserved in many prokaryotes (Nowlin *et al*, 1985), but the signalling network appears to be fine-tuned for each species. A very interesting example is the chemotaxis network of *Rhodobacter sphaeroides* which carries more than one alleles for the *che* genes and has two sites of chemoreceptor localization: one at the cell pole, like in *E.coli*, or at the center of the cell. Although the *che* genes from each locus localize only at one of the two sites (Wadhams *et al*, 2003), the role of multiple *che* genes is even more complicated: since the *cheZ* gene is absent in *R.sphaeroides* the necessity for a phosphatase activity is fulfilled by the existence of an alternative CheY protein which acts as a phosphate sink (Armitage 1999).

It should be noted that from the different receptor domains, the most conserved has been shown to be the cytoplasmic domain, whereas there is great variation in the sensing domain (Wuichet and Zhulin, 2003). This can facilitate the evolution of novel transducers which respond to new environmental stimuli and are readily incorporated in the existing chemotaxis apparatus. However even when all the components of the pathway are conserved, there are still differences, as demonstrated in the case of *E.coli* and *S.typhi*. The dimeric aspartate receptor (Tar) in both cases carries two ligand binding sites and aspartate binding is negatively cooperative. However in *S.typhi* a second aspartate

molecule can still bind, with much lower affinity, to the second site under physiological conditions whereas in *E.coli* it cannot (Biemann and Koshland, 1994).

Such mechanistic differences should be expected to be clarified by the crystal structures of the proteins. However this is far from being true in this particular case. The chemoreceptors have a very high helical content and only parts of them have been crystallized (fig.I-4) Specifically the periplasmic domain of the *E.coli* aspartate receptor ( $Tar_E$ ) in the absence of ligand, the periplasmic domain of the *S. typhi* aspartate receptor ( $Tar_S$ ), both in the presence and absence of ligands (for all the structures see Bjorkman *et al*, 2001) and the cytoplasmic region of the *E.coli* serine receptor have been crystallized (Kim *et al*, 1999).



**Fig. I-4.** (adapted from Weis *et al*, 2003). (A) Schematic representation of a Tar dimer. The two subunits are shown in white and blue (B) Available crystal structures for the periplasmic domain of Tar and cytoplasmic of Tsr. The latter is crystallized as a trimer of dimers. (C) Model of the whole receptor based on the available structures.

However, as will be discussed in more detail in the results and discussion sections, the available structures do not exhibit a significant change in the presence or absence of aspartate, raising the question which is the actual mechanism for signal sensing. Although a lot of different mechanisms have been proposed based on different techniques, with the most widely accepted that of the piston model (Ottemann *et al*, 1999), the real mechanism seems to be unclear as more data are compiled on the interactions between

receptors in the lattice. Furthermore from the vast majority of signals only for some attractants like aspartate, maltose and pH the actual sensing domains have been identified. In the case of repellents the sensing mechanism is completely unknown and it is only extrapolated that it occurs through binding to the receptors, from the behaviour of the attractants. The only proposed mechanisms for repellents is sensing of a) nickel by Tar through the binding of the complex Nickel and Nickel Binding Protein (De Pina *et al*, 1995) b) heavy metal ions through direct binding to the Tar periplasmic region (Scott *et al*, 1993) c) aromatic compounds by binding to the pocket where 1,10-phenanthroline is seen in the crystal structure of the Tar periplasmic domain (Scott *et al*, 1993). However none of these hypotheses has been demonstrated and apart from the necessity of the nickel-binding protein for nickel taxis no binding to Tar of the respective complex has been shown.

The putative structure of the whole receptor, has been generated by combining the crystal structures and modelling of the missing structures (Kim *et al*, 1999). A schematic representation is shown in fig.I-4.

The linker between the second transmembrane region and the cytoplasmic domain is the highly conserved for bacterial transducers HAMP domain (domain present in **H**istidine kinases, **A**denylyl cyclases, **M**ethyl-accepting proteins and **P**hosphatases (Aravind and Ponting, 1999)). The HAMP domain consists of two  $\alpha$ -helices joined by a linker and appears to be crucial for signal transduction, since mutations in this region usually result in locked on or off transducers (Ames and Parkinson, 1988; Appleman and Stewart. (2003)). In the case of the chemoreceptors the HAMP domain plays an additional role: it is involved in the pH sensing (Umemura *et al*, 2002), although the exact mechanism for this is not known.

## Chimeric Systems

Chimeric proteins have proven to be a powerful tool both for elucidating properties of their parental systems but also for generating proteins with novel properties. Roughly, two types of chimeras have been used.

The first type is chimeras of proteins with highly similar function and origin. The most profound examples of this case are the chimeras of the different *E.coli* chemoreceptors with each other. Tar-Tap and Tap-Tar hybrids (Weerasuriya *et al*, 1998) highlighted the importance of the docking site for CheB and CheR in Tar (and Tsr) and Tsr-Tar chimeras shed light on the region of the receptors which is responsible for pH sensing (Umemura *et al*, 2000). In all cases the plasticity of the chemoreceptors well manifested.

The second type of chimeras involves proteins which are not closely related and their properties are emerging from the parental systems, without necessarily being identical. Examples of this case are the chimeras of the chemoreceptors Tar and Trg to the osmosensor EnvZ which are activated, instead of inhibited, upon ligand binding (Utsumi *et al*, 1989; Baumgartner, *et al*, 1994).

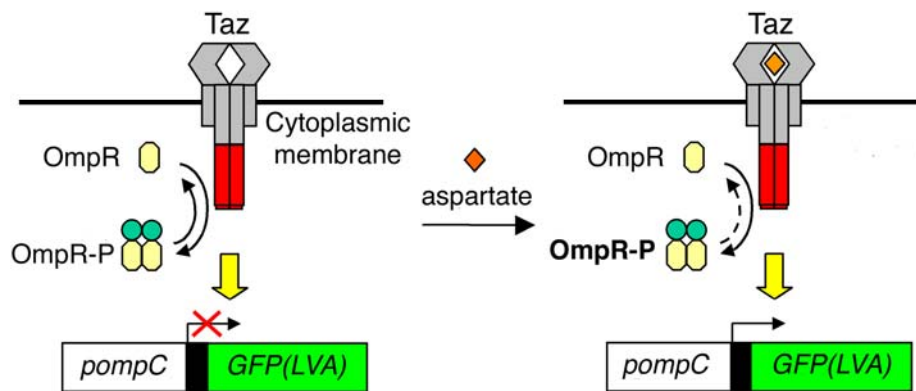
Another example is the chimera of the sensor that is involved in nitrate and nitrite utilization, NarX and Tar (Ward *et al*, 2002). This chimera functions as a chemoreceptor which senses the ligands of NarX, nitrate and nitrite, as repellents.

The most intriguing and extreme example is the chimera between Tar and the insulin receptor which is functional and is activated by Tar's ligand, aspartate (Moe *et al*, 1989).

### The Taz-OmpR system

As mentioned above, using the chemotaxis signalling pathway, for which the signals are known, as a "template" to engineer a signalling pathway fails at the level of coupling the pathway to transcription. The EnvZ-OmpR exhibits exactly the opposite problem: although it is coupled to transcription the exact signal is unknown. These problems can be circumvented by fusing the two pathways so that known signals from the first pathway can lead to an effect on gene expression through the second pathway. Such a fusion of the two pathways has already been demonstrated with the construction of Taz.

Taz is a chimeric receptor which consists of the transmembrane, periplasmic and linker region of the aspartate receptor Tar and the cytoplasmic dimerisation and catalytic domain of EnvZ (EnvZ-C) (Utsumi *et al*, 1989). In the absence of aspartate, the ratio of kinase/phosphatase activities of Taz is such that the level of OmpR-P is insufficient to activate transcription from *pompC*. Upon aspartate binding, the phosphatase activity is down-regulated, while the kinase activity remains constant, leading to an increase of OmpR-P and activation of transcription (Jin *et al*, 1993). Therefore the output of the pathway can be easily monitored by having a reporter gene, like GFP, expressed from *pompC*.



**Fig. I-5.** The Taz-OmpR system. Taz is involved in both phosphorylation and dephosphorylation of OmpR. In the absence of a signal the ratio of the two catalytic activities cannot lead to sufficient OmpR-P levels for transcription from the *pompC* promoter. Upon aspartate binding the ratio changes through a decrease in phosphatase activity leading to transcription from *pompC*, in this case of GFP.

Fusion of the two receptors gives more advantages than simply coupling a pathway to transcription. Since Taz lacks the cytoplasmic region of Tar, it has at least two new very important properties:

- a) the methylation sites are absent and thus Taz is not subjected to adaptation which would take place too fast to allow a response on the transcription level.

- b) the residues which participate in the trimer of receptors dimers and lattice formation are absent, suggesting that Taz probably does not take part in oligomerisation with the chemoreceptors and probably does not cross-talk with them, at least not in the manner that wild-type Tar does.

All the aforementioned advantages could make the Taz-OmpR system appear to be ideal for studying the sensing properties of the periplasmic domain of Tar in an easy way, uncoupled from the effect of the other chemo-receptors. However things are not that simple, as Taz seems not to retain all of the sensing properties of the periplasmic domain of Tar, i.e. Taz fails to sense maltose and the sensitivity of the system for Asp is approximately 1000-fold lower than that of the wild-type chemotaxis signalling pathway (Utsumi *et al*, 1989; Baumgartner, *et al*, 1994). This could be due to various reasons like the fact that the adaptation machinery and lattice formation contribute significantly to the sensitivity of the wild type system. Moreover a subtle conformational change in the periplasmic domain caused by the fusion of the two proteins could alter the affinity for the ligands.

It should be noted that the case of maltose is rather intrinsic. The affinity of the wild type Tar for the maltose-MBP complex is very low ( $K_d = 200 \mu\text{M}$ , Manson *et al*, 1985), but this is compensated by the high concentration of MBP in the periplasm (1 mM Manson *et al*, 1985) and the localisation of Tar, which increases the active concentration of binding sites. In the case of Taz, under the experimental conditions used in the original work on Taz, MBP might not be expressed, since OmpR-P represses expression of the activator of MBP expression encoded by the gene *maltT* (Case *et al*, 1986). Furthermore, a similar decrease in affinity for maltose to that for aspartate would require an extremely high concentration of MBP in order to elicit a response. Therefore it is not completely clear if Taz actually fails to sense maltose or if it is a matter of choosing the appropriate conditions.

Nevertheless, the fact that Taz still responds to aspartate makes the Taz-OmpR system a good “template” to build a novel pathway: the availability of the crystal structure of the aspartate-Tar periplasmic domain gives the opportunity to try to change ligand specificity through rational design and the output of the system can be optimised by building different gene circuits to control it.

*Part II*

*Materials and Methods*

## Bacterial strains and plasmids

The strains used are listed in table II-1.

**Table II-1.** Bacterial strains. For the commercially available ones the supplier is given as a reference

strain	genotype	reference
AT142	<i>F</i> $\Delta$ lacU169 <i>araD rpsL relA thi flhD5301 <math>\Delta</math>envz::<i>Km<sup>r</sup></i></i>	Mizuno and Mizushima, 1987
RP437	<i>thr(Am)1 leuB6 his-4 metF(Am)159 eda-50 rpsL1356 thi-1 ara-14 mtl-1 xyl-5 tonA31 tsx-78 lacY1 F<sup>-</sup></i>	Parkinson and Houts, 1982
UU1250	<i>(aer)</i> $\Delta$ 1 <i>yjgG::Gm (tsr)</i> $\Delta$ 7028 <i>(tar-tap)</i> $\Delta$ 5201 <i>zbd::Tn5 (trg)</i> $\Delta$ 100 <i>leuB6 his-4 rpsL136 thi-1 ara-14 lacY1 mtl-1 xyl-5 tonA31 tsx-78</i>	Ames <i>et al</i> , 2002
VS104	RP437 $\Delta$ cheYcheZ	Sourjik and Berg, 2004
VS181	UU1250 $\Delta$ cheYcheZ	Sourjik and Berg, 2004
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacI<sup>q</sup>ZAM15 Tn10(Tet<sup>r</sup>)]</i>	Stratagene
BL21(DE3)	<i>E.coli B F dcm ompT hsdS(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal <math>\lambda</math>(DE3)</i>	Stratagene
JM110	<i>RspL thr leu thi-1 lacY galK galT ara tonA tsx dam dcm supE44 <math>\Delta</math>(lac-proAB) [F' traD36 proAB lacI<sup>q</sup>ZAM15]</i>	Stratagene
Top10	<i>F mcrA <math>\Delta</math>(mrr-hsdRMS-mcrBC) <math>\phi</math>80lacZAM15 <math>\Delta</math>lacX74 deoR recA1 araD139 <math>\Delta</math>(ara-leu)7697 galU galK rpsL (Str<sup>R</sup>) endA1 nupG</i>	Invitrogen



Plasmids used as a backbone, as a source for genes or provided by a collaborator are listed in table II-2.

**Table II-2.** Plasmids. For the commercially available ones the supplier is given as a reference

Plasmid	ori	resistance	reference
pCL1920	pSC101	Str, Spc	Lerner and Inouye, 1990
pBAD33	p15A	Cm	Guzman <i>et al</i> , 1995
pBluescript KS	pUC	Amp	Stratagene
pET-3a	pBR322	Amp	Novagen
pTAK117	ColE1	Amp	Gardner <i>et al</i> , 2000
pCL113	p15A	Cm	Ames <i>et al</i> , 2002

All receptors and the *ompr* genes from XL1-Blue and BL21(DE3) were cloned under the Plac promoter in pCL1920, which is a very low copy number plasmid (5 copies per cell). Plasmids pBAD33 and pET-3a were used as backbone for the reporter systems. The constructs built in this study are listed in table II-3.

Plasmid pBluescript KS was used as a cloning vector because of its flexibility: it is a high copy number vector of relative small size, with universal primers flanking the MCS and therefore it is a good vector for sequencing genes and correcting/introducing mutations with PCR-ATW.

Plasmid pTAK117 was provided by prof. Collins and used as a source for the gene of the temperature sensitive CI repressor (CIts).

Plasmid pCL113 was provided by prof. Parkinson and used in the chemotactic assays. It carries Tar with the mutation S63G, which does not affect the receptor's function, under the *nahG* promoter. Expression is induced by sodium salicylate which releases the plasmid-encoded NahR repressor from the promoter.

**Table II-3.** Constructs built in this study

<b>construct</b>	<b>Short description</b>	<b>backbone</b>	<b>resistance</b>
pTaz2	TaZ A160V under Plac	pLC1920	Spc, Str
pH1	H1 receptor under Plac	pLC1920	Spc, Str
pH21	H21 receptor under Plac	pLC1920	Spc, Str
pH22	H22 receptor under Plac	pLC1920	Spc, Str
pH3	H3 receptor under Plac	pLC1920	Spc, Str
pBTaz2	TaZ A160V clone	pBluescript KS	Amp
pBTaz	TaZ with a NcoI site	pBluescript KS	Amp
pCOR	TaZ with a Pst I- Nco I cassette for the ligand binding domain	pBluescript KS	Amp
pBTL	TaL receptor clone	pBluescript KS	Amp
pBTS	TaS receptor clone	pBluescript KS	Amp
pBTT	TaT receptor clone	pBluescript KS	Amp
pBTQ	TaQ receptor clone	pBluescript KS	Amp
pBTLM	TaLM receptor clone	pBluescript KS	Amp
pBT69L	TaZ R69L receptor clone	pBluescript KS	Amp
pBT69M	TaZ R69M receptor clone	pBluescript KS	Amp
pBT73M	TaL R73M receptor clone	pBluescript KS	Amp
pBTsz	Tsz receptor clone	pBluescript KS	Amp
pBTsarz	TsarZ receptor clone	pBluescript KS	Amp
pBTSNI	TSNI receptor clone	pBluescript KS	Amp
pBTasZ	TasZ receptor clone	pBluescript KS	Amp
pTL	TaL receptor under Plac	pLC1920	Spc, Str
pTS	TaS receptor under Plac	pLC1920	Spc, Str
pTT	TaT receptor under Plac	pLC1920	Spc, Str
pTQ	TaQ receptor under Plac	pLC1920	Spc, Str
pTLM	TaLM receptor under Plac	pLC1920	Spc, Str
pT69L	TaZ R69L receptor under Plac	pLC1920	Spc, Str

pT69M	TaZ R69M receptor under Plac	pLC1920	Spc, Str
pT73M	TaL R73M receptor under Plac	pLC1920	Spc, Str
pTsz	Tsz receptor under Plac	pLC1920	Spc, Str
pTsarz	TsarZ receptor under Plac	pLC1920	Spc, Str
pTSNI	TSNI receptor under Plac	pLC1920	Spc, Str
pTasZ	TasZ receptor under Plac	pLC1920	Spc, Str
DGB33	dGFP out-of-frame from P <sub>BAD</sub>	pBAD33	Cm
pR1	dGFP under <i>pompC</i>	pBAD33	Cm
pR2	dGFP under <i>pompC</i>	pBAD33	Cm
p33	pBAD33 replacing ClaI-SacI fragment with a promoterless ClaI-AraC-SacI	pBAD33	Cm
pAatII	p33 introducing an Aat II site near the end of araC	p33	Cm
pAORTcor	TetR under synthetic promoter with OR1	pAatII	Cm
pR4	dGFP under <i>pompC</i> with overlapping mutant TetO1	pBAD33	Cm
pR5	TetR under synthetic promoter with OR1	pR4	Cm
pR6	GFP antisense RNA under synthetic promoter	pR5	Cm
pR7	Operon fusion of CIts with dGFP	pR5	Cm
pR5C	pR5 with -33 T->C in synthetic promoter	pR4	Cm
pR6C	pR6 with -33 T->C in synthetic promoter	pR5	Cm
pR7C	pR7 with -33 T->C in synthetic promoter	pR5	Cm
pBOX	OmpR from XL1-Blue clone	pBluescript KS	Amp
pBEX	EnvZ from XL1-Blue clone	pBluescript KS	Amp
pLOX	OmpR from XL1-Blue under Plac	pLC1920	Spc, Str

**Growth Media****LB (1L):**

Bacto-tryptone	10 g
Bacto yeast extract	5 g
NaCl	10 g

Adjust pH to 7.0 and sterilise by autoclaving for 20 min at 15 lb/sq.in on liquid cycle.

**SOC (1L):**

Prepare 950 ml of water containing

Bacto-tryptone	20 g
Bacto yeast extract	5 g
NaCl	0.5 g
KCl	0.186 g

Adjust pH at 7.0 and sterilise by autoclaving for 20 min at 15 lb/sq.in on liquid cycle. Before use add 5 ml of 2M MgCl<sub>2</sub> and 20 ml of 1M glucose (final concentration 20 mM glucose). Filter sterilise using a 0.22-micron filter. Store at 4°C.

**Medium A**

Nutrient Broth (Difco)	7 g
yeast extract	1 g
glycerol	2 g
K <sub>2</sub> HPO <sub>4</sub> (K <sub>2</sub> HPO <sub>4</sub> ·3H <sub>2</sub> O)	3.7 g (4.85 g)
KH <sub>2</sub> PO <sub>4</sub>	1.3 g
water	up to 1L

**Labelling medium (1L):***M9 medium 10x (1L):*

Na <sub>2</sub> HPO <sub>4</sub>	60 g
KH <sub>2</sub> PO <sub>4</sub>	30 g
NaCl	5 g
NH <sub>4</sub> Cl	5 g

Sterilise by autoclaving for 20 min at 15 lb/sq.in on liquid cycle.

*Trace elements 100x (1L):* Dilute EDTA in 800 ml and adjust pH=7.5. Then add the rest and fill to 1L.

EDTA	5 g
FeCl <sub>3</sub> .6H <sub>2</sub> O	0.83 g (0.4982 g anhydrous)
ZnCl <sub>2</sub>	0.084 g
CuCl <sub>2</sub> .2H <sub>2</sub> O	0.013 g
CoCl <sub>2</sub> .6H <sub>2</sub> O	0.010 g
H <sub>3</sub> BO <sub>3</sub>	0.010 g
MnCl <sub>2</sub> .6H <sub>2</sub> O	0.016 g

Filter sterilise using a 0.22-micron filter.

*Labelling Medium (1L):*

M9 10x	100 ml	
Trace elements 100x	10 ml	
Carbon source 20% (w/v)	20 ml	(final concentration 0.4%)
MgSO <sub>4</sub> 1M	1 ml	(final concentration 1 mM)
CaCl <sub>2</sub> 1M	0.3 ml	(final concentration 0.3 mM)
Biotin 1mg/ml	1 ml	(final concentration 1 µg/ml)
ThiaminHCl 40mg/ml	25 µl	(final concentration 1 µg/ml)

Filter sterilise using a 0.22-micron filter.

**Tryptone Broth (1L):**

Tryptone	10 g
NaCl	5 g

Sterilise by autoclaving for 20 min at 15 lb/sq.in on liquid cycle.

**Basic molecular biology techniques****PCR**

The Polymerase Chain Reaction (PCR) was used for amplifying DNA fragments, screening bacterial colonies for constructs, mutagenesis and gene construction.

In all applications the final volume of the reaction mixture is 50  $\mu$ l, containing 3  $\mu$ l of 10 mM solution of each primer and 1  $\mu$ l of 10 mM dNTP mix. PCR screening was carried out using 0.2 units of Taq polymerase, whereas for the rest applications 1-2 units of Pwo polymerase were used.

*DNA amplification*

The desired DNA fragments were amplified using 30-60 ng of template, for plasmid-borne templates, or by lysing a single bacterial colony, by boiling at 98°C for 5 min in water, for genomic templates.

When the primers in use introduce a restriction site, extra bases are added to the 5' end of the primer to facilitate restriction. The exact number of these bases is specified for each restriction enzyme by its manufacturer (NEB). The procedure followed is:

- Heating at 97°C for 2 min (Adding the polymerase in the appropriate volume of 10x buffer at 1.5 min)

- denaturation at 97°C for 30 sec
- annealing at 2°C below the lowest melting temperature of the primers, as calculated by the manufacturer (proligo).
- elongation at 72°C for X min where X is the size of the desired fragment in kb. For fragments smaller than 1 kb the time used was 1 min.
- final elongation at 72°C for 10 min.
- cooling at 4°C.

The number of denaturation-annealing-elongation cycles is 30.

### *PCR-Screening*

PCR screening of colonies was performed as in the same way with the following differences:

- Single colonies were used to inoculate liquid cultures and the remaining cells were lysed by boiling in the appropriate volume of water for 5 min at 98°C.
- The rest of the reaction mix was subsequently added, without performing a hot-start.
- Annealing temperature is 4-6°C below the lowest melting point of the primers.

The primers used for screening usually have a  $T_m=54-58^\circ\text{C}$  and one of them hybridises on the vector backbone while the other on the construct.

### *PCR-mutagenesis-PCR-Around-The-World (PCR-ATW)*

PCR mutagenesis was used to create or destroy restriction sites within cloned genes and to make or correct single point mutations.

The procedure followed is the same as for amplifying a DNA fragment with the following differences:

1. The amount of template is 20-40 ng.
2. The number of cycles is 15.

3. The annealing temperature is 63°C, because of the way the primers are designed: The two primers have a complementary region which consists of the mutation(s), flanked by 10 bases. Each primer consists of 10 bases at the 5' end, hybridising to the template, the desired mutation(s), followed by at least 30 bases, so that the 3' end ends with a c or g, hybridizing to the template. The product generated is the whole vector, carrying the mutated DNA sequence, that is why the method is called PCR-around-the-world.

After the PCR, 1µl (20 units) of DpnI is added directly to the reaction mix and it is incubated o/n at 37°C, in order to degrade the template. DpnI will digest only methylated template, thus not the PCR product. Therefore the strain used for isolating the template is usually XL1-Blue or Top10 which have the methylating enzymes.

#### *Gene construction by PCR*

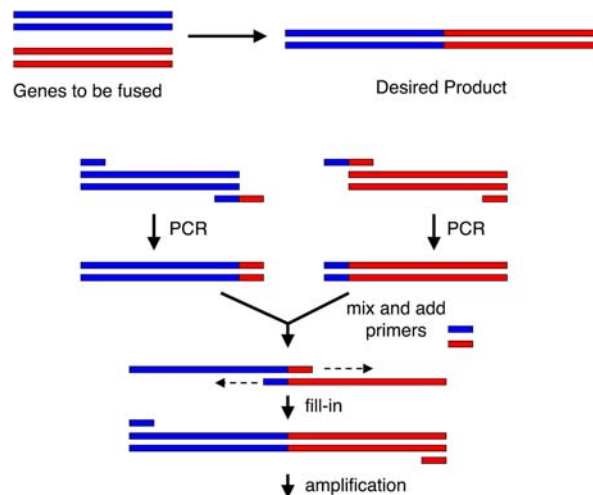
The exact procedure varies for different constructs.

Very small DNA fragments were constructed by using two complementary primers and filling-in with Pwo polymerase, following the same general protocol as for DNA amplification.

Constructs which are the fusion of two DNA fragments were built as follows:

Each fragment is amplified for 10 cycles (if it is plasmid-borne or constructed by primers as described above) or for 30 cycles (if it comes from genomic DNA). The primers used are such, that the 3' reverse primer of the first fragment contains a complementary sequence to the 5' forward primer of the second fragment, marking the fusion of the two fragments. (fig.II-1). The overlap is such that the melting temperature is quite high (above 63°C).

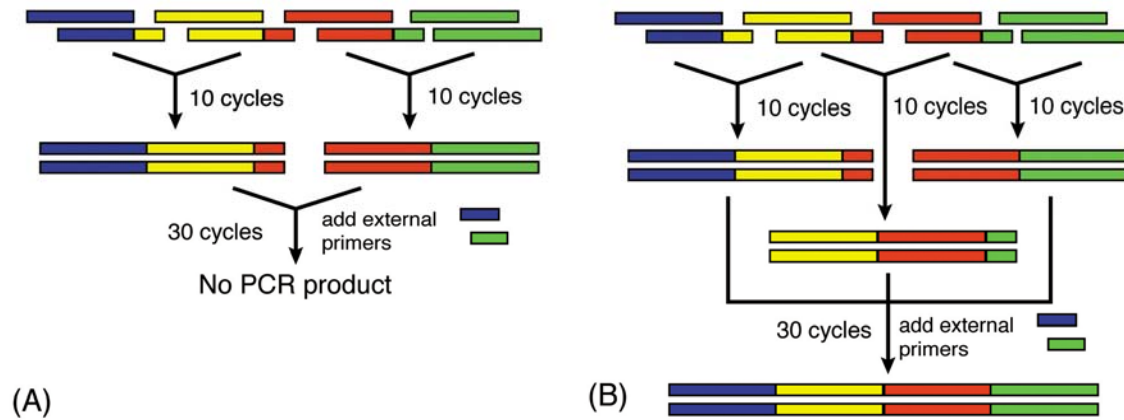




**Fig. II-1.** Fusion of two genes by PCR. The two genes are amplified by using a 3' primer for the first gene and a 5' primer for the second one that contain the sequence at the fusion point and are complementary to each other. When the products of the two PCR reactions are mixed, under PCR conditions, the top strand of the first reaction will hybridize to the bottom strand of the second and the gaps will be filled-in by the polymerase leading to the full-length desired product. Including the external 5' and 3' primers (single colour rectangles) results in amplification of the latter product.

Aliquots from both reaction mixtures are directly mixed in a new tube, with fresh dNTPs, polymerase and the 5' forward primer of the first fragment and the 3' reverse primer of the second. The aliquots are such that the ratio of the aliquots' volume is roughly within the range of the ratio of the sizes of the two fragments e.g for the fusion of a ~150bp synthetic promoter to TetR (~600bp) 0.5 $\mu$ l of the promoter PCR mix and 1.5 $\mu$ l of the TetR PCR mix were used.

When more than two fragments needed to be fused together it was necessary to make a variation in the method, since simple mixing of all the PCR products together didn't result in great (if any) amounts of the desired product. For example for the construction of the ligand binding domain of the mutant receptors 8 overlapping primers were used. Mixing of all the 4 PCR products or of only 2 fusion products didn't was unproductive (fig.II-2A) and it was necessary to make 3 fusion products which were then combined and amplified with external primer (fig.II-2B).



**Fig. II-2.** Fusion of four DNA fragments by PCR. (A) Fusion two-by-two did not give the desired product (B) Fusion two-by-two facilitated by bridging with a fusion product of genes 2 and 3.

In all applications PCR products were cleaned using the Qiaquick PCR purification kit (Qiagen), except when there were by-products. In those cases the desired product was purified by gel-extraction, after agarose gel electrophoresis, using the Gel-Extraction kit (Qiagen).

### *Restriction*

Purified PCR products or plasmids were subjected to o/n restriction at 37°C. Whenever possible double digestion was preferred. Otherwise restriction was done sequentially according to the manufacturers recommendations (NEB). Plasmids were simultaneously dephosphorylated, by adding 1 unit of Shrimp Alkaline Phosphatase (SAP) in the reaction mixture. The amount of restriction enzyme(s) and SAP added was such that the total amount of added glycerol was below 5% of the final volume.

### *Enzymatic reaction clean-up*

Products of restriction reactions were purified using the Qiaquick PCR purification kit, following the manufacturer's protocol for enzymatic reaction clean-up, when the restriction yielded undesirable fragments smaller than 80 bp. When the undesired products were higher than 80 bp the desired products were separated by preparative AGE and purified with the Qiagen gel-extraction kit.

### *Ligation*

Ligations were performed o/n at 16°C using 1 unit T4 DNA ligase, from Roche, in a total volume of 20 µl. The amount of vector used was 200- 400 ng and the molar ratio of insert to vector was always 3:1. The amounts of restricted vector and insert that were needed for the reaction were calculated after determining spectroscopically the concentration of the purified restriction products, with an eppendorf spectrophotometer.

### *Plasmid isolation*

The appropriate kit was used for plasmid isolation, depending on the copy-number and the desired quantity. For high copy number plasmids the Qiagen Mini-prep kit was used, following the manufacturer's protocol, with the following differences:

- the optional wash with PB was always done
- the PE was left on the column for 2-5 min when the preparation would be treated with salt sensitive restriction enzymes.
- the DNA was finally eluted with the kit's Elution Buffer (EB). Water was used for elution only when the preparation would be used for sequencing reactions. When the preparation would be used for restriction reactions with salt-sensitive restriction enzymes, the DNA was eluted either with water or diluted EB 1:1 to achieve higher yield.

Medium copy and low copy number plasmids were isolated using the Qiagen High-Speed midi and maxi kits according to the manufacturer's instructions. The quality of the preparation was usually not adequate for sequencing reactions and an extra purification step was applied, using the columns from the mini-prep kit and the clean-up protocol from the Qiaquick PCR purification kit.

#### *Competent cells-Transformation*

All ligation products and provided vectors were transformed in chemically competent XL1-Blue cells, purchased from Stratagene, or Oneshot Top10 cells, purchased from Invitrogen Life Technologies. Transformations were carried out following the manufacturer's instructions with the following differences: a)  $\beta$ -mercaptoethanol was not used b) the heat-shock was performed in 2-ml reaction tubes c) 20  $\mu$ l of competent cells were used per 10  $\mu$ l of ligation mixture and 10  $\mu$ l per 1  $\mu$ l of plasmid preparation. Transformation of other strains with plasmids was performed with either of the two following methods:

##### *a)TSS method*

The TSS method is a variant of the method described by Chung *et al* (1989) for preparing chemically competent cells. The transformation efficiency achieved with this method is not very high (in the range of  $10^5$ ) so it is appropriate only for transforming with plasmids but not ligation mixtures.

*Materials*

## 2xTSS (100ml)

Bactotryptone	2 g
Bacto yeast extract	1 g
NaCl	2 g
PEG 3350 (I 8000)	20 g (20%)
DMSO	10 ml (10%)
MgSO <sub>4</sub> ·7H <sub>2</sub> O	1.23-2.46 g (50-100 mM)

Mix vigorously for at least 45 min, until the solution is clear.

Adjust pH to 6.50

Filter sterilise using a 0.22-micron filter.

*Experimental procedure*

- A fresh overnight culture is diluted 1:100 into pre-warmed LB and incubated at 37°C at 225 rpm until it reaches an OD<sub>600</sub>= 0.3-0.4.
- Add equal volume of ice-cold 2xTSS and mix gently.
- For long term storage cells are immediately frozen in dry ice/ethanol and stored at -70°C.
- For transformation a 100 µl aliquot is placed in a cold 2 ml reaction tube containing 50-300 ng plasmid DNA and mix gently. (when using frozen cells, the cells are thawed on ice and used immediately).
- Incubate the mix at 4°C for 5-60 min, usually 30 min.
- Add 900 µl LB +20 mM glucose (~20 µl of 20% solution).
- Incubate at 37°C (225 rpm) for 1 h.
- Plate different quantities of the transformation mix ranging from 5 µl to the total transformation mix on the appropriate antibiotic plates, using sterile glass beada.

*b) TB method*

This method is a variant of the method described Inoue *et al* (1990)

*Materials*

TB 1x (100 ml)

10 mM HEPES pH 6.7.	0.2383 g
15 mM CaCl <sub>2</sub> .2H <sub>2</sub> O	0.22053g
250 mM KCl	1.8625 g
55 mM MnCl <sub>2</sub> .2H <sub>2</sub> O	0.89034 g

Mix all component except MnCl<sub>2</sub> and adjust the pH to 6.7 with KOH. Then add the MnCl<sub>2</sub> and filter sterilize the mixture using a 0.22- $\mu$ m filter.

*Experimental procedure*

- Inoculate 3 ml LB medium with a colony of the appropriate *E. coli* strain and incubate the culture overnight at 37°C.
- Add from the overnight culture 150 $\mu$ l (3:500) to 25 ml SOC medium and incubate the culture at 37°C up to OD<sub>600</sub>=0.4-0.6.
- Chill the culture for at least 10 min on ice.

In the following steps, the cell suspension should be kept on ice as much as possible.

- Centrifuge the cell suspension for 10 min at 3,500 at 4°C.
- Gently resuspend the pellet in 5 ml ice-cold TB buffer.
- Incubate the cell suspension on ice for 10 min.
- Centrifuge for 10 min at 3,500 rpm at 4°C.
- Gently re-suspend the pellet in 930  $\mu$ l ice-cold TB buffer and add 70  $\mu$ l DMSO.

- Incubate the cell suspension on wet ice for at least 10 min.
- Aliquot the cell suspension at 100  $\mu$ l per 2 ml reaction tube.
- Shock-freeze the cell suspension in liquid nitrogen and store the tubes at -80° C\* or in liquid nitrogen or use directly.

\*At -80°C the cells will be competent for at least 6 months. In liquid nitrogen they will stay competent indefinitely.

This protocol gives chemically competent cells of the efficiency as commercially available ones ( $\sim 10^8$ ) and can be used for any kind of application. The transformation procedure from this point on is the same as described for the commercial competent strains.

#### *Agarose Gel Electrophoresis (AGE)*

AGE was used for visualising and isolating DNA fragments (PCR products, restriction products) and plasmid preparations using TBE-gels.

TBE 5x (1L)

Trizma base	54 g
Boric acid	27.5 g
EBTA 0.5M pH=8.0	20 ml

The percentage of agarose used, depended on the size of the DNA fragments, although for preparative AGE, 0.7% agarose was preferred in order to minimise the amount of agarose in the sample during the gel-extraction procedure.

*Poly-Acrylamide Gel Electrophoresis under denaturing conditions (SDS-PAGE)*

Cell lysates were analysed by SDS-PAGE with Criterion pre-cast gels (Biorad) Tris-HCl 15%. Gels were run at constant 170 V for 1h.

Loading buffer 2x (8ml)

H <sub>2</sub> O	3.8 ml
0.5 M Tris-Cl pH=6.8	1.0 ml
glycerol	0.8 ml
SDS 10%	1.6 ml
β-mercaptoethanol	0.4 ml
bromophenol blue 1%	0.4 ml

Running Buffer 10x (1L)

Tris	30.2 g
glycine	188 g
SDS	10 g
H <sub>2</sub> O	up to 1L

Visualization of the proteins was done with Western Blotting.



*Western Blotting**Materials*

Transfer buffer 10x without EtOH (1L)

Trizma	30 g
Glycine	144 g

Transfer buffer 1x +EtOH (1L)

Transfer buffer 10x	100 ml
EtOH	200 ml
water	To 1L

PBS (1L)

NaCl	8 g
KCl	0.2 g
Na <sub>2</sub> PO <sub>4</sub>	1.44 g
KH <sub>2</sub> PO <sub>4</sub>	0.24 g

Adjust pH to 7.4 with HCl. Sterilise by autoclaving for 20 min at 15 lb/sq.in. on liquid cycle.

PBST (1L)

PBS	1 L
Tween 20	50 µl (0.05%)

*Experimental procedure*

Western Blots were performed using the Criterion Blotter system from Biorad, according to the manufacturer's instructions. However, optimal transfer conditions were constant current at 380 mA for 26 min.

After the transfer, the nitrocellulose membrane was washed with water and stained with Ponceau S Solution (Sigma), in order to visualise the total amount of protein in each lane. Background staining was removed by washing with de-ionised water. The membrane was then scanned with an Agfa Duoscan *f*40 scanner.

The stain was subsequently removed by washing with PBS. The membrane was blocked at RT for 1h with 5% milk in PBST and then exposed to the primary antibody for 1h at RT and to the secondary antibody, which is for 45 min. The primary antibody was monoclonal anti-GFP from Roche, and the secondary antibody was peroxidase-conjugated donkey anti-mouse purchased from Jackson Immuno Research Laboratories. Each step with an antibody was followed by two 10 min washes with PBST.

After the final wash, GFP bands were visualised with the ECL Western blotting analysis kit from Amersham Biosciences and the exposed films were scanned. Using the program IQMac, the number of pixels for each band on the film was determined and then divided by the number of pixels from the total-protein stain of the corresponding lane. Both values were first corrected for background (by subtracting the number of pixels of membrane/film corresponding to the same area as the lane/band) to adjust for differences in the amount of protein transferred in each lane. The values obtained were subsequently normalised to the value for the control cells.

## Expression Assays

### *Osmotic shock assay for the EnvZ-OmpR system*

A single colony of the desired strain was used to inoculate a pre-culture in medium A, supplemented with the appropriate antibiotics. Cells were grown o/n at 37°C and 200 µl of this culture were used to inoculate 50 ml of fresh medium A with antibiotics (low osmolarity conditions) and 50 ml medium A supplemented with 0.3M NaCl (high osmolarity conditions). The cells were allowed to grow at 37°C until mid-logarithmic phase and then were harvested and lysed by boiling with loading dye at 98°C for 10 min. Samples were subsequently analysed by SDS-PAGE and Western blotting.

### *Compound screening assay for the Taz-OmpR system*

A single colony of the desired strain was streaked on an appropriate antibiotic plate and grown o/n at 37°C. Multiple colonies from this plate were used as the inoculate for a pre-culture in labelling medium in a flask 10x bigger than the culture volume, i.e. 4 ml in a 50 ml falcon tube. Cells were grown o/n at 37°C and 200 µl from this culture are used to inoculate 50 ml of fresh labelling medium with the appropriate antibiotics in a 500 ml flasks. Cells were grown at 200-230 rpm at 37°C until they reach an  $OD_{600} \approx 0.200$  and then aliquoted into 15 ml falcons containing stock solution of the compound dissolved in labelling medium. The amount of stock was such that the final concentration of the compound tested would be 5 mM. The cells were allowed to grow for 1 h and then were harvested and lysed by boiling with loading dye at 98°C for 10 min.

Unlike the osmotic shock assay, exposing growing cells to the compound, instead of inoculating fresh medium which already contains the putative signal, was necessary because in the latter case cells would show a longer, by several hours, lag phase compared to the control cells. Moreover since the compounds are amino acids, short time exposure reduces the amount of amino acid that is metabolised, keeping its concentration

constant at the desired value. It should be noted that the system uses the chromosomal *ompr* gene which is under the control of CRP (Huang *et al*, 1992). Therefore a relatively short exposure time to metabolizable compounds could give more clear answers than growing the cells in the presence of the compound, since the level of OmpR should not change dramatically in 1h, provided that its half-life is not within the order of magnitude of the exposure time.

## **Chemotaxis assays**

### *Chemical-in-plug assay*

The chemical-in-plug assay was done as described by Tso & Adler (1974) with the following differences: Cells were grown o/n at 30°C in tryptone broth containing the appropriate antibiotic and 0.7µM sodium salicylate, for cells carrying pCL113, in order to express Tar. This pre-culture was used to inoculate 100 ml (or 50 ml) of fresh medium at 1:100 and grow to an OD<sub>600</sub><0.600 (usually 0.500). Cells were spun down in 50 ml falcons at 4000 rpm for 7 min at RT (i.e. 24°C), the supernatant was decanted and the cells were resuspended in a couple ml of Chemotaxis Buffer (CB; 10 mM phosphate buffer pH=7.0, 0.1 mM EDTA, 10 mM lactic acid), by gently pipeting up and down. Vigorous handling of the cells was avoided in order to maintain the integrity of the flagella. CB was added up to a volume of 50 ml, the cells were spun down again under the same conditions and resuspended in the remaining CB in the tube after decanting the supernatant. CB was added so that the final volume of the cell suspension was 1 ml.

The cell suspension was mixed with 24 ml 0.3% bacto-agar in CB, which was pre-cooled at 40°C, and poured into a plate which already contained the plugs with the chemical. The plate was incubated for 1h at 30°C and then photographed. Plugs were prepared by using test tubes (16 mm diameter) to cut cylinders out of plates with the desired concentration of each chemical in 2% bacto-agar containing CB.

*Swarm plates*

Swarm assays were done by inoculating semi-solid agar (0.3%) containing different concentrations of the chemicals tested (and 1.4  $\mu$ M sodium salicylate to induce production of Tar from plasmid pLC113) with colonies from fresh o/n LB plates. The plates were incubated for 25 h at 30°C and then examined for swarm formation.

## Preparation of the plates

10x M63 salts for 100 ml:

KH <sub>2</sub> PO <sub>4</sub>	3 g
K <sub>2</sub> HPO <sub>4</sub> .3H <sub>2</sub> O	7 g
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	2 g
FeSO <sub>4</sub> (FeSO <sub>4</sub> .7H <sub>2</sub> O)	0.5 mg (10 $\mu$ l from 50mg/ml solution which is 0.250g/5ml or 0.519g/5ml heptahydrate)

Filter sterilise. The ferrous solution should be freshly prepared because it is not stable.

For 1L for plates

Flask A	500 ml Water
	6 g agar
Flask B	100 ml 10x M63 salts
	2g glycerol
	2 ml Thiamine 50 mg/ml
	1 ml 1M MgSO <sub>4</sub>
	8 ml mix L-Thr, His, Leu, Met, 5 mg/ml each
	390 ml Water (up to 500 ml)

Flask A is sterilised by autoclaving and Flask B by filtration with a 0.22-micron filter.

For each plate 12.5 ml of Flask B contents supplemented with the appropriate antibiotic, sodium salicylate and the appropriate amount from a stock solution of the chemical in water, were mixed with 12.5 ml of pre-cooled at 40°C Flask A contents and poured into petri-dishes.

*FRET experiments.*

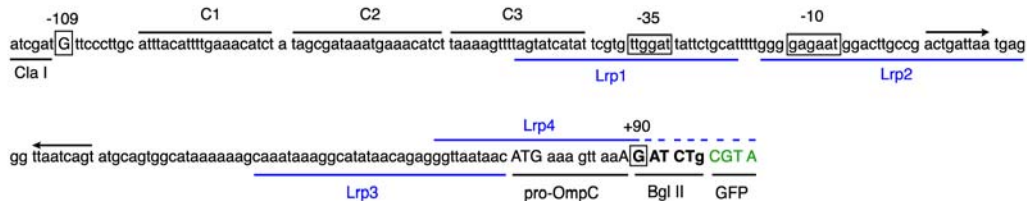
FRET experiments were carried out in collaboration with Dr. V. Sourjik in the University of Heidelberg (ZMBH), as described in reference Sourjik and Berg, 2004, with the difference that experiments were performed on Zeiss Axiovert 200 microscope using a 75 Watt super-quiet Xe-lamp (Hamamatsu) for illumination.

*Part III*

*Results*

### Construction and testing of the core reporter system.

Regulation of our reporter system is based on the OmpR-P-OmpC system. The *pompC* promoter has three binding sites for OmpR-P, C1, C2 and C3 with the most upstream being C1 (Mizuno and Mizushima, 1986). The affinity for each site is decreasing in the order C1>C2>C3 with the affinity for C3 being more than 2 orders of magnitude lower than that for C1 (Head *et al*, 1998), when C3 is considered as an independent unit. However in the context of all three binding sites, binding of OmpR-P is cooperative, lowering the  $K_d$  for the OmpR-P-C1-C2-C3 complex to one order of magnitude higher than C1 (Head *et al*, 1998). Under physiological conditions the concentration of OmpR-P and its affinity for C1 (Cai and Inouye, 2002) are such that C1 should be always occupied, even at low osmolarity. Transcription from the promoter should be activated when the concentration of OmpR-P increases and the binding of additional OmpR-P molecules is facilitated by the co-operativity with the bound OmpR-P moiety at C1. Apart from the activation sites, *pompC* carries 9 sites for the Leucine-responsive-protein, Lrp, spanning a region from upstream the promoter to the beginning of the coding sequence of OmpC (Ferrario *et al*, 1995). Lrp is a decahexamer which dissociates into octamers in the presence of Leucine (Chen *et al*, 2001) and can act both an activator and repressor of a large number of metabolic operons (Calvo *et al*, 1994). It has been shown that Lrp is a repressor for OmpC expression (Ferrario *et al*, 1995).



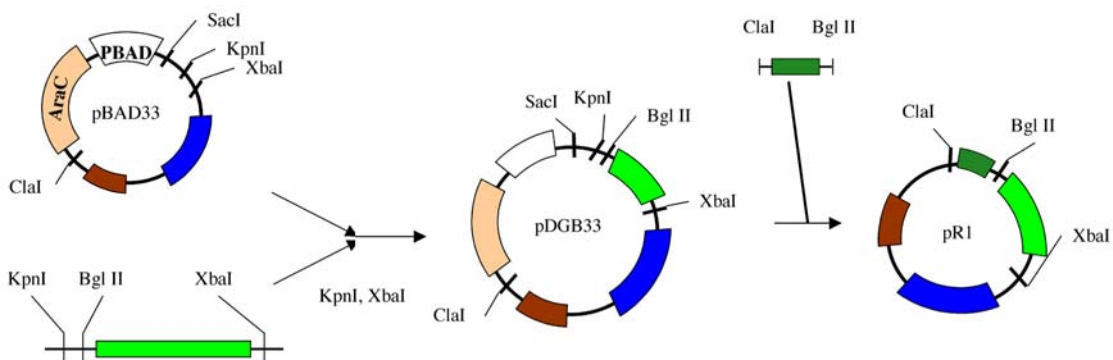
**Fig. III-1.** The core *pompC* promoter that was cloned. In boxes are shown the -109 and +90 of the promoter (with respect to the transcription initiation site). The promoter forms a hairpin depicted by the two convergent arrows and contains three complete Lrp binding sites and a half Lrp site. The *gfp* gene is expressed with the first four amino acids of pro-OmpC and two amino acids encoded by the Bgl II site and an extra base (lower case) introduced to keep the correct reading frame



The core of the reporter system consists of a destabilised GFP, GFP(LVA)), which has a half-life of 40 min (Andersen *et al*, 1998) and a minimal *pompC*. The minimal *pompC* contains only the three binding sites for OmpR-P and spans the region from -109 to +90. This means that the SD and first 4 amino acids of pro-OmpC are kept, but also 4 out of the 9 binding sites for the Leucine responsive protein, Lrp, are kept as well (fig. III-1). The simple reporter system was constructed as follows:

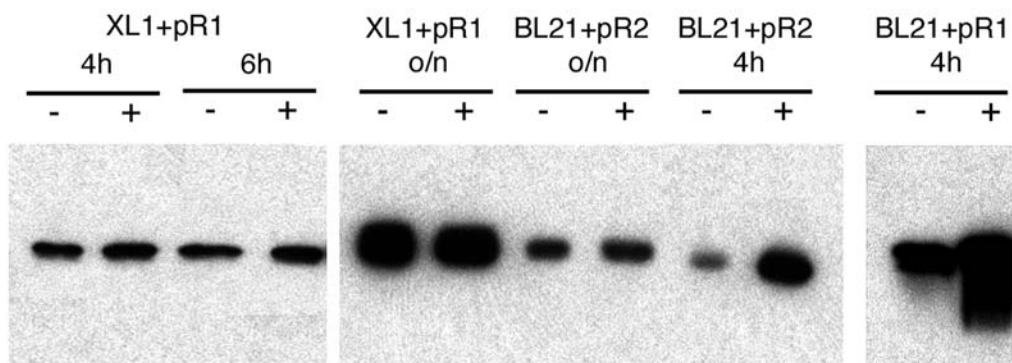
GFP(LVA) was created by amplifying GFPmut3 from Clontech using a 5' primer which carries sequentially a KpnI and Bgl II site and a 3' primer which carries the signalling sequence for the SsrA pathway (RPAANDENVLVA), targeting GFP for degradation (Andersen *et al*, 1998), and an Xba I site. It was cloned to the medium copy vector pBAD33 using Kpn I and Xba I, giving construct pGDB33. Since pBAD33 did not have a Bgl II site, the one inserted together with the PCR fragment is a unique site on pGDB33. As mentioned above GFP(LVA) starts with the first four aa (including the initial Met) of pro-OmpC but also with two extra aa which correspond to the introduced Bgl II site plus a g-c bp which was necessary to keep the coding sequence of GFP(LVA) in the correct reading frame. Thus the exact sequence of the N-terminus of GFP (LVA) is **MKVK-DL-R**.

The minimal *pompC* was amplified from BL21(DE3) having ClaI and Bgl II sites at the ends. Attempts to fuse the *pompC* PCR product to GFP(LVA) or to do a three-fragment ligation (*pompC*+GPF(LVA)+pBAD33) failed, so it was necessary to clone *pompC* into pDGB33. By using ClaI and Bgl II, the *araC* gene and the P<sub>BAD</sub> promoter are replaced by *pompC* (fig. III-2).



**Fig. III-2.** Construction of pDGB33 and pR1.

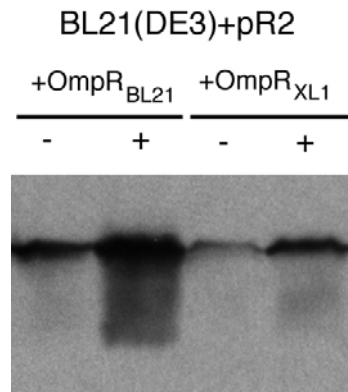
This simple reporter was named pR1. Before assembling the whole pathway, the ability of pR1 to report changes in OmpR-P was tested using the wild-type EnvZ-OmpR system. As a first choice XL1-Blue was picked as a host strain, since it was the strain used for cloning and according to the supplier it should be wild-type for this system. Despite the fact that the sequence of the construct was correct there was no regulation by osmotic pressure; the system was always in the ON state. To test if there was an element in the plasmid that resulted in unregulated expression, the *pompC*-GFP(LVA) construct was extracted from pR1 with Cla I-Xba I digestion and sub-cloned into vector pET-3a, resulting in construct pR2. Vector pET-3a is a pBR322 derivative and thus the copy number should be around 20 and not differ too much from pR1 which should have copy number 10-15 (p15A origin of replication). Transforming BL21(DE3) cells with pR1 or pR2 resulted in a reporter system regulated by osmotic pressure (fig.III-3) suggesting that the observed discrepancy in XL1-Blue was probably due to something intrinsic in the host strain.



**Fig.III-3.** Western blots of strains XL1-Blue and BL21(DE3) carrying the simple reporter system pR1 or pR2 which were grown in medium A with or without 0.3M NaCl for different time frames. pR1 is not regulated in XL1-Blue neither in logarithmic nor in stationary phase. However both pR1 and pR2 are regulated in the logarithmic phase.

In order to account for this observation the *ompR* and *envZ* genes from XL1-Blue were cloned and sequenced. The sequence of the EnvZ protein is exactly the same to the one in the Swiss-Prot databank and thus can be excluded as the cause for unregulated GFP

expression. OmpR was found to carry the single-point mutation D183Y. However when it was cloned into the low copy number pCL1920 and expressed in BL21(DE3) in the presence of pR2, GFP expression was still regulated which means that the specific mutation does not result in a constitutively active OmpR (fig.III-4) Therefore the observed unregulated expression of GFP in XL1-Blue should be due to a third, unidentified, component that interacts with the EnvZ-OmpR system.



**Fig.III-4.** Western blots of BL21(DE3) cells carrying pR2 and expressing OmpR from either BL21(DE3) or XL1-Blue from plasmid pLC1920. Cells were grown for 4 h in medium A with or without 0.3M NaCl

In fig. III-3 the o/n activation of the EnvZ-OmpR system in BL21(DE3) is also shown: cells grown have the same GFP expression level regardless of the osmolarity of the medium.

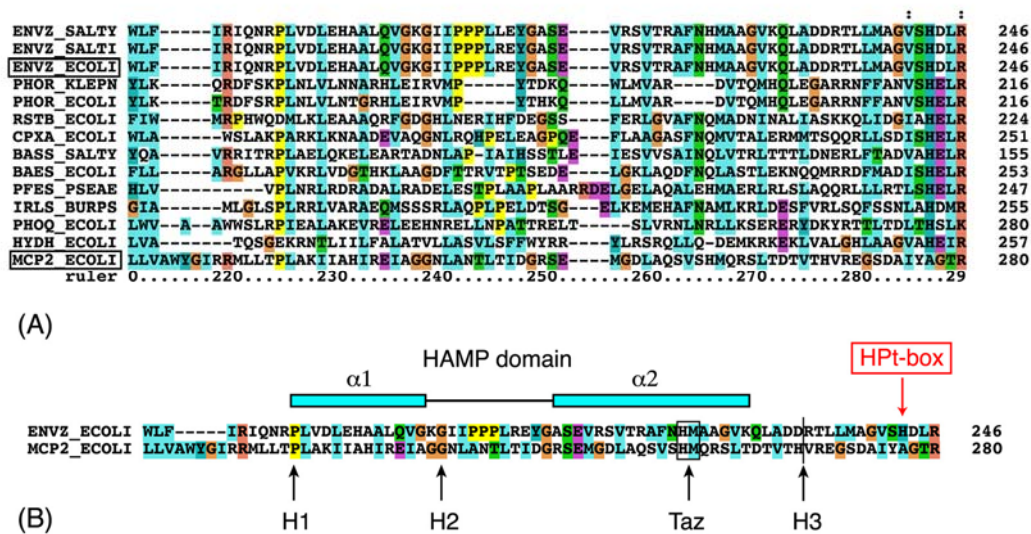
### Construction of Taz and design of fusion variants

It has been shown for the chimeric receptor Trz, which is the respective chimera of the sugar chemoreceptor Trg and EnvZ, that high expression levels of chimeric receptor reduce the sensitivity of the system (Baumgartner *et al*, 1994). Therefore Taz was cloned in the very low-copy number vector pCL1920 under P<sub>lac</sub>, in order to avoid over-expression. Taz was constructed by amplifying the respective fragments of the two

receptors, using BL21(DE3) as a source and fusing the PCR products as described in Materials and Methods. Taz was cloned using Hind III and BamH I. The starting codon of Tar was omitted and the start of the lacZ gene in the vector was used as a start of the protein. In order to keep the reading frame correct an extra **g-c** bp had to be added after the Hind III site so that the exact sequence of the N-terminus is *MTMIPTS-L-IN*.

The clone used for the study carries the mutation A160V, where the numbering corresponds to the sequence of wild-type Tar, but it seems to have no effect on the receptor: even when the mutation is corrected the behaviour of the receptor stays the same (data not shown).

Since the sensitivity of Taz is much lower than that of Tar, we decided to test if shifting the point of fusion of the two receptors could change in a favourable way the sensitivity of the chimera.



**Fig.III-5.** (A) Sequence alignment of EnvZ and its closest homologue histidine kinases with Tar. (B) Close up of the alignment for EnvZ and Tar showing the position of the HAMP domain of both receptors and the Hpt-box in EnvZ. Arrows indicate the point of fusion for the different chimeras. The amino acids (HM) which encode the common NdeI site used originally for the construction of Taz are shown in frame.

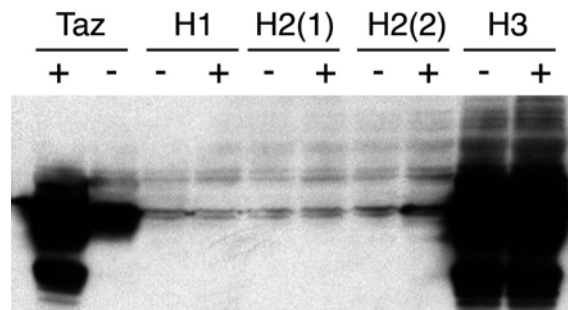
Based on the sequence alignment of the two receptors (fig.III-5), three new points were chosen and the three additional chimeric receptors (H1, H2 and H3) were constructed as Taz with the following differences:

- H1 contains the EnvZ linker instead of the Tar linker and the fusion is done at the conserved Pro219 of Tar.
- H2(1) contains half the linker from Tar and half from EnvZ, having the fusion at the conserved Gly233 of Tar. One of the clones which lost the translation stop codon and included 50 additional aa at the C-terminus, named H2(2), was also tested.
- H3 contains the linker and part of the first cytoplasmic  $\alpha$ -helix of Tar, having the fusion just before the Hpt domain of EnvZ between Thr266 of Tar and Asp233 of EnvZ

### **Testing Taz and the fusion variants.**

AT142 cells carrying the pR2 reporter were transformed with plasmids carrying Taz or either of the three variant receptors and the H2(2) clone. These cells were exposed to 5 mM of L-aspartate at 37°C for 1h as described in the Materials and Methods section and analysed by SDS-PAGE and Western blotting. AT142 have the *flhD5301* mutation and therefore should not express Tar and most of the chemotaxis genes (Liu and Matsamura, 1994). However, even if there is a small level of the wild-type receptors being expressed, Taz contains the dimerization domain of EnvZ which is enough for the formation of homodimers (Tomomori *et al*, 1999) and lacks the cytosolic part of Tar which contributes to the formation of higher order structures (Maddock and Shapiro, 1993; Ames *et al*, 2002). Furthermore, it has been shown that autophosphorylation of EnvZ-C occurs only in *trans* within a dimer and phosphorylation between two different dimers is not possible (Cai and Inouye, 2003). Therefore it is safe to assume that there is no formation of hetero-dimers and the only active receptor species for the system under investigation is the Taz homodimers.

As show in fig.III-6 cells carrying Taz respond in the desired way, whereas cells carrying H1, H2(1), H2(2) result in almost no GFP expression while H3 is not regulated by Asp and seems to be constitutively active showing elevated GFP expression, compared to Taz. The behaviour of cells carrying H1, H2(1), H2(2) could either reflect a defect of these receptors in the phospho-transfer reactions or a problem of their stability and expression. Since there seems to be a very small level of GFP expression the former seems to be the most probable explanation. In either case the fusion variants are unsuitable for further analysis.

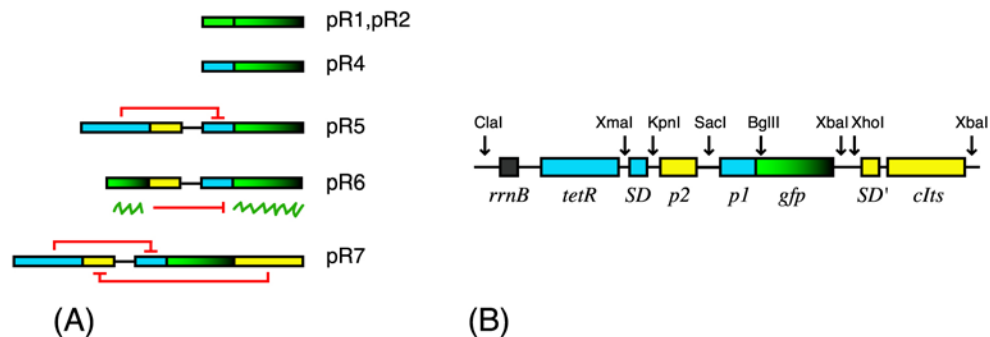


**Fig.III-6.** Western blot of AT142 cells carrying pR2 and the respective receptor in pCL1920 exposed for 1h to 5 mM L-aspartate.

It should be noted that much later from this study, similar fusion variants were shown by Zhu and Inouye (2003) to exhibit the same behaviour as in our case and for some seemingly inactive receptors, like Tez1, activity could be restored by mutations in the linker (in the case of Tez1 Pro185Gln). These results illustrate the exquisite sensitivity of the chemotactic receptors to small conformational changes, but also the importance of the HAMP domain for effective signal transduction: apparently not only the register of the helices in the HAMP domain is important but also the exact sequence, since the inactive H1 has the same register with one of the restored Tez1 mutants but different parts of the Tar and EnvZ sequences.

### Construction of other reporter systems

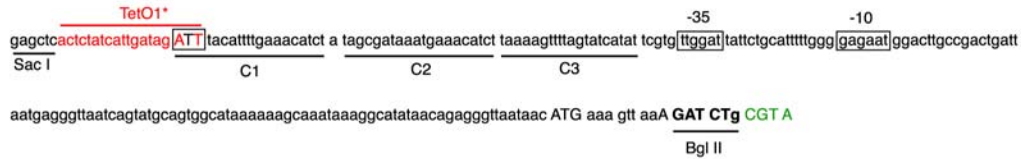
Construct pDGB33 was used as a template to construct more complex reporter systems, in a sequential manner, resulting in a complex construct whose components are in a cassette mode and can be easily exchanged (fig.III-7).



**Fig.III-7.** (A) Schematic drawing of the different reporter systems; pR1 and pR2 have the GFP under the minimal *pompC* (green), pR4 has the *pompC* overlapping with a TetO1 variant (cyan), pR5 expresses also TetR (cyan) from a synthetic promoter that has the OR1 site of the CI repressor (yellow), pR6 yields a 200 bp anti-sense RNA for GFP instead of TetR and pR7 expresses a temperature sensitive CI as a transcriptional fusion to GFP. (B) Schematic drawing of the pR7 construct. Each fragment is flanked by restriction sites so that it can be readily exchanged. The *XbaI* site at the end of the construct is dam methylation sensitive allowing for discrimination between the two sites flanking CI. The *rrnB* terminator was not included in the initial constructs.

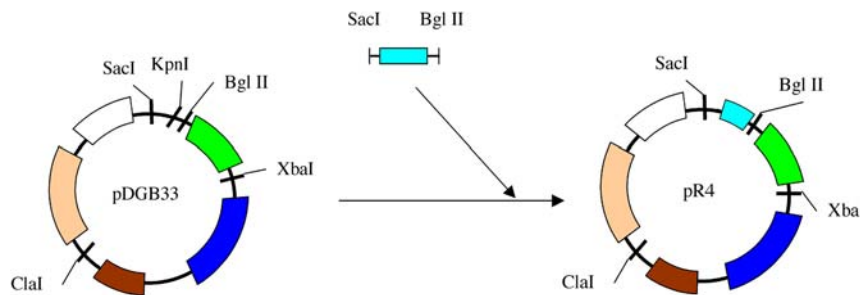
The first intermediate construct is pR4 which was constructed like pR1 with the difference that *pompC* was amplified using a 5' oligo which contained a TetO1 operator, overlapping with 3 bp of the C1. In order to avoid changing the C1 sequence, a mutation in respect to the TetR DNA binding sequence was allowed in the overlapping region, which should reduce the repressing ability of TetR (from Tn10) to 80% of that achieved with the wild-type sequence (Sizemore *et al*, 1990; Wissmann *et al*, 1988). The exact sequence of the mutant TetO1 site is shown in fig.III-8. This position was chosen for the TetO1 operator because there is no other position where it could be placed and compete with OmpR-P binding. Moreover since the affinity of OmpR-P is highest for C1 ( $K_d=7.7 \pm 4.8 \cdot 10^{-9}$  M, Head *et al*, 1998) and its binding to the three sites upstream from the

promoter is cooperative and done in a hierarchical manner (Rampersaud *et al*, 1994), competition with TetR for this site should determine the occupancy of the rest of the sites and activation of transcription.



**Fig.III-8.** Promoter *pompC* with an overlapping TetO1 variant site. The TetO1 site is shown in red, the 3 bp overlap with the C1 site is shown in a box and the base that reduces the TetR binding affinity is shown in black.

The affinity of the TetR for the *tetO1* operator is much higher ( $K_{\delta} \approx 10^{-11}$  M, Orth *et al*, 2000) than the one of OmpR-P for C1 so even with the mutation the strength of the interaction should be sufficient for competition. In fact if TetR binding is too strong, its active concentration can be modulated by adding traces of aTC. It should be noted that Sac I- Bgl II sites were used, instead of the Cla I-Bgl II, for cloning the *tetO1-pompC* into pDGB33. This means that the *araC* gene and P<sub>BAD</sub> promoter are still present (figIII-9)



**Fig.III-9.** Construction of pR4. In contrast to pR1, pR4 retains the P<sub>BAD</sub> promoter and *araC* gene.



However there should be no read-through transcription from P<sub>BAD</sub>, because it should be effectively repressed by AraC and within the *pompC* sequence there is a secondary structure which can act as a transcription termination signal (Mizuno *et al*, 1983).

The *tetR* gene from Tn10 from XL1-Blue was amplified and fused by PCR to a synthetic promoter which carries the OR1 site (Li *et al*, 1997) for the CI repressor between the -35 and -10 regions. The exact sequence of the promoter is shown in fig.III-10.



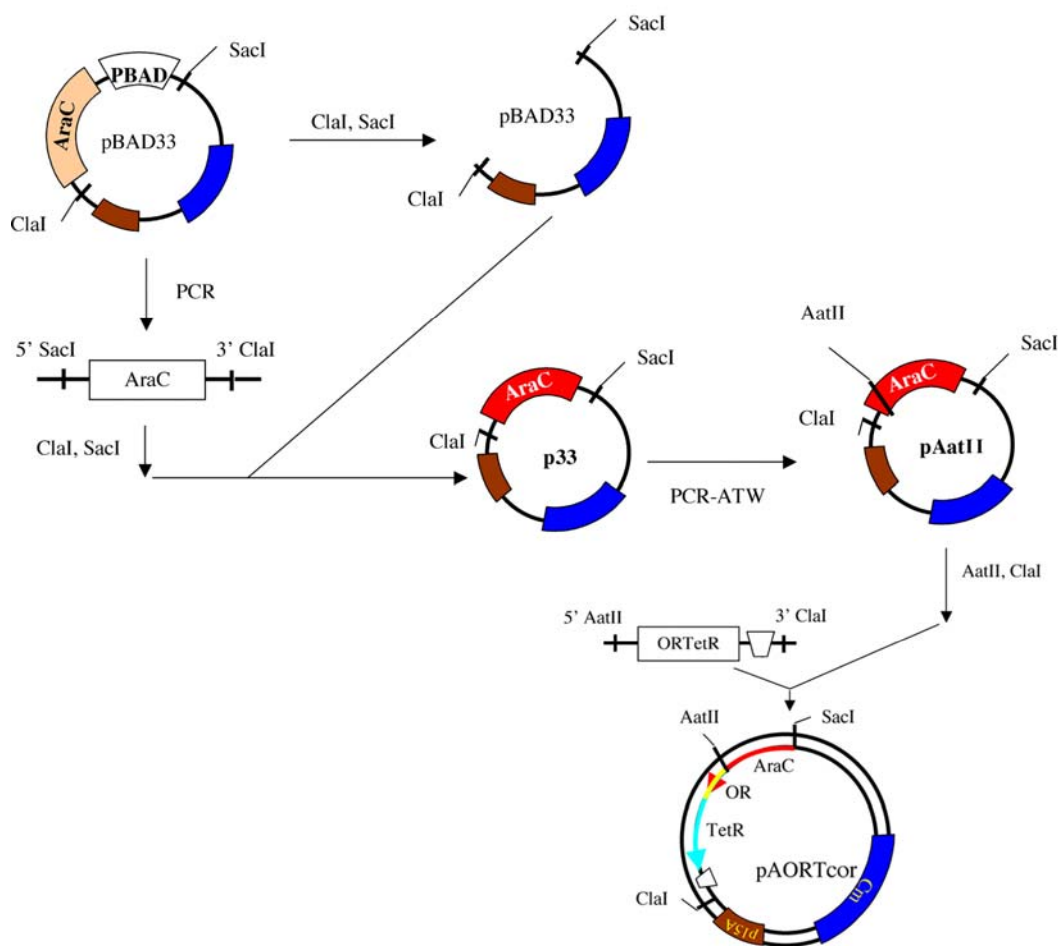
**Fig.III-10.** Sequence of the synthetic promoter for pR5, pR6 and pR7 constructs. The OR1 site is shown in red and the beginning of the *tetR* gene in blue. The silent mutation that was introduced in the second codon of tetR in order to destroy the XbaI site is shown in a box. In the -35 region, above the sequence, is shown the mutation that was also studied

In the construction the last base pair of the second codon of the *tetR* gene has been changed so that the XbaI site which existed in the wild-type sequence is lost, without changing the protein sequence. This was necessary in order to keep the Xba I site and the end of *gfp* unique. The SD sequence chosen provides moderate translation efficiency (Gardner *et al*, 2000). The different parts of the construct (promoter, SD, repressor gene) are thus cloned in a cassette form so that each one can be readily replaced according to the needs of the system.

All attempts to clone the synthetic promoter-*tetR* construct in pR4 gave clones with a lot of mutations and deletions, destroying the synthetic promoter. Since this promoter would be separated only by 30 bp from the C1 site, it could be possible that the supercoiling accumulated by the two divergent promoters (*pompC* and the synthetic one) could have a deleterious effect for the cells – maybe by interfering with plasmid replication and/or expression of the antibiotic resistance gene.

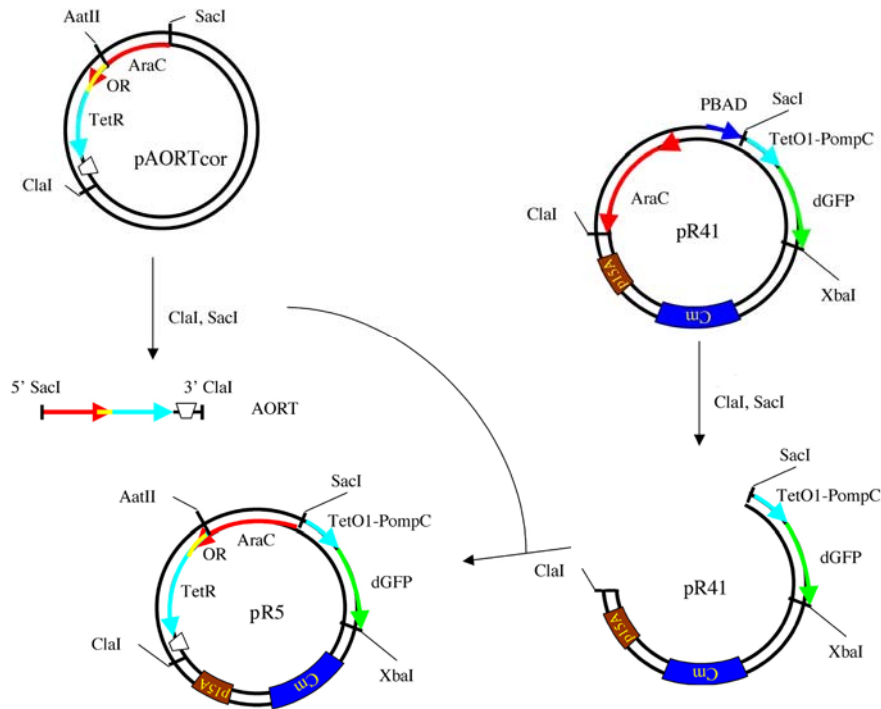
In order to test this hypothesis a spacer of ~700 bp was introduced between the two promoters, because it has been shown that the half-length of transcription supercoiling

wave is ~800 bp (Krasilnikov, *et al*, 1999). As a spacer the promoter-less *araC* gene was chosen so that the sequence is as close as possible to the original one. The construction involved several steps since there were no available restriction sites for direct cloning. The procedure was the following: the coding sequence of *araC* was amplified from the original pBAD33 plasmid having SacI-ClaI sites at its ends and cloned into pBAD33, resulting in p33. An AatII site was introduced by PCR-ATW near the end of *araC* resulting in pAatII. Finally the PCR fusion of the synthetic promoter to TetR was introduced into pAatII by cloning with AatII and ClaI (fig.III-11).



**Fig.III-11.** Construction of the intermediate pAORTcor. The *araC* gene is shown in orange when it has its native promoter and in red when it is promoterless. The blue and brown boxes correspond to the *Cm* resistance gene and origin of replication respectively. The ORTetR rectangle corresponds to the synthetic promoter-*tetR* construct and the trapezoid to the *rrnB* terminator

In this construct the *rrnB* T1 terminator (Artsimovitch *et al*, 2000) was also included to ensure cessation of transcription before the origin of replication. The resulting construct, pAORTcor, was sequenced and the correct clone was used to sub-clone the SacI-ClaI fragment into pR4, resulting in pR5 (figIII-12).



**Fig.III-12.** Construction of pR5. The red arrow corresponds to the promoter-less *araC* gene, the yellow (OR) to the synthetic promoter and the cyan to *tetR*.

The putative toxicity of the construct was not completely abolished, possibly because the spacer was not sufficiently long, but it was low enough to allow cloning and maintenance of the correct sequence-after three passages the construct was sequenced again and there were no mutations.

Apart from the desired sequence, a clone with a promoter with a T->C mutation in the -35 region (pR5C) was obtained. Since this mutation should reduce promoter strength, it

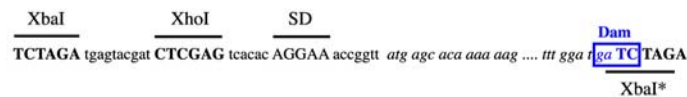
was kept for further analysis, in order to have a broader look at the parameter space of the system.

In pR5 expression of GFP is under the control of both OmpR-P and TetR. When the strength of the synthetic promoter is within a certain region, TetR levels should be such that GFP expression is completely repressed by TetR in the absence of a signal, but upon a positive signal OmpR-P levels should increase, out-compete TetR and activate GFP expression.

Apart from this system of regulation two more systems were constructed to compare the effectiveness of different circuitry.

One of these systems is based on post-translational control: a 200 bp stretch of DNA corresponding to the anti-sense strand of the beginning of GFP was cloned by KpnI-ClaI in pR5 and pR5C, replacing the SD and *tetR* gene and resulting in pR6 and pR6C respectively.

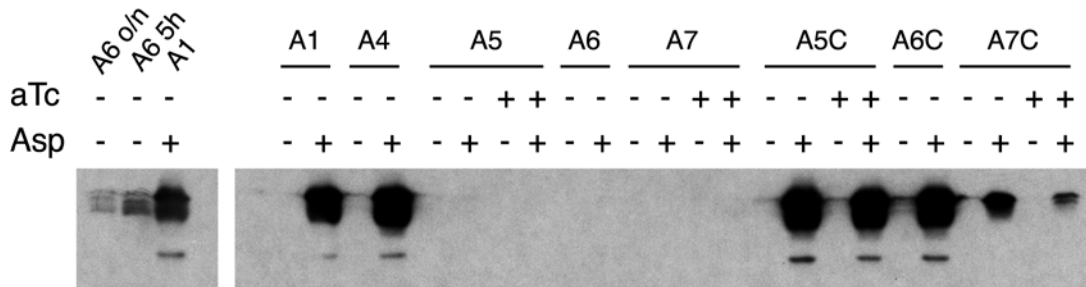
The other one is based on the gene toggle switch: a temperature sensitive form of CI (CI<sub>ts</sub>; Gardner *et al*, 2000) was cloned with a weak SD sequence as an operon fusion to GFP by XbaI-XbaI into pR5 and pR5C, resulting in pR7 and pR7C. CI<sub>ts</sub> has the following mutations in respect to CI: A67T, E118K and S125G. The toggle switch should give the most robust and effective regulation provided that the parameters of the system like promoters' strength, translation efficiency, protein degradation etc, are such that allow the system to be bistable (Gardner *et al*, 2000). The stop codon of CI<sub>ts</sub> was chosen to be tga so that an overlapping dam methylation site with the XbaI site is created, allowing discrimination between the two XbaI sites in plasmid preparations from *dam*<sup>-</sup> strains, since XbaI is sensitive to methylation (fig.III-13).



**Fig.III-13.** The CI<sub>ts</sub> fragment in pR7. The overlapping dam methylation site that renders the last XbaI site sensitive is shown in blue.

### Testing the reporter systems

All the experiments for testing the constructs were done at 37°C, although it is not the optimal temperature for CIts, because at that temperature (30-32°C) the cells needed more than 14 h to grow in labelling medium.

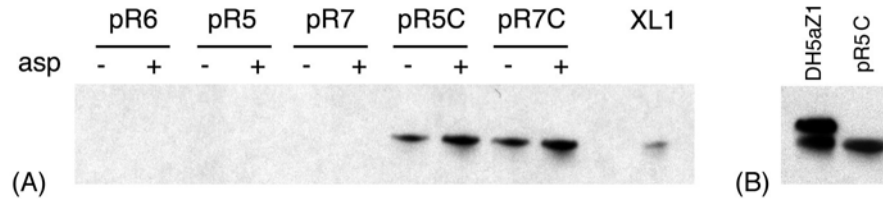


**Fig.III-14.** Western blot with anti-GFP of cells AT142 carrying Taz and the different reporter systems exposed to 5 mM L-Asp for 1h in the presence or absence of aTc. Cells were grown in labelling medium with the exception of the A6 in the first two lanes which were grown in LB in order to see if there is a difference in GFP expression in rich media. Samples A1-A4 carry constructs pR1-pR4 and A5C-A7C have the respective reporters with the mutant synthetic promoter

As shown in fig.III-14 the promoter with the T->C mutation is probably too weak to have any effect in any of the systems tested whereas the opposite holds for the originally designed promoter. Adding increasing amounts of aTc does not seem to affect expression of GFP (data not shown). This suggests that either the level of TetR produced is too high and repression cannot be relieved even at 100 µg/ml aTc or there is a secondary effect, like DNA supercoiling, which prohibits transcription from *pompC*. The possibility that in this case *pompC* has been mutated by the cells to lose completely function, instead of the synthetic promoter can be ruled-out, since cells with pR6 produce low levels of GFP when grown in rich media.

In order to test the amount of TetR produced from every construct samples were analysed as before, using a mixture of two monoclonal anti-TetR antibodies. As a control of the level of expression an o/n culture of XL1-Blue cells grown in LB with tetracycline was used, because XL1-Blue carry the Tn10 in the F episome. As shown in fig.III-15(A)

constructs with the mutant promoter expressed TetR at much higher level than the Tn10 in XL1-Blue, whereas the normal synthetic promoter is completely silent.



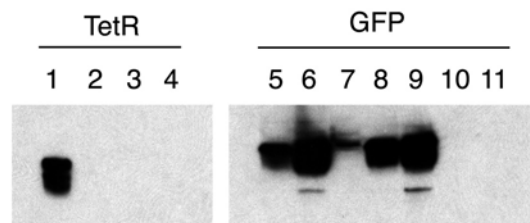
**Fig.III-15.** (A) Western blot using a mixture of two monoclonal anti-TetR antibodies of AT142 cells carrying Taz and the respective reporter in the presence or absence of 5mM L-asp. PR6 was used as a negative control, since it does not carry the tetR gene and XL1-Blue cells, grown in LB+tetracycline, as a positive control because they have the Tn10. (B) Western blot under the same conditions as in (A), for cells carrying pR5C and DH5 $\alpha$ Z1 cells which express enough TetR to regulate P<sub>tet</sub> promoters. The lower band corresponds to the TetR from Tn10 and the upper to TetR which is integrated in the chromosome.

Although the level of TetR produced by the mutant promoter is higher than in the control, the failure to control expression from *pompC* suggests that either competition for binding to C1 is not enough in the presence of C2 and C3 or that the level of TetR is still too low to achieve competition. The latter case was tested by comparing the level of TetR produced by pR5C and strain DH5 $\alpha$ Z1 which carries both Tn10 and a chromosome-integrated TetR and has been shown to regulate promoters carrying two *tet* operators. As shown in fig. III-15(B) the amount of TetR produced in DH5 $\alpha$ Z1 is more than double than that produced by pR5C. The two bands in the blot correspond to the TetR from Tn10 and the chromosome-integrated TetR which has 18 additional amino acids at the N-terminus. Therefore it seems possible that the amount of TetR produced by the mutant promoter is insufficient to repress expression from *pompC*, which has only one mutant *tet* operator, despite the fact that the affinity of TetR for its operator is much higher than the affinity of OmpR-P for C1.

The fact that in the constructs with the designed synthetic promoter there was expression neither of GFP nor of TetR raised the question of the integrity of the construct. The promoters and the genes were sequenced again and there was no mutation,

insertion or deletion. However, by doing diagnostic PCR, a DNA fragment of approximately 1.2kb was mapped after the *gfp* gene. This DNA fragment is absent in the parental plasmid (pR4) and also in the constructs with the mutated promoter and therefore it appears to be a good candidate for silencing both promoters. Its origin is not known, since construction of pR5 from pR4 does not involve that region of the vector. Partial sequencing of this fragment reveals that it is inserted at the end of the *gfp* gene and that it corresponds to a fragment of the transposase gene from Tn10, containing only part of the enzyme and several stop codons, encoded on the same strand as *gfp*. The way in which this fragment could silence the two promoters is unclear, but it could account for the fact that the DNA sequence was tolerated by the cells and not mutated like in the other cases: if a strong synthetic promoter is unfavourable for the cells, then indirect silencing, instead of mutating the promoter, could give a survival advantage to the cells while keeping the integrity of the DNA sequence of the construct.

The latter is further demonstrated by the following experiment: since the construct with the insertion had the correct DNA sequence, the fragment containing the spacer, the synthetic promoter, the *tetR* gene and *rrnB* terminator was cut-out with Sac I and Cla I and re-inserted into pR4, yielding pR5X. In all of the colonies sequenced the vector had undergone recombination, in a different way for each colony:-using primers designed to sequence the construct, yielded instead the sequence of different regions of the plasmid which are not in the vicinity of the cloned genes, e.g. in one colony the sequence of part of the antibiotic resistance gene was obtained instead of *tetR*.



**Fig.III-16.** Western blots using either anti-TetR or anti-GFP to determine the level of each protein in two different colonies of pR5X. Lane 1 DH5 $\alpha$ Z1, lanes 2,5 SA with no amino acid and 6,7 with L-aspartate and L-leucine, lanes 8,9 pR5X, Taz carrying cells colony #3 with or without L-aspartate and lanes 10, 11 colony #4.

Two of the clones were transformed into AT142/pR2 cells and were tested by SDS-PAGE and western blotting for TetR and GFP expression in the presence or absence of L-aspartate (figIII-16). Only one of the clones expressed GFP, but none of the clones expressed TetR, although the presence of the *tetr* gene was verified by PCR.

### **Design of receptors with mutated ligand binding domain**

#### *Analysis of the Crystal structures used for computer Design*

The success of protein design depends on the quality of the template structure that is being used. In this case, the best crystal structure is that of the periplasmic domain of Tar from *Salmonella typhimurium* in complex with one aspartate molecule (pdb code 2LIG). In table III-1 the available apo structures of Tar and the aspartate bound form from *S.typhi* (Tar<sub>S</sub>) and *E.coli* (Tar<sub>E</sub>) are listed. Structures of the apo form had a monomer as the crystal lattice unit, due to the dimer's symmetry. Thus the structure of the dimer was re-constructed with the programme SwissPdbViewer using the instructions given in each pdb file. The RMSD of the structures with respect to the apo-Tar<sub>S</sub> (1LIH) was calculated either for the whole structure or excluding the last helix of the periplasmic domain, which has been proposed to move upon aspartate binding, according to the piston model (Ottemann *et al*, 1999). 1LIH was chosen as a reference because it is the only apo structure with an available aspartate bound structure of high resolution. In fact the latter structure, with only one site occupied by aspartate, should be the most representative because in *E.coli*, in contrast to *Salmonella*, negative co-operativity allows binding of only one aspartate molecule per receptor dimer, within the physiological range of aspartate concentration (Biemann and Koshland, 1994).



**Table III-1.** Available structures for apo Tar of *S.typhi* and *E.coli* and the highest resolution ligand bound structure of Tar of *S.typhi* (Björkman *et al*, 2001)

<b>Pdb code</b>	<b>Description</b>	<b>ligands</b>	<b>Resolution (Å)</b>	<b>RMS<sup>a</sup> (Å)</b>	<b>RMS<sup>b</sup> (Å)</b>
1LIH	<i>Salmonella</i> , cross-linked with disulphide bonds (xx, yy)	phenanthroline	2.2	-	-
2LIG	<i>Salmonella</i> , cross-linked with disulphide bonds (xx)	L-aspartate Phenanthroline 1 sulphate	2.0	2.76	2.60
1WAS	<i>Salmonella</i> , wild-type (zz)	-	2.7	2.65	2.66
1VLS	<i>Salmonella</i> , wild-type (ww)	-	1.85	2.54	2.57
2ASR	<i>E.coli</i> , wild-type (qq)	2 sulphates	2.3	2.11	2.26

<sup>a</sup> RMS deviation of the backbone atoms of the whole periplasmic domain, with 1LIH as a reference.

<sup>b</sup> RMS deviation of the backbone atoms of the periplasmic domain without the most C-terminal helix, with 1LIH as a reference

As shown in table III-1 the RMS deviation is approximately the same for the aspartate-bound structure and the various apo structures, even when the “in theory moving” helix is excluded. Thus it appears that the “movement” assigned to the last helix upon aspartate binding, based on structures 1LIH and 2LIG, is actually within the structure refinement error and probably reflects a difference in crystallisation conditions and packing rather than a conformational change with biological significance. Of course this raises the questions which is the true apo conformation of the receptor and which is the actual mechanism of signal transduction.

However it should be noted that since the available structures corresponds only to the periplasmic domain, it could be possible that the actual structure in the wild-type Tar and/or the chimera is altered because of constraints rising from the transmembrane and cytoplasmic regions.

This poses a problem for a computer design exercise because we do not know the structure of at least one relevant state and therefore when making mutations we could create unwanted compensatory effects.

#### *Testing the prediction capability of the design algorithm*

Before trying to change the specificity of the receptor, it is necessary to test how well the computer design can predict the effect of mutations on aspartate binding in wild-type Tar. Perla algorithm (Angrand *et al*, 2001) was used to create the structures of the complex aspartate-Tar for different mutants for whom the  $K_d$  has been experimentally measured. The structures generated were subjected to two cycles of energy minimization using the programme SwissPdbViewer 3.7 and the energy of the complex was calculated using the Fold-X algorithm (Guerois and Serrano, 2000; Guerois *et al*, 2002 ).

As shown in table III-2. The effect of most mutations can be predicted reasonably well.

**Table III-2.** Experimental  $K_d$  and swarming rates, in mm/h when the cells are exposed to 0.1 mM L-Asp from (Björkman *et al*, 2001) and predicted  $K_d$  for mutations of the residues interacting with the ligand.

mutation	$K_d$ ( $M^{-1}$ )	swarming	Predicted $K_d$ ( $M^{-1}$ )
WT	0.000003	0.27	0.000001
R64A	0.047	0.04	0.001
R64C	>0.100	-	0.002
R64D	0.021	0.02	2.24
R64K	0.030	0.03	0.0001
R64S	0.040	0.04	0.0009
S68C	~0.000002	-	0.000003
S68G	~0.000002	-	0.000003
S68H	~0.000002	-	0.000002
S68I	~0.000002	-	0.000003
R69A	0.008	-0.02	0.005
R69C	0.002	-	0.004
R69H	0.005	-	0.0001
R69K	0.0002	0.34	0.0002
R69D	-	0.025	25
R69S	-	0	0.008
R69P	0.0039	-	-
R69M	-	-	0.0002
R73C	0.007	0.06	0.0015
R73K	0.0004	0.18	0.00006
R73S	0.007	0.04	0.0015
R73D	-	0	0.04
R73W	0.003	-	**

The possibility of other amino acids binding at the same pocket was tested by calculating the  $K_d$  values for the respective complex. Amino acids that have reasonable predicted  $K_d$  values (that is without large Van der Waals clashes) are shown in table III-3.

**Table III-3.** Predicted  $K_d$  for different amino acids as ligands in the place of aspartate. Values only for amino acids which did not have large Van der Waals clashes are shown.

Amino Acid	$K_d$ ( $M^{-1}$ )	Amino Acid	$K_d$ ( $M^{-1}$ )
D	0.000001	S	0.001633
E	0.000007	T	>0.100
Q	0.000831	V	0.001689
A	0.011777	I	0.004204
G	>0.100	L	0.129576
C	0.104035		

As expected aspartate and glutamate have the lowest  $K_d$  values and the rest have at least 10-fold higher  $K_d$  values than glutamate, with glutamine being the next amino acid with low  $K_d$ . Experimentally it has been shown that glutamate is sensed by Tar with lower affinity than aspartate, although the experimental difference was approximately 1000-fold (Utsumi *et al*, 1989; Baumgartner *et al*, 1994) instead of the predicted 10-fold. Glutamine has been shown to have a  $K_d > 10mM$  and appears not to be a chemoattractant with the classic chemotaxis assays (Clarke and. Koshland, 1979). The value of the  $K_d$  is at least 1 order of magnitude higher than glutamate which is in agreement with the prediction

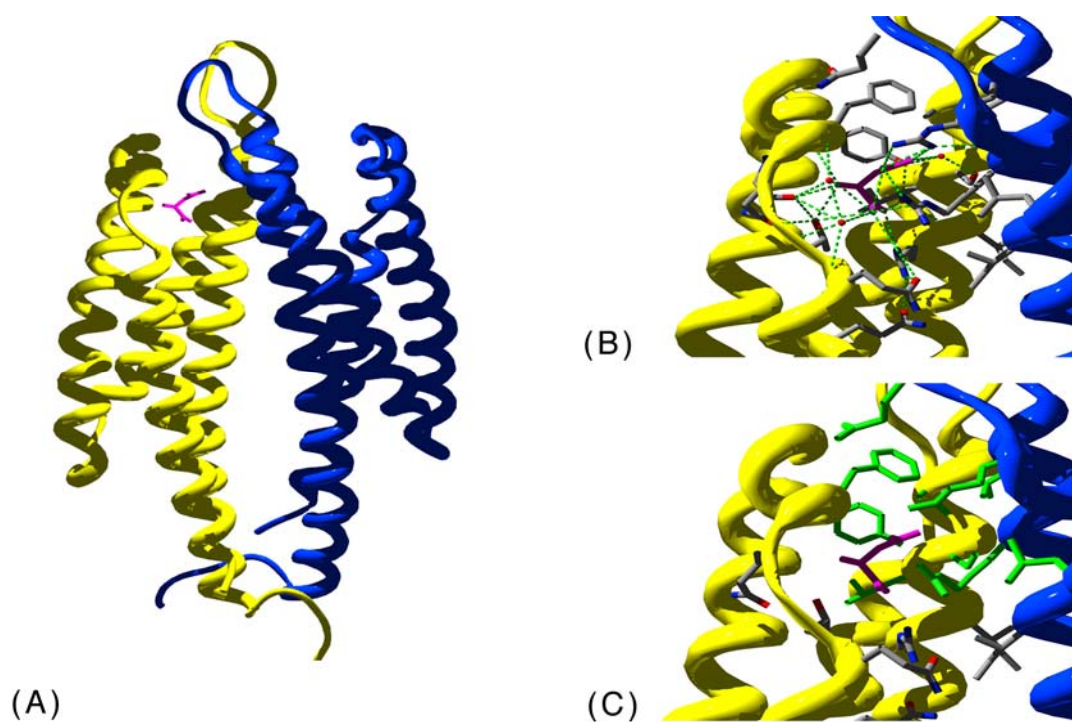
Thus with all the caveats associated to the fact that we do not know the structure of one of the states, the design algorithms seem to perform well when confronted to simple cases.

### *Designing new specificity*

Before engineering a receptor with completely different specificity from the parent receptor, it is necessary to test how feasible it is to change the specificity towards stimuli which are close from a structural point of view, to the original one, i.e. amino acids. This means that the residues which contribute to binding of the ligand backbone remained unaltered and only the residues contributing to side-chain binding were mutated *in silico*. The network of the native interactions in the wild-type complex is shown in fig.III-17.

As candidates for new ligands four amino acids were chosen:

- Leucine, because its side-chain has a similar structure to aspartate but it is a hydrophobic residue.
- Glutamine, because it is close in structure and nature to glutamate which is also a ligand for Tar.
- Serine and threonine, because the serine receptor, Tsr, has a binding pocket which differs only in a few amino acids, mainly 2, from that of Tar. Thus in this case we just computationally changed those residues, plus a few ones in the case of Thr.



**Fig.III-17.** (A) Crystal structure of the periplasmic domain of Tar from *Salmonella taphimurium* with a single L-aspartate molecule (magenta). (B) The hydrogen bond network for binding of aspartate to the receptor (C) The ligand binding pocket. Residues which were mutated are shown in green.

The name given to each receptor was representative of the amino acid it was designed to bind, i.e. TaL for leucine, TaQ for glutamine, TaS and TaT for serine and threonine respectively. Structures of the putative complexes were generated with Perla and treated the same way as the Tar mutants. For TaS and TaT the mutations chosen were such that they would recreate the binding site of Tsr, with the difference that there were extra mutations for TaT, in order to have space for accommodating the methyl group. For TaL and TaQ residues shown in table were randomly mutated and only the mutations with the best energy were chosen for experimental test.

**Table III-4.** Residues in the binding pocket for the designed receptors. Mutations are shown in red.

Residue	Taz	TaL	TaQ	TaS	TaT
67	L	L	I	L	L
68	S	I	S	N	N
149	Y	F	N	F	F
69B	R	L	L	R	K
72B	V	M	A	V	V
73B	R	M	R	R	M
82B	Q	A	Q	Q	Q
89B	L	W	W	L	L

**Table III-5.** Predicted Dissociation constants ( $M^{-1}$ ) for D and the respective ligand for each receptor

	D	L	S	T	Q
WT	0.000001	0.129576	0.001633	0.689670	0.000831
TaL	0.395005	0.001311	-	-	-
TaS	0.000013	-	0.001633	-	-
TaT	0.001605	-	-	0.032994	-
TaQ	0.006860	-	-	-	5.2

As shown in table III-5, there is a shift in the specificity for TaL, although the absolute value of the  $K_d$  for Leu is not very satisfying. Surprisingly TaS (and TaT) is predicted to have higher affinity for aspartate than serine despite the fact that the introduced mutations were chosen specifically to re-constitute the binding site of Tsr. In the design of TaQ there was a design error, but the mutations from the prediction were anyway experimentally tested, because in the predicted structure the substituted residues take up almost the whole binding pocket which could lead to an interesting behaviour.

## **Construction of receptors with mutated ligand binding domain**

In order to construct the receptors which carry several mutations in the ligand binding domain, two restriction sites were introduced within the Tar coding sequence to facilitate cloning. A Pst I site was introduced at position 281, where the numbering corresponds to the DNA sequence of the chimera, by changing with PCR-ATW the caa codon to cag, causing no change in the protein sequence. Taz2 cloned in pBluescript KS (pBTaz2) was used as a template. Subsequently, a Nco I site was similarly introduced at position 578, restoring at the same time the A160V mutation. The resulting construct was named pCOR. The fragments with the mutations were constructed using overlapping oligos as described in the Materials and Methods section and cloned in pCOR, substituting the wild-type ligand binding domain. The constructs were sequenced and correct clones were sub-cloned into pCL1920 using Hind III and BamH I.

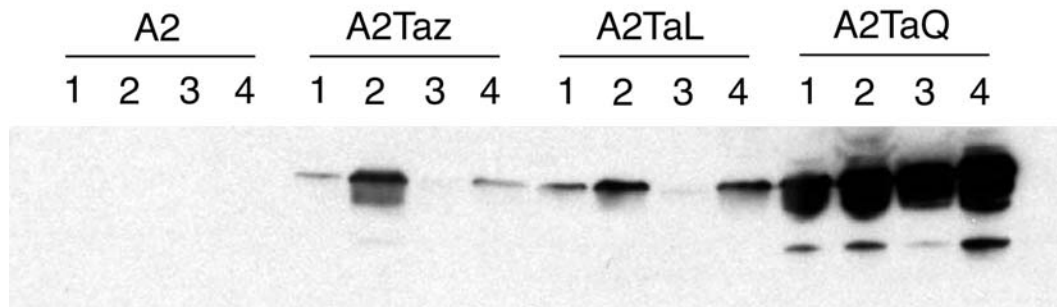
Single point mutants were generated by PCR-ATW using pBTaz2 as a template and then sub-cloned into pCL1920.

Plasmids pCL1920 carrying the different receptors were named after the receptor that they are carrying, e.g. plasmid pCL1920 with TaL was named pTaL. These constructs were transformed into AT142 cells harbouring pR2 by using the TSS method.

### **Testing the mutant receptors**

TaL and TaQ receptors were tested by exposing AT142 with pR2 carrying the respective constructs to either L-Leu, L-Asp, L-Gln or no amino acid. As controls cells with Taz were used, but also AT142 cells with pR2 but no receptor, in order to check the background level of expression in the absence of an EnvZ catalytic domain.

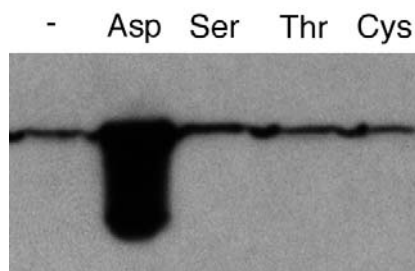




**Fig.III-18.** Western blot using, anti-GFP, of AT142 cells carrying pR2 (A2) or pR2 and a receptor (Taz, TaL or TaQ). Cells were exposed for 1h to either no stimulus (lanes 1), 5 mM L-Asp (lanes 2), 5 mM L-Leu (lanes 3) or 5 mM L-Gln (lanes 4).

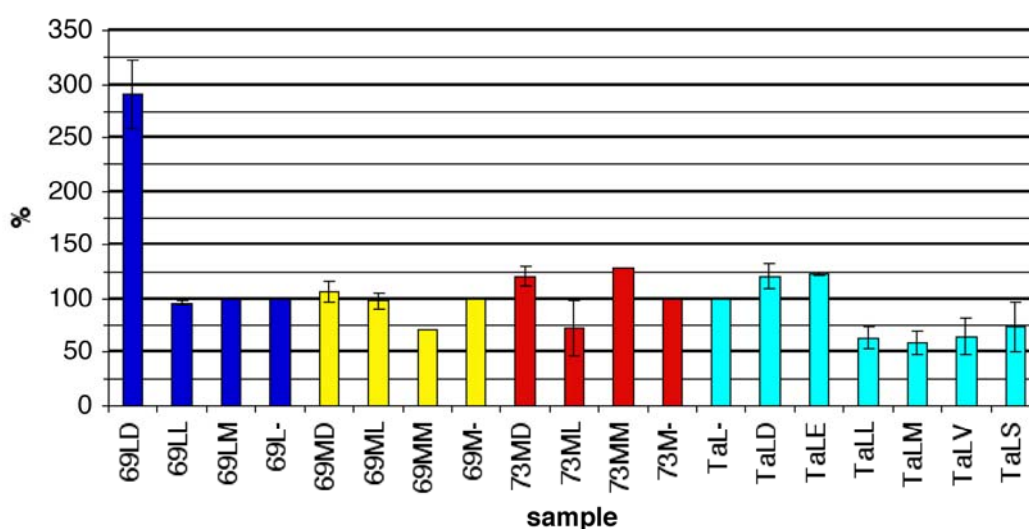
As shown in fig.III-18 the cells without a receptor do not have detectable GFP levels and therefore in this strain and under the experimental conditions the presence of a functional EnvZ-C is required for GFP expression. TaQ is locked in the active state and cannot respond to any of the signals tested. The fact that the locked-on TaQ results in the same levels of GFP expression for all the amino acid tested, shows that under the experimental conditions, there is no significant effect on OmpR levels, through possible changes in its transcription by CRP. L-Gln does not have an effect on either Taz or TaL, but surprisingly L-Leu seems to have an inhibitory effect on both TaZ and TaL.

When TaS and TaT were tested for L-Ser, L-Asp, L-Thr and L-Cys only L-Asp activated TaS as computationally expected (fig.III-19), whereas the other aa had no effect on GFP expression and TaT was locked-off (data not shown).



**Fig.III-19.** Western blot using, anti-GFP, of AT142 cells carrying pR2 and TaS, exposed to 5 mM of L-amino acids for 1h

TaL was re-designed, aiming to achieve activation by L-Leu. The new receptor was named TaLM because it had only the mutations R69M and R73M. The predicted dissociation constants for aspartate and leucine were  $\sim 150$  mM and  $573$   $\mu$ M respectively. Surprisingly this mutant was locked-off and not partially responsive like TaL. In order to check if one of the mutations in TaLM was responsible for the complete loss-of-function the single mutants R69M, R73M were constructed. Since TaL has the mutation R69L, the respective single mutant was also constructed.



**Fig.III-20.** GFP levels for the different receptor mutants and TaL, exposed to Asp, Leu and Met. The data of each series, which correspond to the average of three experiments, are normalized to the value of the respective control

As shown in fig.III-20 all single-point mutants have a non-detectable basal level but respond to L-Asp. Therefore the basal level observed in the case of TaL should be the result of partial activity recovery by one or the combination of the rest of the mutations introduced.

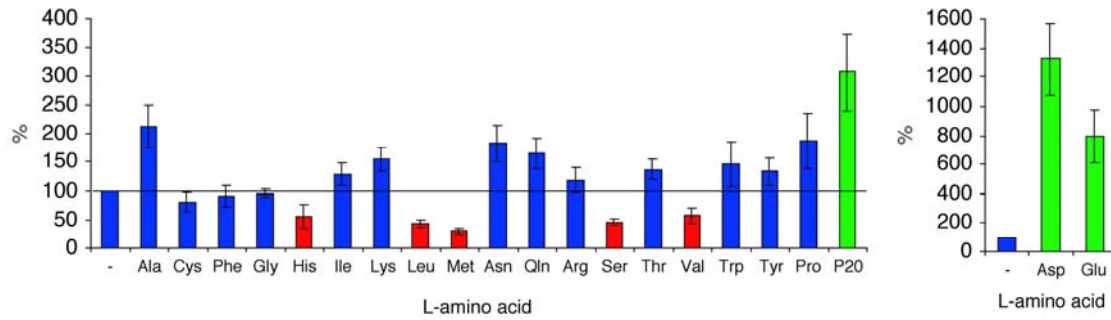
In conclusion, the designed mutants were not able to respond to the target amino acids. In the case of Leu, an inhibitory effect was seen which later was also seen for the WT receptor (see following chapters) and could mask any activation. Replacement only of the

conserved Arg73 locked the receptor in an off conformation which could be explained by the hydrophobic residues been more favourable in the inactive conformation than when bound to Leu. For Ser although the mutations introduced were the ones that make the Tar binding pocket different from that of Tsr, was not abolished. This is interesting because it is the expected behaviour from the computer prediction (this will be discussed later on in the following chapters). Finally, the receptor designed to bind Gln did not respond to it, as expected when we realized our mistake. However, this mutant proved to be very interesting for understanding the chimera as explained later.

It should be noted that the effect of the single point mutations in the ligand binding domain of Taz cannot give any answer to the question if leucine binds the same way as aspartate, since it has already been shown that different mutations in the binding pocket can have two very different effects (Yang *et al*, 1993): the R64C substitution renders Taz constitutively active, whereas the R69H or R73Q substitutions eliminate the sensing properties of Taz without changing its baseline kinase and phosphatase activities.

### **Responsiveness of Taz to L-amino acids**

These results and especially the fact that L-leucine seems to inhibit the wild-type Taz suggested that it is necessary to examine carefully the response of the Taz system to all natural aa before trying to modify the receptor's specificity. To do so, the system was exposed to 5 mM of each amino acid for 1h at 37°C, as described in the Materials and Methods section. As shown in fig.III-21, L-Asp and L-Glu clearly activate the system and L-Pro can elicit a stimulatory effect only at very high concentration (20 mM). Using such a concentration for L-Pro was based on earlier chemotaxis studies which suggested that L-Pro could elicit a moderate attractant response only at high concentrations and maybe through its metabolism (Mesibov and Adler, 1972).

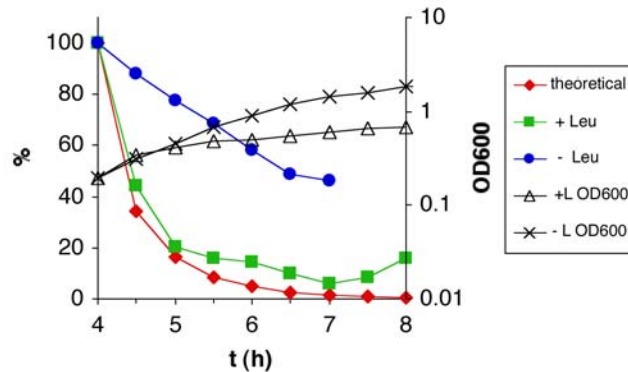


**Fig.III-21.** Response of SA cells to exposure for 1h at 37°C to 5 mM of L-amino acid and also to 20 mM L-Pro (P20). Activating amino acids are shown in green and inhibitory in red. The values represent the average of three experiments and were normalized to the value of the control (no stimulus).

Although some amino acids like L-Ala seem to have a very small stimulatory effect, it is not comparable to that of L-Asp and L-Glu and is within the noise. Surprisingly L-Leu, L-Met, L-Ser and L-Val had a clear inhibitory effect whereas in the case of L-His there is inhibition but not as strong as for the other aa. The 40-50% GFP level measured for these samples at a first glance does not seem very important. However if one considers the 40 min half-life of GFP(LVA), cell growth and the small time-frame of the exposure to the signal (1h) it appears that this level of GFP corresponds to what would be expected if GFP production ceased instantaneously upon addition of the signal. This is more obvious with the following experiment: cells are allowed to grow without L-Leu and when they enter into the logarithmic phase of growth they are divided into two flasks. In one of them the cells continue growing without any signal and in the other they continue growing in the presence of saturating L-Leu (5 mM). Samples from both flasks were taken every 30 min. If GFP production ceased completely then the amount of GFP that should be detected at a given time point should be given by the exponential decay of GFP, with  $t_{1/2} = 40$  min, corrected for cell growth – i.e for time-point  $i$  where the cells have reached an absorbance of  $OD_{600}^i$  the % of GFP detected should be given by the equation:

$$GFP(\%)_i = \frac{OD_{600}^{i-1}}{OD_{600}^i} 100 e^{-\frac{t}{1.5 \ln 2}}$$

Comparing the theoretical values to the experimental ones (fig.III-22) it is obvious that L-Leu shuts down completely production of GFP.



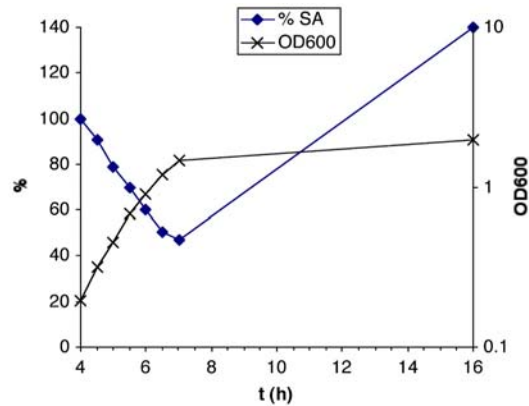
**Fig.III-22.** Growth curves (X and  $\Delta$ ) and GFP levels ( $\bullet$  and  $\blacksquare$ ) in SA cells grown at 37°C in the absence of a stimulus or at constant saturating L-Leu concentration, once they entered into exponential phase, respectively. The theoretical decrease of GFP, in the case of complete cessation of GFP production, correcting for cell growth is shown in red ( $\blacklozenge$ ).

However, different questions are raised by this experiment: since the wild-type EnvZ-OmpR system is activated after mid-logarithmic phase by an unknown mechanism (Martinez-Flores *et al*, 1999) why does the Taz-OmpR system remains regulated in the logarithmic phase and could the chimeric system respond independently of the state of cell growth?

### Dependence of the system's responsiveness on cell growth

These questions were addressed by following the GFP levels in cells that were growing in the absence of signal and by exposing cells that are at different points of the growth curve for 1h to L-Asp and L-Leu.

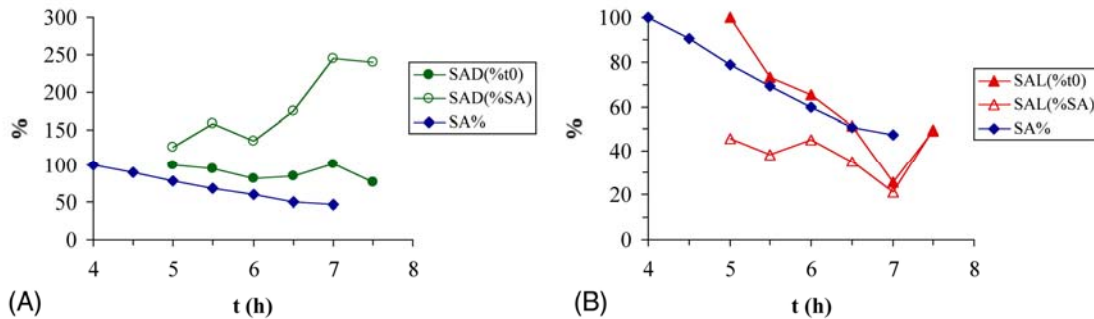
In the absence of a signal the GFP levels decrease throughout the whole growth curve, even at early stationary phase and increase only after o/n growth (fig.III-23).



**Fig.III-23.** Growth curve of SA cells in labelling medium the absence of any stimulus. The  $OD_{600}$  values (x) are plotted against the secondary Y axis. The levels of GFP, normalized to the first data point, are shown with (♦), plotted against the primary Y axis.

When cells from the o/n cultures are exposed to either L-Leu or L-Asp they do not respond (data not shown) which means that activation of the system by products of cell lysis is improbable. Thus the system seems to be activated only at very late stationary phase but probably through a different mechanism.

Following the responsiveness of the system through-out the logarithmic phase it seems that the system retains its ability to be regulated by the amino acids. Activation by 5 mM of L-Asp seems to be increasing with time when each data point is normalised to the respective point of non-induced cells (figIII-24(A)). However this is an artefact produced by the fact that the level of GFP in the non-induced cells decays with time. When the data points are normalised to the initial value of GFP for the non-induced cells it is clear that the amount of GFP is approximately the same for all cases which means that 5 mM of Asp saturate the system and this happens independently of the state of cell growth. Similarly responsiveness to L-Leucine seems to be constant (figIII-24(B)), because L-Leu stops completely GFP expression and thus the difference between the induced and non-induced cells at any time point remains the same. However since the basal GFP level decreases with time the sensitivity of the measurement will also decrease at points later in the growth curve.

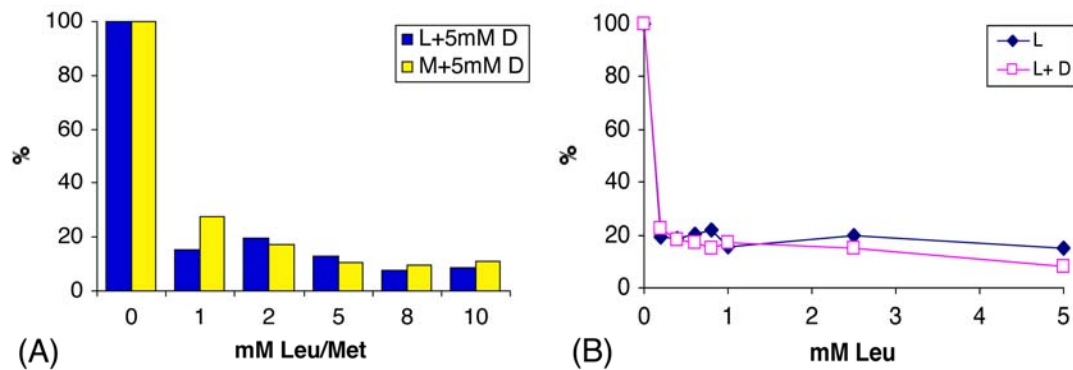


**Fig.III-24.** Responsiveness to L-Asp and L-Leu at different points of the growth curve. Cells were grown in labelling medium and starting at  $t_0 = 4$  h, which corresponds to the beginning of the exponential phase, aliquots were taken and exposed to 5 mM of each amino acid for 1h. In both plots the GFP levels in non-exposed cells are shown with ( $\diamond$ ), normalized to the first data point. (A) Responsiveness to L-Asp; in ( $\bullet$ ) values are normalized to that of control cells at  $t_0$  and in ( $\circ$ ) values are normalized to the corresponding value of the control cells at the specific time-point (B) Responsiveness to L-Leu; in ( $\blacktriangle$ ) values are normalized to that of control cells for  $t_0$  and in ( $\triangle$ ) values are normalized to the corresponding value of the control cells at the specific time-point.

These mean that although the system responds equally well through-out the whole logarithmic and early stationary phase, the sensitivity of the method is best at early points of growth for studying repression and later points for studying activation.

### **The inhibitory effect of Leu is applied on the first level of the pathway, the receptor**

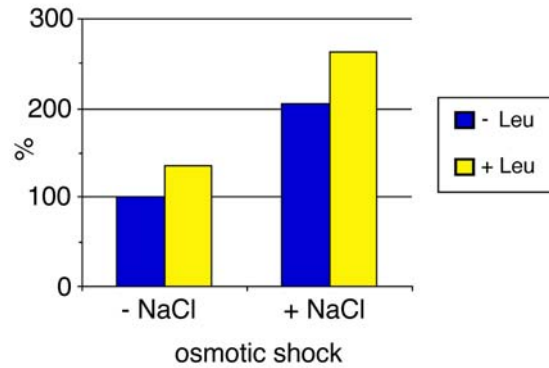
As already mentioned, since the cloned *pompC* carries 4 sites for Lrp, the observed effect of Leu inhibition could be indirect through this protein. To test this hypothesis cells were exposed to different amounts of L-Leu, since it is the only amino acid from the ones for which we have observed an inhibitory effect that is reported to bind to Lrp, in the presence/absence of saturating L-Asp (5mM). At the range of concentrations tested L-Leu had a dominant effect over L-Asp (fig.III-25). The same was true when L-Met was used instead of L-Leu.



**Fig.III-25.** (A) Competition between saturating L-Asp (5 mM) and different concentrations of L-Leu (blue bars) or L-Met (yellow bars). (B) Competition between L-Asp and L-Leu at concentrations of Leu below 1 mM (□). Cells treated only with L-Leu.(♦) are shown as a control. In all cases data are normalized to the value for non stimulated cells.

This together with the fact that the locked-on TaQ receptor shows no change of GFP levels in the presence of L-Leu show that an active receptor can produce enough OmpR-P to out-compete any putative effect of Lrp and therefore L-Leu, as well as the other amino acids, should be acting on the receptor. It should be noted that the conclusion drawn with the locked-on TaQ is valid only provided that the level of OmpR-P produced by activated Taz and locked-on TaQ is the within the same range than in the wt Taz. In order to check if this inhibition was due to an interaction of the amino acids with EnvZ-C, BL21(DE3) cells carrying pR2 were subjected to osmotic shock in the presence and absence of 5 mM L-Leu in medium A. The response was the same regardless of the presence of L-Leu (figIII-26), indicating that L-Leu and the other inhibitory amino acids are probably interacting with the Tar part of the chimera.

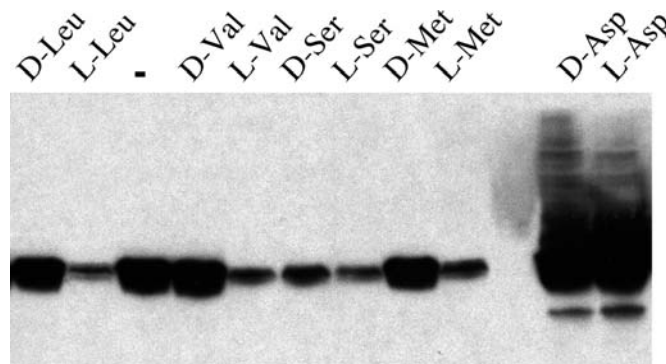




**Fig.III-26.** Osmotic response in the context of L-Leu. BL21(DE3) cells carrying pR2 were grown in medium A with or without 0.3M NaCl in the presence (yellow bars) or absence (blue bars) of 5 mM L-Leu.

### Stereo-specificity in sensing the amino acids which elicit a response

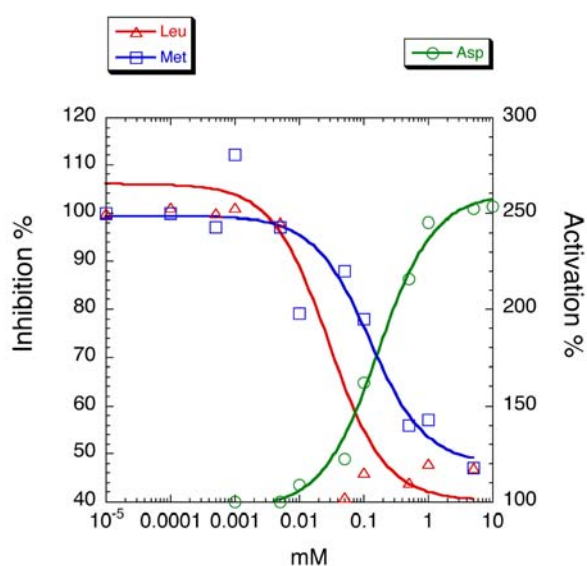
Since the majority of the inhibiting amino acids have a hydrophobic side-chain, the possibility of non-specific hydrophobic interactions with the receptor had to be considered. This question was addressed by exposing cells to either D- or L- amino acids. As shown in fig.III-27 the inhibitory effect is stereo-specific for the L- form of the hydrophobic amino acids, whereas it seems not to be stereo-specific for polar ones regardless if they are activating (DL-Asp) or repressing (DL-Ser) the system. It should be noted that L- and D-His are also discriminated (data not shown).



**Fig.III-27.** Western blot, using anti-GFP, for cells exposed to 5 mM of L- and D- amino acids for 1h.

### Apparent affinities for L-Asp, L-Leu and L-Met

The competition experiments had already shown that the system has a greater apparent affinity for L-Leu and L-Met than for L-Asp. In order to have a more representative image of the apparent affinities cells were exposed to different concentrations of the respective amino acid, the data were fitted to a sigmoidal curve (fig.III-28) and the IC<sub>50</sub> was calculated.



**Fig.III-28.** Dose response curves for L-Asp (○), L-Leu (△) and L-Met (□).

The equation used for fitting the data was equation 1 for L-Leu and L-Met and equation 2 for L-Asp.

$$y = \min + \frac{\max - \min}{1 + 10^{-(\log IC_{50} - \log C)}} \quad (1)$$

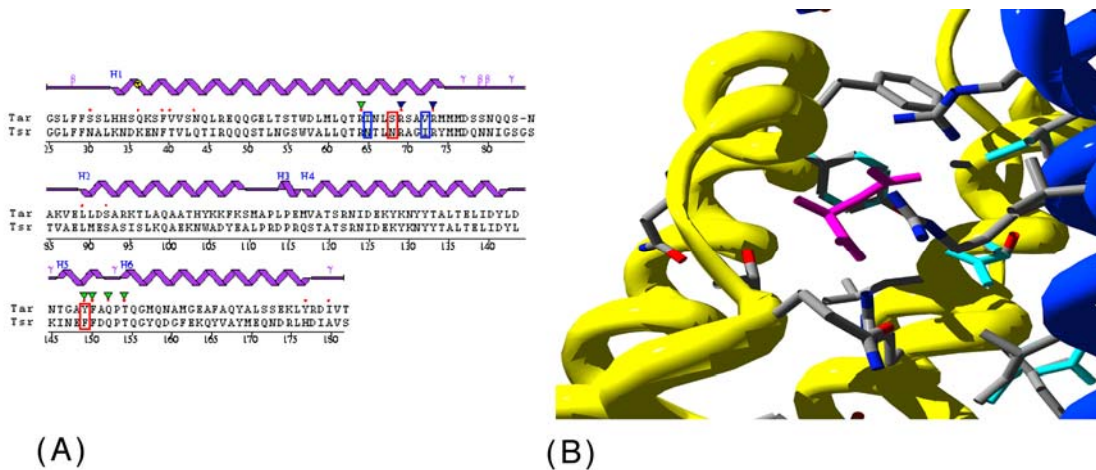
$$y = \min + \frac{\max - \min}{1 + 10^{\log IC_{50} - \log C}} \quad (2)$$

For each amino acid the experiment was repeated three times and the final apparent  $K_d$  represents the average of the IC50 values, calculated from each experiment.

The apparent  $K_d$  for L-Leu is  $39.6 \pm 27.9 \mu\text{M}$ , for L-Met  $127 \pm 82 \mu\text{M}$  and for Asp  $266 \pm 103 \mu\text{M}$ . Although the values have a substantial error, they follow the trend of the competition experiments: L-Leu and L-Met can out-compete in descending order L-Asp.

### Construction and testing of Tsr-Tar-EnvZ chimeras

The puzzling behaviour of TaS raised the question whether the two mutations introduced in the Tar ligand binding domain were enough to re-construct the Tsr binding domain. Taking a closer look at the crystal structure of the aspartate occupied binding site it was considered that positions 72 (Val in Tar, Ile in Tsr) and 65 (Ile in Tar, Asn in Tsr) - although not interacting directly with the ligand could influence its binding (figIII-29).



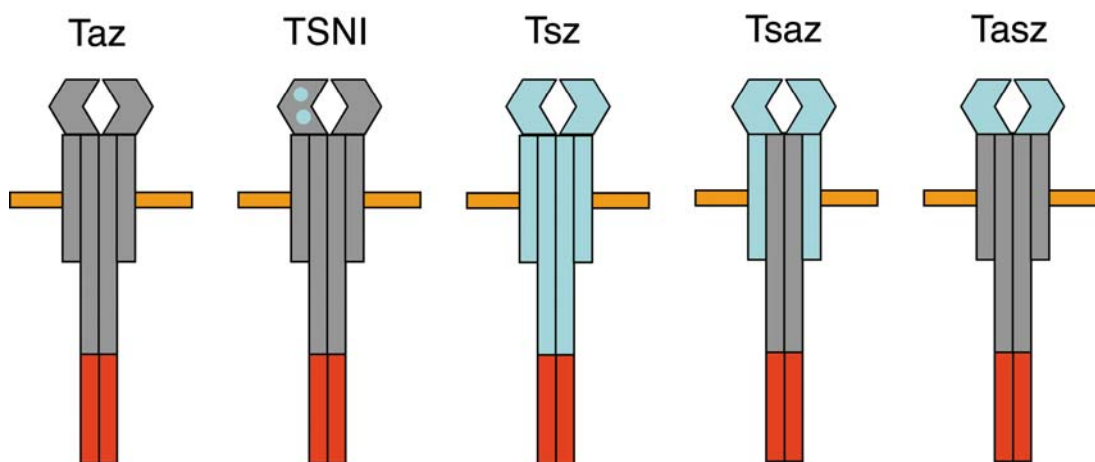
**Fig.III-29.** The ligand binding pockets of Tar and Tsr. (A) sequence alignment of the periplasmic domains. Triangles indicate the residues involved in ligand-binding for Tar. In red rectangles are marked the residues which were initially considered for TaS and in blue rectangles the two extra residues close to the binding pocket which are different (B) Crystal structure of Tar-L-Asp complex (2LIG). The residues which are different in Tsr are shown in cyan.

For example although residue 65 is not directly involved in binding, it can affect the number of water molecules present in the pocket, which take part in the hydrogen-bond

network that contributes to the binding of the amino acid. However, when the mutations were modelled and the binding of aspartate and serine was calculated, aspartate was found again to be recognised with higher affinity than serine, with respective binding energies  $-7.38$  kcal/mole and  $-2.14$  kcal/mole. The mutant receptor, TSNI, showed a peculiar behaviour which is described below, together with that of Tsr chimeras.

The chimera between Tsr and EnvZ, Tsz, was constructed, in order to see if it would be activated by L-Ser like Taz is activated by L-Asp. TsZ was constructed by amplifying the respective part of Tar with HindIII and NdeI sites at the ends and replacing the respective part of Tar in the Taz construct. However cells with Tsz showed a very low basal level of GFP and no responsiveness to any amino acid as will be shown later.

This was a surprising result taking into account the high degree of homology between Tsr and Tar. To solve the problem Tar-Tsr-EnvZ chimeras were constructed, hoping that the signal transduction ability of the Tar-EnvZ fusion could be combined with the sensing properties of Tsr. As shown in fig.III-30 two different triple chimeras were constructed:



**Fig.III-30.** Schematic drawing of different chimeras combining parts of Tar (grey), Tsr (cyan) and EnvZ (red). In TSNI the mutations that were introduced in order to reconstitute the Tsr binding domain are depicted as cyan dots.

- TsaZ which has the N-terminus, first transmembrane part (TM1) and ligand binding domain of Tsr and is fused to Taz at position 179, where the numbering corresponds to wild-type Tar. Construction of Tsaz was based on the observation that the TM2 of

Tar and Tsr are interchangeable (Tatsuno *et al*, 1994). Tsaz was constructed as follows The N-terminus of Tsr up to the second EcoR V site of the coding sequence (535) was amplified by PCR, since this site is at the same position as the unique EcoR V site in Tar. This fragment was fused by PCR to a fragment corresponding to the coding sequence of Tar which is flanked by the unique EcoR V site for Tar, and the NdeI site. The Tsr-Tar fragment was subsequently used to replace the Tar moiety of Taz.

- TasZ which has the ligand binding domain of Tsr fused at positions 61 and of 162 Taz, where the numbering corresponds to the wild-type Tar. Construction of Tasz was done by taking advantage the Taz construct which has the ligand binding domain in the form of a PstI-NcoI cassette.

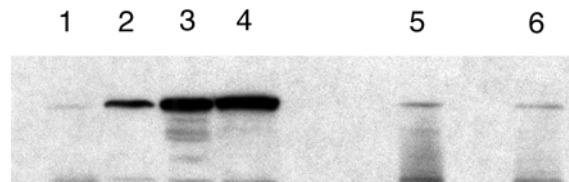
### **Checking the suitability of the genetic background for the expression of the new receptors**

It should be noted that as aforementioned the strain used for these experiments (AT142) has the *flhD5301* mutation and therefore transcriptional units like the *tar-tap* operon, which are controlled by the FlhD/FlhC activator should be silent (Liu and Matsumara, 1994). Although it has been assumed that all chemoreceptors are under the control of this transcription factor it has been clearly demonstrated for Tar, Tap (Liu and Matsumara, 1994) and Aer (Pruss *et al*, 2003) but not for Trg and Tsr. Therefore it seems possible that in this genetic background Tsr expression is not abolished and formation of hetero-dimers prevents the formation of an active chimera. The study of the chimera between Trg-Envz, Trz, carried by Baumgartner *et al* was done in a similar genetic background without however any similar complications. This could be due to the fact that the expression level of the chimera was higher than in this study but also because Trg is a low abundance receptor whereas Tsr is the most abundant of the chemoreceptors, being at least at 20-fold higher levels than Trg.

Therefore before studying the chimeras mentioned above it is necessary to clarify whether Tsr is being expressed in the cells. This possibility was tested by growing

overnight cells which contain no, some or all of the receptors and strain AT142 and comparing their profile in western-blots with antibody against residues 290-470 of Tsr which are conserved in the MCPs. The control cells were:

- RP437 which has all the MCPs. This strain was grown in Trp broth, which is the medium commonly used for chemotaxis assays, in order to ensure expression of the MCPs and avoid effects from the metabolism. All the other strains were also grown in Trp broth in order to have comparable results, apart from AT142 which was also grown in labelling medium to check if its profile is different in the medium used for the chimera assays.
- UU1250 which has none of the five MCPs.
- UU1250 carrying plasmid pLC113. These cells express Tar at levels which can elicit taxis to aspartate only when they are induced with 0.7  $\mu$ M sodium salicylate, as previously described (Ames *et al*, 2002). They were grown both in the absence of inducer, in order to check the basal level of expression from the promoter and in the presence of inducer, to check the level of receptor that is required for a response.



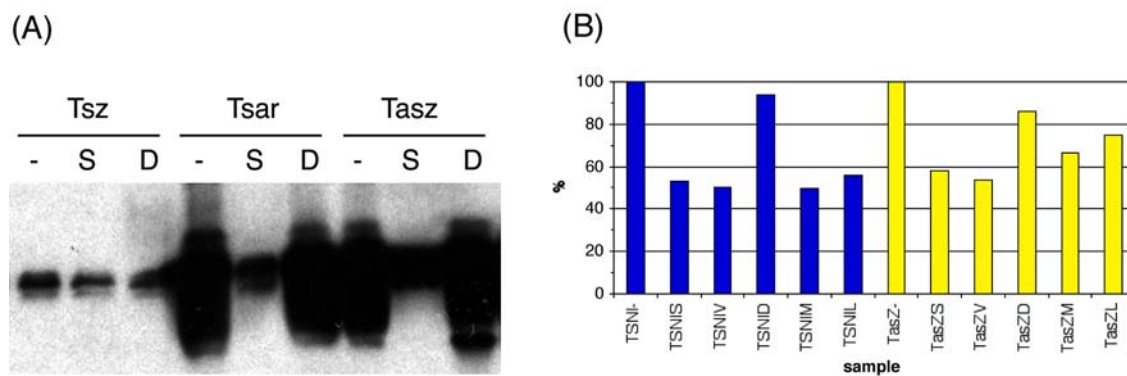
**Fig.III-31.** Western blot, using polyclonal antibody against the 290-470 region of the MCPs. Cells were grown o/n at 37°C in TB apart from AT142 which were grown both in TB and labelling medium. Lane 1 corresponds to UU1250, lanes 2 and 3 to UU1250/pLC113 without or with induction with 0.7 $\mu$ M sodium salicylate, lane 4 to RP437 (wt) and lanes 5 and 6 to AT142 grown either in TB or labelling medium.

As shown in fig.III-31 the basal level of the inducible promoter is quite high and the amount of receptor induced from the plasmid is similar to the sum of all the receptors in the wt. The receptor-less strain shows a very faint band which seems not to be specific. The profile of AT142 in either medium is similar to that of the receptor-less strain. Therefore it is safe to assume that the *flhD5301* mutation is sufficient to limit expression

of the MCPs and even if Tsr is expressed at all, its level would be too low and probably insufficient to sequester the chimeric monomers.

### Behaviour towards amino acid of the new receptors

Once ensured that wild-type Tsr is not expressed in our cell lines the behaviour of all constructs was analyzed, when exposed to L-Ser and L-Asp (figure III-32).



**Fig.III-32.** (A) Western blot of cells with pR2 and either Tsz, Tzar or Tasz, exposed to 5 mM of L-Asp and L-Ser.(B) Responsiveness of TSNI (blue bars) and Taz (yellow bars) to L-Asp and the inhibiting for Taz amino acids.

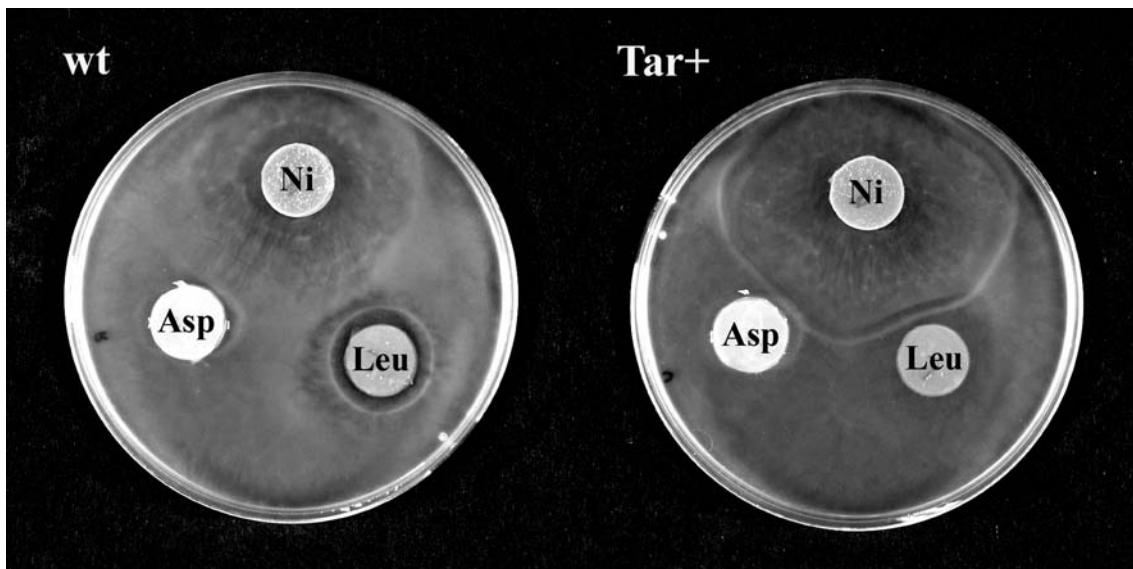
The chimeras Tsz and Tzar are locked in a non-responsive very low basal level state, but the re-designed TSNI mutant (see first paragraph of this section) and the chimera Tasz show a new pattern: The basal level of the receptor seems to be saturating like to that for activation of Taz by Asp. L-Asp doesn't seem to have any effect, but L-Ser inhibits both receptors.

Looking into the effect of the rest of the inhibitory aa for Taz (fig.III-32B), they seem to have an inhibitory effect on both TSNI and TasZ which is however more profound for TSNI. Therefore just by mutating 4 residues in the ligand-binding domain of Taz to the respective ones in Tsr, it is possible to imitate the behaviour of the whole ligand binding domain of Tsr.

## Chemotaxis studies

### *Leucine taxis*

Assuming that the conformational changes in Tar and Taz are co-related, then since aspartate, which is an attractant for Tar activates Taz and leucine inhibits Taz it is possible that leucine might be sensed as a repellent by Tar. Leucine has been described in very early chemotaxis studies as a repellent and the response has been assigned to the serine receptor, Tsr (Reader *et al*, 1979 ). However, more recent studies with capillary (Mao *et al*, 2003) and tethered cell assays (Khan and Trentham, 2004) have shown that in a  $\Delta tsr$  background leucine can elicit an attractant response. Moreover these studies have implicated Tar as being responsible for this response. However, in all of them the conclusions drawn were based on the behaviour of mutants which still contained different combinations of the other chemoreceptors. Given the extensive functional interactions among the different chemoreceptors (Ames *et al*, 2002; Gestwicki and Kiassling, 2002), it was considered necessary to test the effect of leucine in a Tar-only background.

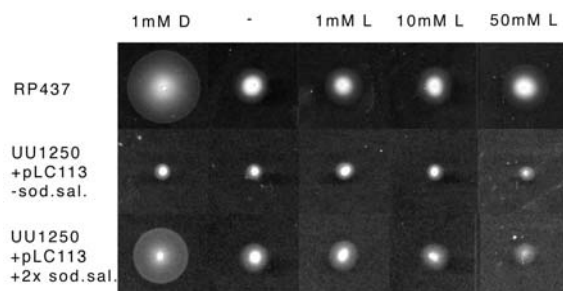


**Fig.III-33.** Chemical-in-plug assay. RP437 (wt) and UU1250/pLC113 (Tar+) cells were allowed to swim away from plugs containing 0.1 M of chemical for 1h at 30°C.



The chemical-in-plug assay was used to determine whether leucine can be recognized as a repellent by Tar. As described in the Materials and Methods section, wild-type RP437 cells and receptor-less UU1250 cells expressing Tar from plasmid pLC113 were exposed to plugs containing 1 mM of either the known Tar repellent  $\text{Ni}^{2+}$ , as a positive control, aspartate, as a negative control and leucine. As shown in fig.III-33 wild-type *E.coli* swim away from nickel and leucine, forming especially in the case of leucine a characteristic hallow, whereas the Tar-only cells respond only to nickel. This means that Tar is expressed and functional in these cells and therefore failure to swim away from leucine represents failure of Tar to sense leucine as a repellent

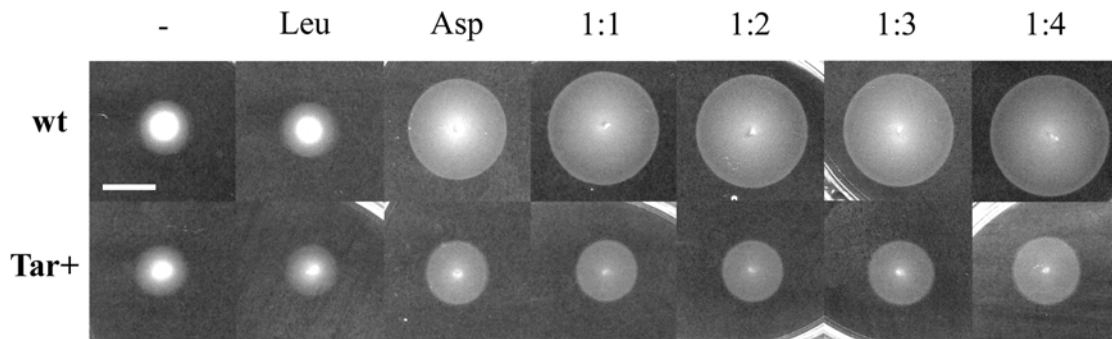
Swarm plates were used in order to test whether leucine can act as an attractant for Tar. Plates were prepared as described in the materials and methods section and were inoculated with either wild-type RP437 or UU1250/pLC113 cells.



**Fig.III-34.** Swarm plates of wt and UU1250/pLC113 cells without inducer or with double the amount of inducer compared to liquid cultures. Plates were allowed to develop at 30°C for 25h.

As shown in fig.III-34 wild-type cells form a characteristic disk in aspartate plates, while in leucine plates they form a much smaller and different pattern disk, which is exactly the same to the disk formed in control plates. UU1250/pLC113 cells behave exactly like the wild-type when Tar expression is induced, at any concentration of the inducer of sodium salicylate. In the absence of inducer there is absolutely no ring formation in any plate. The lack of a disk similar to the one in control plates of RP437 or UU1250/pLC113 induced cells can be simply accounted for by the fact that in the latter cases there is a residual taxis to the additives in the plates, while in non-induced UU1250/pLC113 there is no receptor to mediate any type of taxis.

Since both the chemical-in-plug and leucine swarm plate assays did not show any response to leucine through Tar it was considered possible that the effect of leucine is too subtle to be seen in these assays. To test this hypothesis swarm plates containing different ratios of aspartate and leucine were tried, in order to see if leucine can have an effect on the aspartate-disk formation. As shown in fig.III-35 Leucine had no effect at any ratio, suggesting that under the given experimental conditions there is no sensing of leucine through Tar and no significant competition of leucine and aspartate for binding to Tar.



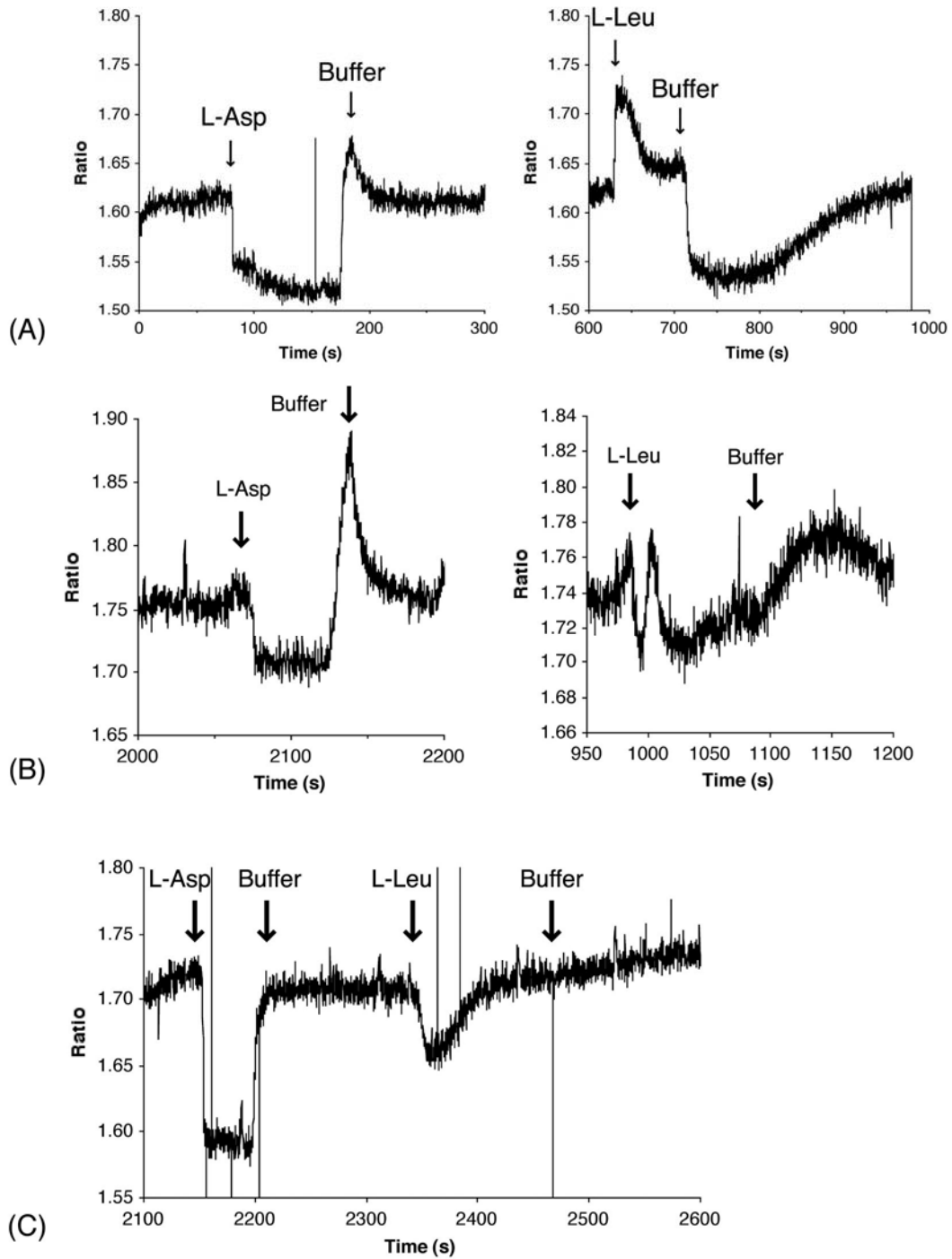
**Fig.III-35.** Swarm plates of wt and UU1250/pLC113 (Tar+) cells containing different ratios of L-Asp:L-Leu with 1 mM L-Asp in all plates. The white bar corresponds to 1 cm.

However it should be noted that the swarm plate method might not be suitable for studying leucine taxis, since there are two opposing factors in this particular case, which makes interpretation of the results very hard: On one hand, since leucine is not utilised by the bacteria metabolism as effectively as aspartate (see discussion), formation of the necessary gradient for taxis, might be impaired in the range of leucine concentrations used. On the other, all available strains for the study are leucine auxotrophs, which means that a high enough leucine concentration is required for normal cell growth. Balancing these two opposing driving forces is not straightforward. Therefore different methods were used to test whether leucine is an attractant for Tar.

## FRET experiments

Most chemotaxis assays reflect the effect of chemicals on the motor through the chemotaxis signalling apparatus, at different time scales. However Fluorescence Resonance Energy Transfer (FRET) can be exploited to see directly the effect of chemicals on the chemotaxis signalling apparatus in the following way: CheY is phosphorylated to CheY-P by CheA and CheY-P is dephosphorylated by CheZ. Therefore at steady state conditions for the formation of CheY-P, the level of the CheY-P:CheZ complex should be representative of CheA activity. The levels of the complex can be monitored using plasmid-borne CheY-YFP and CheZ-CFP fusion proteins in a *ΔcheYZ* genetic background, since excitation and fluorescence of CheZ-CFP will lead to excitation of CheY-P-YFP only when they are complexed, changing the levels of fluorescence for both proteins. When plotting the ratio of fluorescence of YFP/CFP, the plot follows the effect of chemicals on CheA activity i.e. attractants, which down-regulate CheA activity, reduce the YFP/CFP ratio, while repellents have the opposite effect. As shown in figure III-36 VS104 (wild-type) cells show a decrease in the YFP/CFP ratio upon aspartate addition and then show a pseudo-repellent behaviour upon removal of aspartate (washing with buffer) caused by the adaptation machinery. Leucine, in accordance to the other assays, acts as a repellent for the wt cells.

However for receptor-less cells (VS181) which express only Tar from pLC113 the pattern is more complicated: Although aspartate gives the same behaviour as for the wt, leucine gives a kind of biphasic response: it first elicits an oscillatory repellent response (2 peaks), but with time instead of adapting, the sign of the response is inverted into that of an attractant. The attractant part of the response seems to be greater than the repellent. Removal of leucine, by washing with buffer, results in pattern which looks like the

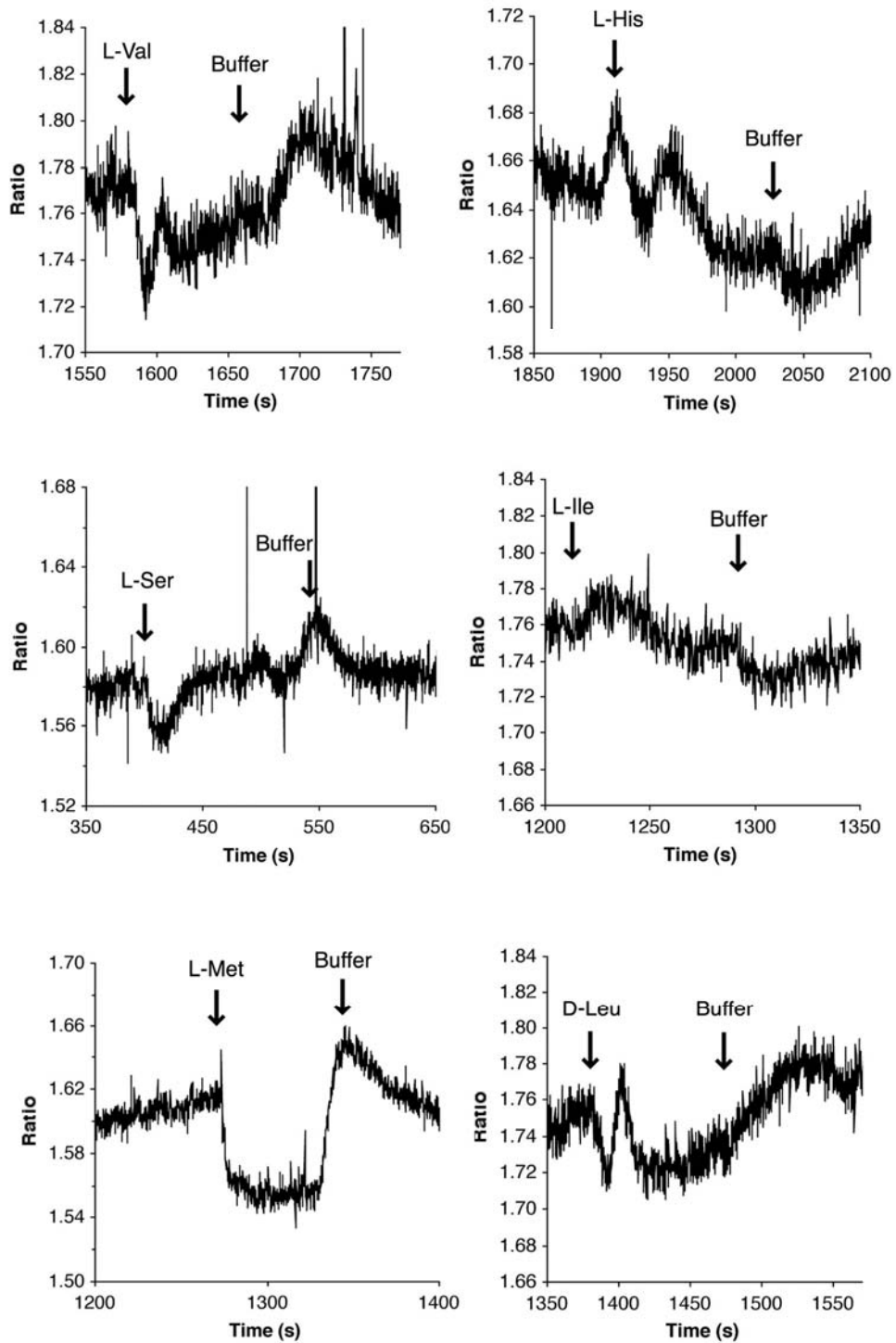


**Fig.III-36.** FRET experiments cells exposed to 10  $\mu$ M L-Asp and 10 mM L-Leu (A) wt cells (B) Receptor-less cells expressing Tar from pLC113 (C) Receptor-less and  $\Delta cheBcheR$  cells expressing Tar from pLC113

inverted response, suggesting that leucine is initially “remembered” the way it evoked the very first response. This could reflect an over-shoot or generally an implication of the adaptation machinery. Therefore the same experiment was repeated with cells carrying only Aer and expressing Tar, which are  $\Delta cheBcheR$  (VS164). In this case cells failed to adapt to aspartate, as expected, but behaved in a surprising way towards leucine: the response was that of an attractant, but it was followed by adaptation of the cells in the same way that cells adapt to aspartate in the presence of CheB and CheR. Furthermore, when Tar expression is not induced in these cells, they do not respond at all to leucine. These results clearly shows that Tar is essential for mediating the response to leucine, but the mechanism by which leucine is sensed is different and much more complex than that for aspartate. Moreover there seems to be an additional adaptation pathway, which is specific for only some stimuli.

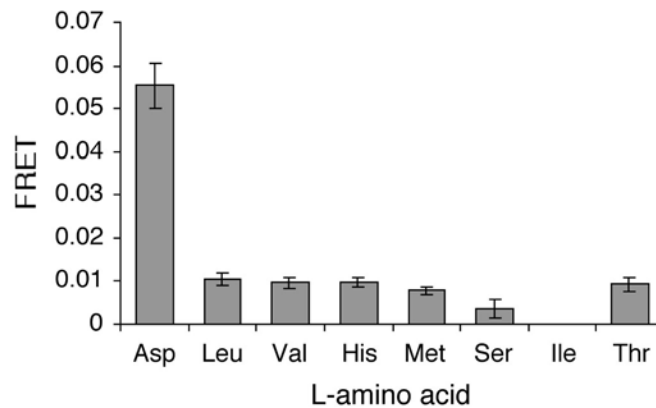
This interesting finding raises some questions like whether leucine is binding directly to Tar or through a binding protein and whether the alternative adaptation machinery acts on Tar or on a different protein binding to Tar. Competition experiments with the adaptation deficient cells between L-aspartate and L-leucine showed that in fact there is no competition, i.e. at all ratios of aspartate/leucine tried the response was that for aspartate (data not shown). This fact not only indicates that aspartate and leucine are sensed in a different way, but also the mechanism by which adaptation takes place for leucine in the  $\Delta cheBcheR$  cells does not have any effect on aspartate sensing, since in the presence of both amino acids the signal for aspartate is the same as in the absence of leucine.

Although this finding could suggest that leucine is acting through a periplasmic binding protein, no evidence was found to support this hypothesis. The only two known candidates, which are involved in amino acid transport, seem not to be involved: one of them, LivJ, is specific for leucine transport and the other, LivK, is responsible for the transfer of leucine, valine and isoleucine (Adams *et al*, 1990). Valine gives a similar pattern to leucine which rules out the possibility that LivJ is responsible for the phenomenon, while isoleucine does not give the same pattern, making it highly unlikely for LivK to be involved.



**Fig. III-37.** FRET experiments with receptor-less cells, expressing Tar from pLC113, exposed to 10 mM of the respective amino acid.

From the amino acids that were found to inhibit Taz, L-valine, L-serine and L-histidine showed a similar response to L-leucine, although the response to serine was not as profound as for the others. However L-methionine acted as a normal attractant (fig.III-37), L-valine, L-serine and L-histidine apart from giving a complex pattern in adaptation wt cells, were also sensed as attractants by Tar in  $\Delta cheBcheR$  genetic background (fig.III-38) and showed adaptation (data not shown).



**Fig.III-38.** Response in FRET units, which also reflects the degree of CheA inhibition, of receptor-less  $\Delta cheBcheR$  cells expressing Tar from pLC113 to 20  $\mu$ M L-Asp and 10 mM of the other L-amino acids. The results represent the average of three experiments.

It is worth noting that all these responses are evoked only in the mili-molar range and not in the micro-molar as for aspartate and as expected for chemoeffectors, although with different techniques leucine has been shown to elicit a response even in the micro-molar range (Mao *et al.*, 2003; Khan and Trentham, 2004). However this does not affect the significance of the response, since it is only seen when Tar is expressed and thus it reflects a true sensing property of the receptor. Moreover it is rather intriguing that although the affinity of the chimera Taz for Asp is lowered to the mili-molar range, its affinity for the other amino acids seems to increase by one order of magnitude. As far as the stereospecificity is concerned D-aspartate was recognised by Tar but also D-leucine elicited a response quite similar to the L- optical isomer. Therefore, there seem to be some common features between the behaviour of the chimera and Tar, as shown by

FRET experiments, but also distinct differences. Discrepancies between FRET experiments and chemotaxis assays are considered in the discussion.



*Part IV*

*Discussion*

### **The Taz-OmpR-ompC system**

In the original study by Utsumi *et al* the chimeric system was found only to be activated by some ligands like L-aspartate. The aim of this study was to use this system as a template in order to build a novel signal transduction pathway whose output would be regulated by a gene network. However intrinsic properties of the parent systems and unexpected results arising during the study, like the fact that some amino acids, like L-leucine, can down-regulate expression of GFP from *pompC*, made it necessary to re-address some of the basic properties of the system and to refocus the objectives of the Thesis into a re-examination of the chemotactic process in *E. coli*.

#### *Responsiveness of the Taz-OmpR-pompC system in respect to cell growth*

It has already been shown by Martinez-Flores *et al* that the wild-type EnvZ-OmpR system in *E.coli*, retains its responsiveness until the mid-logarithmic phase, after which it is activated by an unknown mechanism. Although in that study the rate of production and degradation of the porins was not measured or taken into account, in order to distinguish between net accumulation of the porins and actual activation of the system, in this study it is clearly shown that this activation indeed takes place: BL21(DE3) cells carrying the reporter plasmid pR2 with the destabilised GFP(LVA) under a minimal *pompC* were responsive to osmotic shock only until mid-logarithmic phase and cells in stationary phase exhibited the same levels of GFP, independently of exposure to 0.3 M NaCl or not.

Such a cross-talking system could have serious implications for the chimeric system, since it is not known how it would behave in the context of Taz. Therefore, as a starting point all experiments had to be carried out at the beginning of the logarithmic phase. In contrast to the osmotic shock experiments, when minimal media containing the putative signal were inoculated, cells showed a lag phase of 5-6 hours longer than the control cells. In order to overcome this problem cells were inoculated in media without any putative signal, allowed to grow until they enter into exponential phase ( $OD_{600} = 0.150-0.200$ ) and subsequently exposed for 1h to the chemical, so that cells do not exceed the mid-logarithmic phase. This way the cultures retained their synchrony in growth, while

being exposed to the signal for a short time-frame, sufficient for gene expression, but too short for great changes in the signal concentration by metabolism.

In the context of the chimeric receptor, instead of EnvZ, cells appeared to be less sensitive to this activation. Cells at any point of cell growth, ranging from early exponential phase to stationary phase were equally responsive to signals (L-aspartate and L-leucine). This is manifested for aspartate by the fact that when the GFP levels at each time point are normalized to the first data point of the time series, they are approximately the same, indicating that the activation of the system is the same regardless of the phase of cell growth. However when monitoring the change in levels of GFP in non-stimulated cells with time, it is clear that cells at the beginning of exponential growth have already accumulated a substantial amount of GFP which decays until the stationary phase. Therefore, in the case of leucine, normalizing each data point to the respective data point for the control cells, shows that the decrease in GFP levels is the same at any point of the growth curve.

Overnight cultures show an increase in GFP and lose their responsiveness to any stimuli (L-aspartate or L-leucine), which means that somehow the system is activated at late stationary phase. Further accumulation during the lag phase cannot be excluded either. Activation by release of amino acids through cell death can probably be excluded, since the number of activating amino acids is much smaller than that of the inhibitory and the effect of the inhibitory amino acids is dominant over the stimulatory effect of L-aspartate even at 10-fold lower concentrations. Taking into account the quantile distribution of amino acids in *E.coli* proteins (Karlín *et al*, 1992), it is almost impossible for the concentration of free aspartate and glutamate to be enough to overcome the inhibitory effect of other amino acids. For example, in strain *E.coli* B/r under aerobic conditions and in rich media the sum of the inhibitory amino acids (leu, met, ser, val and his) is 1271  $\mu\text{mol/g}$  of dried cells, whereas the sum of activating (asp and glu) and their precursors (asn and gln) is only 958  $\mu\text{mol/g}$  (Neidhart *et al*, ). On the other hand, release of a different, uncharacterised, dominant signal rising from cell death cannot be excluded.

The most likely possibility appears to be that the system which activates porin expression in the wild-type system is also responsible for GFP accumulation in the chimeric system. However, since this activation is not as efficient as in the case of the

wild-type system and since it is absent in cells without EnvZ or Taz, it seems that it requires at least EnvZ-C in order to take place and that Taz either lacks some important element of EnvZ or has an altered structure of EnvZ-C so that the activation cannot take place earlier than in the very late stationary phase.

The change in the growth-dependent activation of the system gives several advantages to the chimeric system. First of all accumulation of GFP until the entry into exponential phase, in combination with the short half-life of the GFP used, allows detection of inhibition of the system. Otherwise there would not be a detectable difference between the control and the inhibitory signals as was the case in the original study of Utsumi *et al*, where the long half-life of the reporter, LacZ, (Bergquist and Truman, 1978) masked the inhibitory effect of L-serine. Moreover the chimeric system is freed from the time constraint as far as responsiveness is concerned: exposure to any signal can be done at any point of the growth curve. However there is an extra advantage for the sensitivity of the measurements: since the level of GFP is decaying in non-stimulated cells, signals which activate the system will show an increasing difference from the control at a given time point as cells progress through the growth curve, while the opposite stands for inhibitory signals. Therefore the effect of inhibitory signals can be monitored with higher sensitivity at the beginning of the growth curve, while the effect of activating signals can be monitored with higher sensitivity at latter time points, e.g. the mid-logarithmic phase.

### **Complex reporter constructs**

As expected by the noise in gene expression, non induced cultures should contain a significant amount of fluorescent cells and cultures exposed to a signal should contain a significant number of cells with low or zero fluorescence (Becskei *et al*, 2001). Therefore different types of gene circuitry were designed in order to control more tightly the output of the pathway. Unfortunately one of the constructs (pR5), which would also serve as the basis for the others, appeared to be toxic.

In this construct TetR was supposed to be expressed from a synthetic promoter, which contained the OR1 site for CI between the -35 and -10 regions and repress expression

from *pompC* by binding to a mutant TetO1 site that overlapped with C1. Activation would take place only when OmpR-P levels would be high enough to out-compete TetR for binding to C1 and if the level of TetR expression would have been too high, activation could be fine-tuned by adding traces of aTc. However all initial attempts to clone the synthetic promoter-*tetR* fragment failed, giving either no clones or clones in which the synthetic promoter was destroyed by mutations and deletions in the conserved regions.

This peculiar phenomenon was initially attributed to the fact the synthetic promoter and *pompC*, were divergent promoters with a short spacer in between, possibly affecting the system through accumulation of negative supercoiling (Wu *et al.*, 1988; Wu *et al.* 1995). In order to test this hypothesis the promoter-less *araC* gene was introduced so that the spacer was approximately 800 bp long. This fragment was chosen simply because it pre-existed in the plasmid backbone and was removed during the cloning of *tetR*. The clones obtained still had mutations but it was possible to obtain a single point mutant and to correct it with PCR-ATW. In order to be sure that the PCR did not introduce any mutations in the rest of the construct, the spacer-promoter-*tetR* fragment was excised and re-cloned into pR4. Sequencing of the construct, even after several generations showed that the sequence remained intact, suggesting that the problem of toxicity had been overcome. Therefore the other constructs, pR6 and pR7 were also constructed using pR5 as a starting material.

Moreover, the single point mutant promoter was also used for making the respective constructs, since the mutation T-36C is expected to only reduce promoter strength (Vo *et al.*, 2003), allowing to explore a bigger part of the parameter space. However when functionally tested, the constructs with the mutant promoter showed similar behaviour to the simple systems pR1, pR2, suggesting that the mutant promoter is too weak to have an effect. Construct pR7C, which has the temperature sensitive CI as a transcriptional fusion to *gfp*, gave much lower levels of GFP. A possible reason for this is that the cells did not grow in the specific medium at the permissive temperature (30°C) and the experiment had to be carried out at 37°C, which is close to the non-permissive temperature (42°C). Thus, for pR7C, lower GFP levels could be due to a fitness problem of the cells caused by partially denatured CI.

On the other hand cells with the desired synthetic promoter showed no GFP expression, even when activated with aspartate. Although this could be due to too tight repression, it was rather puzzling that repression could be so efficient for the anti-sense RNA construct (pR6) and that addition of aTc could not relieve repression by TetR. TetR expression from both mutant and wild-type promoter was tested by western blot analysis using a mixture of monoclonal anti-TetR antibodies and surprisingly only the mutant promoter expressed TetR. As expected, the expression level was much lower than that for control cells (DH5aZ1) which produce enough TetR for tight repression of TetR-regulated promoters (Lutz and Bujard, 1997). However it was not clear why in pR5 both the designed promoter and *pompC* were completely silent. Thus pR5 was sequenced again and no mutation, insertion or deletion was found in either promoter or gene. However diagnostic PCR with different primers revealed the insertion of a 1.2 kb DNA fragment downstream of *gfp*. This fragment was partially sequenced and was found to correspond to part of the transposase gene from Tn10, containing several stop codons in-frame with the gene. Although it is puzzling how this fragment was inserted, the mechanism by which it silences the two promoters is even less clear. However this insertion seems to be the only plausible explanation why the sequence of the construct remained intact: silencing the system indirectly probably relieved the cells from the toxicity problem, without needing to mutate the cloned sequences.

The reason for the toxicity of the construct remains elusive, but it is further demonstrated by the fact that when the correct sequence of spacer-promoter-*tetR* is excised from pR5 and re-cloned in pR4, all of the clones tested have undergone recombination, which affects expression of either TetR or both TetR and GFP, although the cells used are *recA*<sup>-</sup>.

### **Alternative fusion points**

Since the sensitivity of Taz is approximately 1000-fold lower than Tar, it was tested if shifting the point of fusion of the two receptors can change the sensitivity of the chimera in a favourable way. It should be pointed out however that in any case it would be highly

unlikely to reach the same levels of sensitivity as wild-type Tar, since its sensitivity is enhanced by two factors which are absent in the case of the chimera: the lattice formation and the adaptation machinery. Based on the sequence alignment of the two receptors, three new points were chosen resulting in the three additional chimeric receptors H1, H2 and H3, which contain increasing fragments of the Tar linker region. From these receptors H3 had a basal level of GFP expression and did not respond to aspartate, while H1 and H2 showed a barely detectable GFP expression level.

The behaviour of H1 and H2 could either reflect a defect of these receptors in the phospho-transfer reactions or a problem of their stability and expression. Since there seems to be a very small level of GFP expression the former seems to be the most probable explanation.

Furthermore, while we were finishing our study, similar fusion variants were shown by Zhu and Inouye to exhibit the same behaviour as in our case and for some seemingly inactive receptors, like Tez1, activity could be restored by mutations in the linker (in the case of Tez1 P185Q). Apparently, as shown in the latter study, the register of the helices in the linker, but also the sequence itself is crucial for the function of the chimera. Since in our case the fusion is based on a sequence alignment and does not change the register of the helices, it seems that the exact sequence of the linker plays a dominant role.

### **Rational design of Taz mutants with altered specificity**

According to the original study on Taz by Utsumi *et al* L-serine and other ligands were not sensed by Taz, while the only effect observed was activation by L-aspartate and its  $\alpha$ -methyl-ester. Therefore it was assumed that it could be possible to switch specificity of the receptor from L-aspartate to either L-leucine, L-glutamine, L-serine or L-threonine through rational design. These signals were chosen on the basis of similarity to the original signal according to their structure (leucine), structure and polarity (glutamine) and the fact that the serine receptor has an almost identical binding site for serine. In order for any design to be successful it is necessary to use a template of good quality.

As a template the crystal structure of the Tar periplasmic domain from *Salmonella typhimurium* in complex with one aspartate molecule was chosen, because it is the best available structure and it is probably closer to the structure of Tar from *E.coli*, where only one aspartate molecule can bind due to negative co-operativity. The quality of the structure and its predictive power were assessed by using the Perla, SwissPdbViewer and Fold-X algorithms to calculate the dissociation constants of aspartate for various mutations in the binding pocket which have already been experimentally measured. The predictions follow the trend of the experimental data and at least for the wild-type system it seems that the predictive power of the algorithms with this template is quite good.

When other amino acids are placed in the position of aspartate the  $K_d$  values for the respective complex are much higher than those for aspartate and glutamate, suggesting that if the receptor has any affinity for other amino acids it would be too low compared to the normal signals. Therefore trying to switch affinity towards leucine, glutamine, serine and threonine seems reasonable.

*In silico* mutations in the binding pocket were done using the Perla algorithm. The energy of the generated structures was minimized with SwissPdbViewer and the energy of interaction, from which the  $K_d$  was calculated was determined with Fold-X. In the case of leucine and glutamine all different combination of amino acids at the critical residues were tried and only the best structure was chosen to be tested for each receptor. However the design for TaQ was not very good and looking at the putative structure it seems that the binding pocket is filled by the chosen residues, without leaving space for a ligand. Even the best structures, although they have increased affinity for the respective ligand, are predicted to have weaker interaction with the new ligand compared to the aspartate-wild-type Tar interaction. In fact in the case of serine and threonine the serine (or threonine)- mutant Tar interaction is predicted to be weaker than the aspartate-mutant Tar, although the mutations in this case were not random but specifically selected so that they re-constitute the binding pocket of the serine receptor, Tsr. This raises the question how selectivity is achieved in Tsr and whether there is a different conformation or conformational change that could account for serine specificity in Tsr.



### **Behaviour of the designed receptors**

The behaviour of the designed receptors was far from what was expected. TaQ is constitutively active and none of the amino acids tested had an effect on GFP levels. Cells expressing TaS were not activated by L-Ser, L-Thr but were activated by L-Asp and cells expressing TaT showed a low basal level of GFP and were not responsive to any amino acid tested. In the latter case probably the R69K and R73M substitutions abolished ligand-binding rather than providing more space for the methyl group of L-Thr. However the biggest surprise was TaL which had a significantly high basal level and in the presence of L-leucine there was a drastic drop in GFP levels which unexpectedly was also seen in the case of Taz. The discrepancies between predictions and experimental results can be explained if a conformational change in the periplasmic domain is induced by the fusion to the cytoplasmic domain of EnvZ. This hypothesis could be tested in the future by the simple fact that the predictive power of the method is acceptable for the wild-type Tar and therefore making the respective substitutions to wild-type Tar should shift the affinity of the wild-type receptor for the respective ligands.

#### *TaS and the Tsr-Taz variant*

The failure of TaS to recognize L-Ser and the ability to recognize L-Asp, although they were in agreement with the theoretical prediction, were rather puzzling, since the mutations made should re-constitute the core of the ligand binding pocket of Tsr. Although the differences in the binding pocket are few and we have converted one into another, there are differences outside which could modify the structure of the receptor and provide specificity towards Ser. In order to check this, different fusions of Tsr to EnvZ or Taz were constructed and also 2 more mutations (in the vicinity of the binding site) were introduced in order to match as possible the binding pocket of Tsr. Surprisingly Tsz, which is the equivalent to Taz Tsr-EnvZ chimera, was not functional. Although there was a basal level of GFP expression, which suggests that the chimera is expressed, there was no effect of any of the amino acids tested. A possible explanation would be the following: the strain used for the study, AT142, has the *flhD5301* mutation

and therefore does not express Tar and most of the chemotaxis genes. However it has not been shown whether Trg and Tsr expression is also dependent on the FlhC/FlhD activator and thus it is plausible that there is expression of Tsr and formation of hetero-dimers with chimeric monomers which would prevent normal signal transduction. Such a hypothesis cannot be rejected on the basis that the Trg-EnvZ chimera, Trz, is functional (Baumgartner *et al*, 1994) since Trg, in contrast to Tsr, is a low abundance receptor and its levels are probably too low to interfere with the chimera. However the western blots with anti-MCP serum for strains with different or no receptors and AT142, clearly show that there is no Tsr expression in AT142. The only bands observed corresponded to non-specific binding. Therefore it seems that the orientation of the extracellular part of the receptor in Tsr is very different from that in Tar, and therefore linking at the same position abolishes functionality of the receptor, despite the apparent similarity in the binding pocket.

This hypothesis is supported by the fact that using the structure of Tar as a template the designed Tsr binding domains (TaS and TSNI) are predicted to have a much higher affinity for aspartate than for serine and by the behaviour of the other constructs. Tsaz is locked in a non-responsive very low basal level state, like Tsz, but TSNI and Tasz show an intrinsic pattern: The basal level of the receptor seems to be saturating, like to that for activation of Taz by Asp. L-Asp does not seem to have any effect, but L-Ser inhibits both receptors. Furthermore, the rest of the inhibitory aa for Taz, seem to have an inhibitory effect on both TSNI and TasZ which is however more profound for TSNI. Therefore just by mutating 4 residues in the ligand-binding domain of Taz to the respective ones in Tsr, it is possible to approximate the behaviour of the whole ligand binding domain of Tsr.

#### *TaL and its variants*

The fact that cells expressing TaL showed down-regulation of GFP expression upon exposure to leucine, was exactly the opposite of what was desired. Therefore the possibility of changing the sign of the response was tested by introducing only mutations R69M and R73M, which should favour the interaction with the hydrophobic side-chain of leucine in a similar way that the respective arginines interact with the charged side

chain of aspartate in the wild-type case. However the resultant receptor, TaLM, showed a very low basal level and no responsiveness to leucine. In order to understand the significant difference between TaLM and TaL, single mutants were tested (R69M, R69L, R73M) which unfortunately could not give more information apart from demonstrating the importance of residues 69 and 73 in signal sensing. The only plausible explanation seems to be that the combination of mutations in TaL result in a conformational change of the receptor which has a high basal level of activity and retains a certain degree of responsiveness to leucine. This explanation, versus a change of the interactions of the mutated residue with the ligand, is supported by the observation that mutations in the ligand binding domain can have very different and drastic effects on Taz (Yang *et al*, 1993): the R64C substitution renders Taz constitutively active, whereas the R69H or R73Q substitutions eliminate the sensing properties of Taz without changing its baseline kinase and phosphatase activities

### **Re-examination of the sensing properties of Taz**

The fact that cells with either TaL and Taz showed down-regulation of GFP expression upon exposure to leucine raised a lot of questions about the actual sensing properties of Taz. In the original work by Utsumi *et al*, Taz failed to recognize maltose. However under both their and our experimental conditions, expression of the activator which is encoded by *malT* and is responsible for expression of the maltose response genes, including the Maltose-Binding-Protein (MBP), is repressed by OmpR (Case *et al*, 1986). Therefore the responsiveness of Taz was re-examined only for the 20 naturally occurring amino acids. Apart from L-aspartate, L-glutamate activated the system and L-proline elicited a weak activation at much higher concentration (20 mM). Most of the other amino acids had no significant effect with the exceptions of L-Leu, L-Met, L-Ser, L-Val and L-His which down-regulated GFP expression. Within the time-frame of the experiment this decrease was in the order of 40-50%, but when cells were exposed to leucine for longer time periods, then the levels of GFP decayed exactly as theoretically

predicted in the case of complete cessation of GFP production upon exposure to the signal, i.e. leucine, while taking into account cell growth.

This means that the inhibitory amino acids and especially leucine manage to completely shut-down GFP production. However there is a possibility that this effect is caused by proteins other than the receptor. This seemed quite possible especially for leucine, since the cloned region of the *pompC* contains four binding sites for Lrp. The latter possibility was excluded on the basis that the locked-on TaQ was not affected by leucine, whereas in the competition experiments with Taz, the inhibitory effect of leucine was dominant even at 25-fold lower concentrations than aspartate. These two findings suggest that the effect of leucine cannot be in the part of the signalling pathway that is below the receptor-level and that Lrp cannot compete with the level of OmpR-P produced by an active receptor, provided that the levels of OmpR-P generated by activated Taz and the locked-on TaQ are within the same range. This assumption is quite safe to make, since as shown by the response curves, the wild-type chimeric system is 10-fold more sensitive to leucine than aspartate and thus if there were an effect through Lrp, the locked-on mutant receptor would have to produce 10-fold more OmpR-P than Taz. The latter is highly unlikely since it has been shown that the amount of OmpR-P needed to activate transcription from *pompC* is 10% of the total amount of OmpR molecules inside the cell (Cai and Inouye, 2002).

Furthermore, the wild-type EnvZ-OmpR system was shown not to be affected at all by leucine, suggesting together with the aforementioned results that leucine interacts with the Tar moiety of the chimera. Such a result, would have interesting implications for the wild type chemotaxis system, if the conformational changes upon ligand binding in Taz and Tar are co-related.

### **Examination of leucine effect on chemotaxis**

If the conformational changes in Taz and Tar, which are induced upon ligand binding, are co-related then since aspartate is an attractant for Tar and elicits an opposite response to leucine through Taz, leucine would be expected to be sensed as a repellent by Tar.

However, early chemotaxis studies have shown that Tsr recognises leucine as a repellent and more recent ones have shown that leucine can elicit an attractant response in a  $\Delta tsr$  background, possibly through Tar. Since all of these studies used deletion mutants which still had different combinations of some chemoreceptors and as the extensive functional interactions between the different types of chemoreceptors are well established, it was necessary to test the effect of leucine on cells expressing only Tar from a plasmid (UU1250/pLC113).

In this context, the chemical-in-plug assay, which is a typical experiment for repellents, showed that although Tar is expressed and functional under the experimental conditions (cells swimming away from nickel), it does not sense leucine as a repellent.

The swarm-plate assay, which is a typical assay for metabolizable attractants, again showed the expected pattern of an attractant response for aspartate and the pattern for leucine was exactly the same to the control, for both wild-type and Tar-only cells. The pattern for the control was a very small disk, different from that for aspartate and probably it can be attributed to basal taxis towards additives in the plates, since non – induced UU1250/pLC113 cells, which have no chemoreceptors, failed to move further than the point of inoculation. Swarm plates with different ratios of aspartate and leucine also showed no difference to aspartate-only plates, suggesting that there is no competition between aspartate and leucine and that leucine cannot affect the response to aspartate.

It should be pointed out that failure of cells to form a disk in leucine plates similar to that in aspartate plates does not necessarily exclude the possibility that leucine can act as an attractant. The problem in interpreting the results for leucine arises from the differences in amino acid metabolism and also from the fact that all strains used are leucine auxotrophs. In order to form a disk the cells must metabolize the chemical with a certain rate, so that a gradient can be formed and act as the driving force for taxis. Leucine cannot be utilised as efficiently as aspartate: aspartate can be used either as a carbon or nitrogen source through the reactions  $L\text{-asp} + 2\text{-oxolutarate} \rightarrow L\text{-glu} + \text{oxaloacetate}$  and  $L\text{-asp} \rightarrow \text{fumarate} + \text{NH}_4^+$  (Neidhart *et al*; Goux *et al*, 1995), whereas leucine is mainly used for protein synthesis. Therefore a gradient will probably form only at much lower concentrations of leucine than aspartate. However, since the cells are leucine auxotrophs, they already require a substantial amount of leucine for growth, which could

be limiting the gradient formation. Balancing these two opposing needs is not straightforward and thus the swarm plate assay is probably not a reliable method in the case of leucine.

FRET experiments with cells which are also  $\Delta cheYcheZ$ , but express the plasmid-borne fusions CheY-YFP, CheZ-CFP can by-pass this obstacle. Cells carrying all receptors respond as previously described: aspartate acts as an attractant and leucine as a repellent. In this case, when cells are exposed to aspartate, the ratio of fluorescence for YFP/CFP (R) decreases because CheY phosphorylation is down-regulated and as time progresses cells adapt and the ratio returns to the basal level. If aspartate is removed by washing with buffer then the cells give an attractant-removal response, which looks like a repellent response. This pseudo-repellent response is manifested as an increase in R and is caused by the adaptation machinery. However when cells expressing only Tar are used, although the response to aspartate remains the same, leucine generates an intrinsic, “biphasic” response: cells respond in the beginning as if leucine is a repellent and as time progresses, instead of adapting, they switch the sign of the response to that of an attractant. Upon leucine removal the pattern generated is the inverted one. This means that cells keep in “memory” the very first reaction to leucine and give first an attractant removal response followed by a repellent removal response. . L-Valine, L-Serine and L-Histidine had a similar effect, whereas L-Methionine behaved as a normal attractant.. In cells without Tar there was no response which indicates that Tar is essential for the response.

Since this peculiar pattern resembles an over-shoot in adaptation, the same experiment was carried out with cells lacking the known adaptation machinery ( $\Delta cheBcheR$  cells), to test if/how the adaptation machinery is involved in this complex behaviour. When the cells are exposed to aspartate, they show the expected drop in R, fail to adapt and do not give the attractant-removal response when washed with buffer. When they are exposed to leucine the response is that of a normal attractant, but surprisingly there is adaptation in a time-frame and manner similar to that for aspartate in wild-type strains. The same is true for valine, serine and histidine.. This finding clearly demonstrates that the CheB-CheR machinery plays an important role in the biphasic response, but also that there is an additional adaptation mechanism. When the same strain is exposed to different ratios of

aspartate to leucine the behaviour of the cells is exactly the same to that for the respective concentration of aspartate, as if leucine has no effect, even at 10-fold higher concentrations of leucine. An obvious explanation for this could be that aspartate out-competes leucine for binding either preventing binding to the same site or changing the conformation of the receptor in a way which prevents leucine binding to a different site. However there are still more possibilities which could account for this phenomenon and should be considered.

One alternative explanation is that leucine interacts with Tar through one or more different proteins and that the second adaptation machinery acts on that level. Therefore when leucine and aspartate are used together in  $\Delta cheBcheR$  cells, adaptation for aspartate is not seen because the adaptation machinery works not directly on Tar, but on the proteins involved in the leucine response. The only known periplasmic proteins which bind leucine are LivJ and LivK, which are both involved in amino acid transport inside the cell (Adams *et al.*, 1990). LivJ is specific for leucine, whereas LivK is specific for leucine, valine and isoleucine. Since cells, of either adaptation background, show similar responses for leucine, valine and histidine, but not for isoleucine, it seems unlikely that any of these two binding proteins is mediating the response. However the possibility that non-characterised periplasmic and or transmembrane proteins are involved cannot be excluded.

Another explanation is that leucine binds indirectly to Tar or even directly either at the same or a different site to aspartate, but induces a different conformational change which renders Tar susceptible to a different type of adaptation machinery. This conformation and modification of Tar would be specific for leucine (valine, serine and histidine) but would not have an impact on aspartate sensing. In other words this type of modification for adaptation to leucine would not infer adaptation to aspartate. This hypothesis can be partially tested by trying to identify possible modifications of Tar in the presence of leucine other than methylation. The fact that leucine and the other amino acids are sensed in a different way than aspartate is further supported by the fact that in the FRET experiments the sensitivity of the wt system is within the micro-molar range for aspartate and within the mili-molar range for the other amino acids, whereas in the chimera the

affinity for aspartate is within the mili-molar range and the affinity for the other amino acids is slightly increased.

In any case it is not possible to distinguish between leucine binding directly to the same site as aspartate, to a different site or indirectly through a protein complex, by competition. Mutagenesis of the aspartate binding pocket is also not very informative or straightforward to explain, since in either context (Taz or Tar) mutations can lead to global conformational changes affecting the signalling properties of the receptor, instead of simply interfering with ligand binding.

It is clear that leucine sensing through Tar is a very complicated phenomenon, involving proteins which are not yet characterised. The fact that leucine gives a complex response in FRET experiments, but a more simple attractant response in capillary, tethered cell and some swarm-plate assays could be easily accounted for the fact that the latter assays reflect the behaviour of the whole system, including the motor, whereas FRET reflects only what happens at the signal transduction level and possibly the net effect under the experimental conditions of the other assays is that of the attractant, which would be the same for the FRET experiments if the two phases of the response were integrated.

### **Implications of Tar responses for Taz**

Taking into account all the aforementioned possibilities it is clear that leucine, valine and histidine sensing by the chimera is a remnant from Tar. However the exact sign of the response by Taz is not related to that in Tar: attractants like aspartate and glutamate activate Taz, while attractants like leucine and methionine and can inhibit Taz. This indicates that the conformation of the periplasmic domain of Tar in the context of the chimera is different to that of wild-type Tar. Such a possibility could also account for the 1000-fold lower apparent affinity of Taz for aspartate than Tar, although the fact that the sensitivity of Tar is enhanced through the lattice formation and the adaptation machinery should be taken into consideration. Moreover this conformational change could also account for the failure of the design to change ligand specificity and reflects the problems



in rational design rising from having resolved structures of only fragments of transmembrane proteins. As discussed above there were several discrepancies between theoretical predictions and experimental results with the most intriguing being the predicted failure of the reconstituted Tsr binding domain to bind serine with higher affinity than aspartate. Such discrepancies imply that the available crystal structures do not provide the whole ensemble of conformations of the chemoreceptors, making rational design very difficult. As already seen in the results section, the apo and ligand bound structures exhibit differences which are within the error of structure determination and therefore appear to be probably crystallisation artefacts, rather than true conformational changes. Taking as well into account the fact that the structures correspond only to the periplasmic domain, it is impossible to tell which is the true apo conformation in the whole receptor and especially in the case of the chimera, where the different cytoplasmic region can change the orientation of the subunits even more.

Despite these problems the fact that Taz responds, although in an intrinsic manner, to signals of Tar displays the flexibility of the chemoreceptors. This flexibility is highlighted by different chimeras either between the chemoreceptors themselves (Tap-Tar, Tar-Tsr) or chemoreceptors and other receptors (Tar-insulin receptor, Trg-EnvZ, NarX-Tar). In the former case the properties of the chimera are more close to the N-terminal parental receptor, i.e. the Tap-Tar hybrid senses dipeptides exactly like Tap. In the other case the effect of the signalling molecule(s) can be either closer to the response of the N-terminal receptor, i.e. the NarX-Tar chimera has higher affinity for nitrate than nitrite like wt NarX (Ward *et al*, 2002), or closer to the way that the C-terminal receptor would interpret it. For example, in the cases of Trz, Taz and the Tar-insulin receptor chimera, ribose and aspartate respectively are sensed by the correct sensing domain but result in a net increase of the kinase activity of the receptor as is expected during sensing through wt EnvZ or the insulin receptor.

In any case, while the opposite is not true, any signal sensed by a chimeric receptor appears to be a signal for at least one of the parental receptors regardless of its net effect. However, depending on the affinity of the parental receptor and the limitations of the methods used for studying the parental system, sometimes such ligands remain uncharacterised. This is very well manifested in this study where amino acids which were

thought not to be recognised by Tar according to classical chemotaxis techniques, were shown to be Tar ligands with FRET experiments. In fact the FRET experiments revealed new aspects of chemotaxis:

a) signals can have a complicated response which is seen as a net attractant, repellent or neutral response with other assays because of integration of the whole response with time

b) there is an additional adaptation mechanism to that of methylation, which is specific for some but not all of the ligands of a chemoreceptor and acts within the same time range as the methylation dependent adaptation. How this is achieved is not clear but periplasmic binding proteins do not seem to play a role, as discussed earlier.

The peculiarities of the crystal structures together with the results of FRET experiments with cells expressing only one receptor clearly demonstrate that although there are a lot of data on bacterial chemotaxis, we actually know much less than we thought about the molecular mechanism of signal transduction and chemotaxis itself in *E.coli*. Actually since most of the experiments in the past have been done with single or double deletion mutants of the chemoreceptors or with not well defined mutants it is necessary to re-evaluate the data on chemotaxis using strains with better defined genotype, expressing a single chemoreceptor species. It should be noted that deletion mutants which have been used up to now usually express non-functional parts of the deleted chemoreceptors. Comparing the response of these strains, carrying the chemoreceptor in a plasmid, to that of “clean” deletion mutants, the amplitude of the response is much higher (Sourjik, V., personal communication). This further demonstrates the little knowledge that is available on the receptor lattice and signalling complex formation and should be taken into account when looking at the properties of single receptors.

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