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Transcriptional regulators à la carte: engineering new effector specificities in bacterial regulatory proteins

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For many regulators of bacterial biodegradation pathways, small molecule/effector binding is the signal for triggering transcriptional activation. Thus, regulation results from a cross-talk between chemicals sensed by transcriptional factors and operon expression status. These features can be utilised in the construction of biosensors for a wide range of target compounds as, in principle, any regulatory protein whose activity is modulated by binding to a small molecule can have its effector/inducer profile artificially altered. The cognate specificities of a number of regulatory proteins have been modified as an astute approach to developing, among others, bacterial biosensors for environmentally relevant compounds.

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Introduction

Bacterial biosensors [1,2] have been developed that take advantage of regulator specificity. Environmental pollutants can be detected by fusing the promoters of biodegradative routes for the pollutant of interest to easily measurable reporter genes. Transcriptional regulators activated by the target contaminant interact with the promoter triggering production of the quantifiable marker (Figure 1) [3–11]. In this way, it is possible to envisage the construction of biosensors for a great number of priority compounds, defined by the number of molecules acting as effectors for regulators. However, if regulator specificity is engineered so as to become novel and/or narrower, possible issues arising from cross signals resulting from heterogeneous contaminants in a sample can be avoided, and open the possibility of constructing modular libraries of regulators with specific detection characteristics. In principle, any transcriptional regulator whose activity is modulated by binding to a small molecule can have its effector/inducer profile altered.

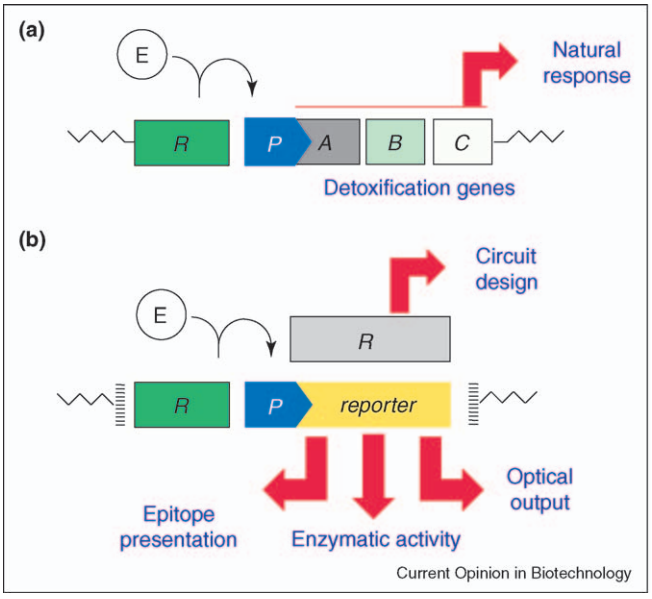
Engineering of effector-binding sites could yield more specific regulators with higher sensitivity. Alternatively, regulator variants can be pursued that broaden the effector profile of a given transcriptional factor. To date, specificity mutants for several regulatory proteins have been generated, namely variants of XylR, DmpR, XylS, NahR and TetR. In this review the properties of these engineered regulators will be described, with respect to their range of specificity and sensitivity to effector concentration. The biotechnological applications of such regulators à la carte and the prospects of blending genetic 'wet' procedures with computational approaches to predesign effector-binding proteins is also examined.

Methodology

Methods employed for diversity generation in effector-binding transcriptional regulators include DNA shuffling (XylR [12,13], DmpR [12] and TetR [14,15]), error-prone PCR (DmpR [16] and NahR [17]), chemical mutagenesis (XylS [18] and XylR [19]), saturation mutagenesis (TetR [14,15,20]), and the identification of spontaneous mutants (DmpR [21]). Computational design is also poised to make its contribution, as the crystal structure of the transcriptional regulator DntR has been solved to allow modelling of mutations leading to activation upon 2,4-dinitrotoluene binding [22]. Figure 2 illustrates strategies of diversity generation and mutant isolation that have been or could be used. Some DmpR mutants were isolated ingeniously, by recovering *Pseudomonas putida* cells carrying DmpR from soil amended with 4-methylphenol [21]. Here, the exposure of cells to amended soil led to the quick selection of subpopulations with improved ability to degrade 4-methylphenol. Enhanced growth was caused by mutations in the DmpR effector-binding domain, which allowed greater levels of transcription of the degradative gene cluster. This result suggests that regulator mutation is, at least in this case, the easiest route to catabolic fitness.

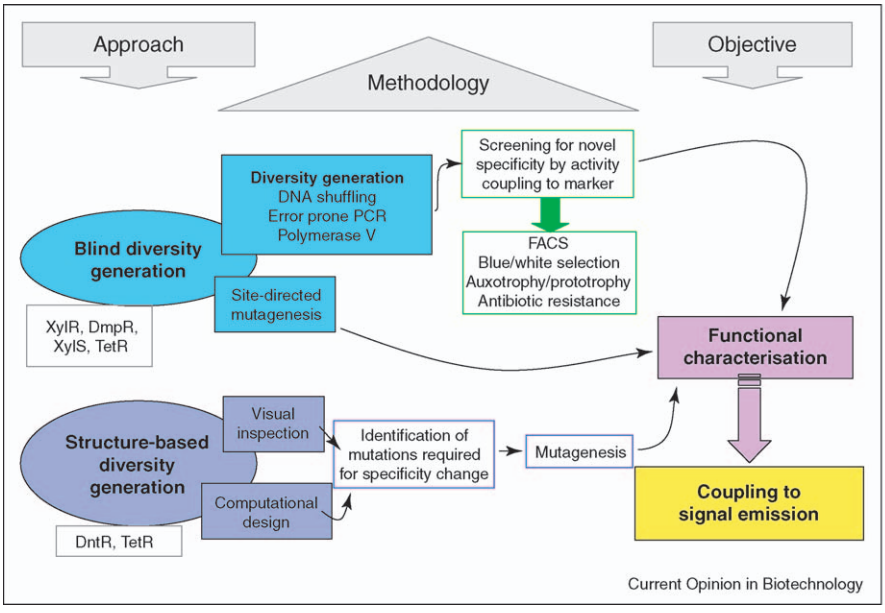
Mutagenesis procedures have been coupled to a range of screening setups, including blue/white colony selection/β-galactosidase activity in the presence of distinct effectors [14–17,19,20], growth on select effector as sole carbon source coupled to promoter activation and antibiotic resistance in presence of the effector [21], and coupling of antibiotic resistance (XylS [18] and XylR [13]) or a counterselection marker [13] to the cognate promoter of the activator of interest.

Figure 1



Basic set up of a biosensor using effector-binding bacterial regulators for the detection of recalcitrant and/or contaminant compounds. The gene coding for the regulator (*R*) is often found next to the genes under its control (*A,B,C*). **(a)** In the environment, effector binds to the regulator which in turn binds to promoter (*P*) and triggers transcription of the catabolic route, leading to detoxification. **(b)** In a biosensor, effector binding triggers transcription of a reporter gene (e.g. *β-gal*) that provides a measurable signal (light or enzymatic activity) or of surface-displayed epitope tags. Alternatively, a circuit or regulatory cascade can be designed so that effector-mediated activation is coupled to expression of another regulator, whose downstream promoter activation leads to signal amplification.

Figure 2



Possible strategies for the generation and isolation of regulators with novel specificities. Regulators for which no structural information is available require employment of methods to generate diversity at random — blind diversity generation — followed by screening methods powerful enough to discern the best possible variants with novel specificities over a background of constitutive, non-functional, and intermediate phenotypes. When structural information is available, a structural framework reveals possible amino acids that can be changed to result in altered and/or improved specificities for a desired compound. Mutants are then assayed for the desired function. FACS, fluorescence-activated cell sorting.

XylR/NtrC family mutants with novel specificities

Transcriptional activators of the NtrC family are involved in a range of cellular functions. They act by binding to enhancer sequences located approximately 100 base pairs upstream from the transcription start site, and depend on the alternative sigma factor σ^{54}. The structural hallmarks of σ^{54}-dependent activators are a so-called AAA^+ domain, for ATP hydrolysis and σ^{54} interaction, and a C-terminal helix-turn-helix domain for DNA binding [23,24]. XylR-type NtrC family proteins that regulate biodegradation operons share an N-terminal domain for small molecule/effector binding, such that environmental signals are sensed directly by the transcriptional activator [19,25]. It is thought that effector binding releases repressive interactions between the A (effector binding) and C domain (AAA^+) [26,27], leading to a cycle of DNA binding, multimerization and ATP hydrolysis [28,29], in conjunction with σ^{54} binding and formation of a transcriptionally competent open complex [30]. One well-studied NtrC family member that binds directly to effector molecules is XylR, a regulator of the upper operon for toluene, *m*-xylene and *p*-xylene biodegradation borne by the so-called TOL plasmid pWWO of *P. putida* mt-2 [30]. Thus, XylR lends its name to the subfamily of σ^{54}-dependent bacterial regulators of aromatic compound biodegradation routes — the XylR-like proteins. Indeed, the pathways regulated by XylR-like proteins degrade a wide range of aromatic compounds. The effector profiles of a few regulators have been delineated and are surprisingly broad (Table 1), probably reflecting the rapid evolution of biodegradative routes to take advantage of whatever carbon source is available.

A marked feature of XylR mutants that arises during screening *in vivo* for new effector specificities is their increased range of effectors, with both loss and gain in degree of specificity (Table 1). The same is true for DmpR, a close relative of XylR, which activates an operon for the degradation of methylphenols in *Pseudomonas* sp. CF600. For example, XylR mutants XylR1 and XylR5 gain the ability to activate transcription in the presence of phenol, but they also have increased responsiveness to every other aromatic effector tested (except for XylR5 with *m*-xylene, which shows reduced levels of responsiveness; Table 1). Most mutants display a broader spectrum of effectors, accompanied by an increase in activation levels. On the other hand, some regulators have increased effector affinity, as judged by activation at concentrations neutral for the wild-type regulator [13,16]. One DmpR mutant, D9, has improved response to 2,4-dimethylphenol only at low concentrations (75 μM) concomitant with reduced activation by phenol [16], perhaps reflecting a change in the affinity of the protein for either effector (Table 2). Another feature of some XylR mutants concerns activation by 3-nitrotoluene, which binds to wild-type XylR without productive interactions, as judged by its ability to inhibit activation by the inducer 3-methylbenzylalcohol [13,31]. Yet, all of the XylR mutants are active in the presence of this compound, suggesting that their mutations did not alter specificity, but instead facilitated the multimerization cycles required for activation. Such effects are likely to be related to interdomain repression, whereby effector binding releases a repressive physical interaction between the A and C domains, poising the regulator to activate transcription [28,29]. It is possible that XylR-type regulators require not only effector binding but also subsequent signal transmission. In other words, effector binding and signal transmission to the central ATPase domain are connected features in activation, but mutation can alter each independently of the other.

Table 1

Effector range of XylR mutant regulators.

	wt	E172K	D135N	D135N/E172K	XylR1	XylR2 (F65L)	XylR3	XylR4 (L184I)	XylR5
Toluene	+++	+ (↓)							
3-Methylbenzylalcohol	+++	++	+++ (↑)	− (↓)					
m-Xylene	+++	+− (↓)			++++ (↑)	++++ (↑)	++ (↓)	++ (↓)	++ (↓)
2-Nitrotoluene	+	++ (↑)	++++ (↑)	++ (↑)	++++	+++	+++	+++	++++
3-Nitrotoluene	−	++ (↑)	+++ (↑)	+ (↑)	+ (↑)	+ (↑)	+++ (↑)	+− (↑)	++ (↑)
4-Nitrotoluene	++	+ (↓)			+++ (↑)	++++ (↑)	+++ (↑)	+++ (↑)	+++ (↑)
2-Aminotoluene	−	−							
3-Aminotoluene	++++	− (↓)							
4-Aminotoluene	−	−							
4-Chlorobenzaldehyde	++	− (↓)							
Phenol	−				++ (↑)	−	+− (↑)	−	+− (↑)
Benzene	+				++++ (↑)	+− (↓)	+	+++ (↑)	++ (↑)
Biphenyl	−				+ (↑)	+− (↑)	+− (↑)	−	+ (↑)
References	[61]	[19]	[31,62]	[62]	[13]	[13]	[13]	[13]	[13]

The response of each mutant is shown with respect to the wild-type (wt) regulator as indicated by the up or down arrows. XylR1 to 5 are mutants generated with a mutagenic shuffling procedure; XylR2 and XylR 4 are point mutants (as shown). XylR1: V124A, XylR 1–160, shuffle 161–166, DmpR 167–220; XylR3: XylR 1–45, shuffle 46–50, DmpR 51–220; XylR5: XylR 1–160, shuffle 161–166, DmpR 167–220 [12].

Table 2

Effector range of DmpR mutant regulators.

	wt	F42Y	R109C	L113V	D116N	F122L	E135K	B9 (K6E, F42S)	B23 (Q10R, K117M)	B24 (Q10R)	D9 (D116G, K117R)	F17 (D116V)
Phenol	+++						++ (↓)	+++	+++	+++	++ (↓)	+++
2-Methylphenol	+++						+++					
3-Methylphenol	+++						+++					
4-Methylphenol	++	+++ (↑)	+++ (↑)	+++ (↑)	+++ (↑)	++	+++ (↑)					
2,4-Dimethyl- phenol	–	++ (↑)	++ (↑)	+ (↑)	–	++ (↑)	++ (↑)	+ (↑)	++ (↑)		++ (↑)	–
3,4-Dimethyl- phenol	++	+++ (↑)	++	+++ (↑)	+++ (↑)	++	+ (↑)			++ (↑)		–
2-Nitrophenol	–							+ (↑)	++ (↑)		–	
4-Nitrophenol	–	–	++ (↑)	–	++ (↑)	–	+++ (↑)	–	–		–	–
2-Chlorophenol	+++							++ (↓)	+++		+++	
2,4-Dichloro- phenol	–	+++ (↑)	+++ (↑)	+++ (↑)	+++ (↑)	+++ (↑)	+++ (↑)	–	+++ (↑)	++ (↑)	–	++ (↑)
4-Chloro-3- methylphenol	–							–	–	++ (↑)	–	–
4-Ethylphenol	–	–	+ (↑)	–	+ (↑)	++ (↑)	++ (↑)					
References	[63,64]	[21]	[21]	[21]	[21]	[21]	[21,64]	[16]	[16]	[16]	[16]	[16]

The response of each mutant is shown with respect to the wild-type (wt) regulator as indicated by the up or down arrows. In data from [16], levels of activation shown are relative to wild-type DmpR activity at 25 or 75 μM of effector compounds.

A model for the structure of the XylR A domain has been generated, and the mutations in XylR variants map at several sites [13,32]. Althoug h some of the XylR and DmpR mutations fall in regions implicated in effector binding [12], others do not. Thus, the release of inter-domain repression and the subsequent structural changes as steps in the activation process may be more important than previously considered. The data from chimeric (originated from DNA shuffling) and point mutations might be better interpreted if specificity reflected not only the geometry of the binding site, but also the protein conduit through which the effector has to proceed to reach the site. Although not yet proven experimentally, there could well be a tunnel of this sort for effector binding, shaped by surfaces of the A and C domains. This notion is not without precedent, as it has been shown that alteration of an amino acid at the entrance tunnel of a haloalkane dehalogenase conferred changes in specificity to the enzyme [33••], arguing for binding-site-independent specificity mechanisms.

LysR family mutants with novel specificities

The LysR-type transcriptional regulator (LTTR) family is very large, with as many as 100 members identified by sequence homology and/or domain prediction. The structure of four LTTRs have been solved, CysB [34], CbnR [35], OxyR [36] and DntR [22]. LTTRs have a 60–70 amino acid N-terminal helix-turn-helix motif and a C-terminal region that confers effector specificity and contains tetramerisation determinants. Effector binding to LTTRs triggers changes in the DNA-binding interface [37] and in this way promotes transcription, possibly through interactions with the RNA polymerase and open complex formation [23]. Effectors for LTTRs of aromatic degradation pathways are usually pathway intermediates rather than leading substrates [38].

Mutants of NahR, a salicylate-responsive LTTR that regulates expression of the *nah* and *sal* naphthalene degradation operons in several bacteria [39], have been obtained with novel specificities. Using error-prone PCR and blue/white colony selection in the presence of benzoate, a non-cognate NahR inducer, Cebolla and colleagues [17] isolated two mutants responsive to this compound, NahR3 (Arg248Cys) and NahR4 (Asn169Asp). Although the wild-type protein is not activated at all by benzoate, both mutants activated transcription to levels comparable to that of wild-type regulator with salicylate. Also, NahR3 showed much higher affinity for 3-chlorobenzoate and 3-methyl salicylate than wild-type NahR, as judged by the induction of transcription at low effector concentrations [17] (Table 3).

The importance of residues Arg248 and Asn169 in effector recognition was highlighted by the observation that of six benzoate-responsive mutants (with low basal activity levels), four had the Asn169Asp mutation and two carried the Arg248Cys mutation. These mutants also carried other changes, but the recurrence of amino acid changes at positions 169 and 248 argues for a prominent role in effector recognition [17]. Indeed, the crystal structure of DntR, activator of the 2,4-dinitrotoluene (2,4-DNT) degradation route of *Burkholderia cepacia* R34, revealed central roles for these residues in salicylate binding. DntR shares 60% amino acid sequence similarity with NahR; Arg248 of DntR is located in the hydrophobic effector-binding pocket and His169, equivalent to Asn169, forms hydrogen bonds with salicylate [22]. The crystal structure of DntR was solved to allow the rational design of a 2,4-DNT biosensor: modelling of 2,4-DNT in the DntR structure directly implicated mutations in His169 and Arg248, among others, as being necessary to allow 2,4-DNT binding by DntR [22]. Thus, for the case of NahR

Table 3

Effector range of NahR mutant regulators.

	wt	NahR3 (R248C)	NahR4 (N169D)	NahR7 (R132C)	NahR5 (M116I)	NahR6 (M116T)	NahR8 (M116V)
Benzoate	–	+++ (↑)	+++ (↑)	+ (↑)	+ (↑)	+ (↑)	+ (↑)
Salicylate	+++	++++ (↑)	+++	+++	+ (↓)	+ (↓)	+ (↓)
Salicylamide	–	+ (↑)	–	+++ (↑)	+ (↑)	–	+ (↑)
2-Hydroxy benzylalcohol	–	–	–	–	–	–	–
2-Chloro benzoate	–	+++ (↑)	+++ (↑)	+ (↑)	+ (↑)	+ (↑)	+ (↑)
3-Chloro benzoate	++	+++ (↑)	++	++	+ (↓)	+ (↓)	+ (↓)
4-Chloro benzoate	–	–	–	–	+ (↑)	+ (↑)	+ (↑)
3-Methylsalicylate	+++	++++ (↑)	+++				
References	[17]	[17]	[17]	[17]	[17]	[17]	[17]

The response of each mutant is shown with respect to the wild-type (wt) regulator as indicated by the up or down arrows.

and DntR, experimental data from blind diversity generation with error-prone PCR and structure determination have coincided in pinpointing key residues for specificity determinants. Still, when a wider range of amino acid substitutions were tried in positions Asn169 and Arg248 (separately or together), changes in promoter affinity were observed [40], suggesting that mutations in effector pocket residues can also influence other steps in activation, such as multimerisation.

Outside of biodegradative route regulators, novel specificity mutants were isolated of XapR, an LTTR of xanthosine phosphorylase (*xapA*), a purine nucleoside phosphorylase of *Escherichia coli*. XapR induces *xapA* transcription upon binding to xanthosine, a purine nucleoside, but plating on a range of nucleosides as sole carbon sources led to the isolation of XapR variants that induced transcription to very high levels in the presence of xanthosine or of select nucleosides (inosine, adenosine and deoxyadenosine) [41].

AraC/XylS family mutants with novel specificities

Although comprising a very large family of regulators [42], little is known about the specificity determinants for members of the AraC/XylS family that regulate catabolic operons. Although the structures of two AraC/XylS-type regulators have been solved [43,44], because of low sequence conservation the structural basis of effector binding remains unclear. The conserved features of AraC/XylS activators are a C-terminal stretch of around 100 residues containing two helix-turn-helix motifs [42], sufficient for transcriptional activation [45], and a non-conserved N-terminal region that has been implicated in effector binding, dimerization, and polymerase contacts [23]. Sequence comparison shows that homology of the effector binding N-terminal region is low, even within regulators of biodegradative routes. Like XylR-type regulators, effector binding triggers interdomain signalling that leads to activation, as mutations in either the C- or N-terminal domains can give similar phenotypes with respect to the ability to activate transcription [46,47]. The AraC protein is known to function through a 'light switch mechanism', in which the protein exists in two states — on or off. In the model, in which AraC exists as a dimer, in the absence of the effector arabinose an arm at the N terminus of each monomer bridges to the DNA-binding domain of the other monomer. On binding arabinose, the arm shifts to cover the effector-binding site, releasing the DNA-binding domains and enabling activation [48]. It is presently unclear whether this mechanism operates in all AraC/XylS regulators.

Of the AraC/XylS-type proteins for biodegradative routes, the most thoroughly studied is XylS. Upon binding benzoate and *m*-toluate (intermediates of toluene and *m*-xylene biodegradation), this protein activates the *Pm* promoter of the pWW0 plasmid of *P. putida* mt-2 (see above) for transcription of a lower catabolic operon for these aromatics [49]. Of many mutations in the N-terminal region of XylS that have altered response to effectors, two are markedly different from XylS, namely Arg41Gly [18] and the variant known as XylS2 [50]. XylS2 shows remarkable levels of activation in the presence of benzoates substituted mostly at the 2 and 4 positions, and with very different R groups (2- and 4-ethylbenzoate, 4-chlorobenzoate, 4-bromobenzoate, 3,4-dichlorobenzoate, 2-hydroxybenzoate, 2-hydroxy-3-chlorobenzoate, 2-hydroxy-5-methylbenzoate, 2-methoxybenzoate, and 4-methoxybenzoate) [50]. Mutant Arg41Gly is interesting in having increased responsiveness to benzoate, while its ability to respond to 2-methylbenzoate, 4-methylbenzoate, and 3-methoxybenzoate is nearly abolished [18].

The XylS2 mutant (together with NahR4 mutant, also of altered specificity range [17]) has been used in a transcriptional cascade for amplifying gene expression. In the system reported by Cebolla *et al.* [51], NahR4 activates transcription of the cognate promoter *Pnah* (which naturally drives expression of naphthalene-degrading genes in yet another *Pseudomonas* strain). In turn, *Pnah* is fused to the *xylS2* gene. Because both variants are activated by salicylate, the presence of this compound yields levels of activation of a marker gene under the control of *Pm*

(activated by XylS) several orders of magnitude above those obtained with activation of the *Pnah* or *Pm* promoters alone. Thus, mutants responding to the same effector allow signal amplification in response to environmental signals by cascade circuits, which may be used for engineering biosensors.

TetR mutants with novel specificities

The TetR repressor of tetracycline resistance is one well-studied bacterial regulator with respect to its potential for biosensor design. This is because TetR has been used in eukaryotic systems of heterologous gene expression, where stringent control levels are achieved by fusing the highly sensitive TetR repressor (nanomolar binding constants for tetracycline) with activation domains of viral or eukaryotic origin [52]. A combination of biochemical and structural studies have allowed detailed characterisation of the regulator's mode of action [53]. De-repression of the target promoter takes place when effector binding causes allosteric changes in the protein, leading to repositioning of the DNA-binding motifs and loss of affinity for the binding site. The effector- and DNA-binding residues are separated by an interface region that transmits structural changes to the DNA-binding head. Effector specificity variants can carry mutations either in the effector-binding pocket or in the interface region [14,15,20,54].

A TetR variant all but unable to de-repress transcription upon tetracycline binding has been identified through a combination of DNA shuffling and saturation mutagenesis; the variant yields high levels of promoter activity upon binding suboptimal or non-effectors [14]. The variant carried two mutations only, one in the effector pocket (His64Lys) and the other in the pocket secondary shell (Ser135Leu). Mutation of Ser135 seems to change the stability and/or conformation of the effector-binding site. In the same study, two tetracycline analogues, San and Cmt1, elicited different responses from two classes of mutants. Variants induced to different degrees by San bind this effector with similar binding constants. By contrast, variants induced by Cmt1 have very different effector-binding affinities, while maintaining similar levels of inducibility. In other words, directed evolution captures the requirements for activation by each compound, but each effector needs the variant regulator to have a very definite set of characteristics that allow binding, allostery and DNA head affinity loss, leading to transcription. Specificity of the His64Lys/Ser135Leu variant for another tetracycline analogue, 4-Ddma-atc, was achieved through saturation mutagenesis of a position encoding an amino acid predicted to obstruct 4-Ddma-atc binding owing to steric hindrance [15].

The altered specificity of effector pocket mutants of TetR comes from changes in effector recognition [14], whereas in interface region mutants altered specificity is a product of remodelled allosteric changes upon binding of suboptimal or non-effectors [20]. Thus, the detailed study of TetR mutants in a structural context gives a detailed account of how changes away from the binding pocket lead to different specificities.

Wet versus computational generation of effector-responsive regulators

As discussed elsewhere [55], the acquisition of specificity for a new effector in a transcriptional regulator requires a pre-existing regulator with a certain escape (i.e. responsiveness to non-legitimate effectors), upon which new specificity can be built by natural (or artificial) rounds of mutagenesis and selection. Yet, the move from specificity for compound A to specificity for compound B rarely takes place in a single step, even if A and B are related structurally. *In vivo* screenings for new specificities systematically result in regulator variants that broaden the range of effectors towards the new one, rather than mutants that exchange specificities altogether (see above). But why is this the case? It has been calculated that the number of combinatorial possibilities to alter the geometry of an average effector-binding pocket in a regulatory protein is in the order of 10^{76} (in the range of the number of atoms in the universe) [56••]. As the best technologies for the generation of molecular diversity in the laboratory hardly go beyond 10^{12}–10^{14}, it is clear that experimental mutation/selection procedures to select novel variants are unable to cover all the combinatorial space of any given effector-binding pocket and would tend to pick variants with a relaxed specificity. Although this can be compensated for *in vivo* by making various rounds of mutation/selection, it is indeed rare to find mutants with a *bona fide* change of effector specificity. This probably reflects the fact that natural evolution of the specificities of transcriptional factors does not occur in a single step. By contrast, it is probable that moving between peaks of effector specificity is bound to occur through valleys of non-specificity. In fact, regulators of microbial pathways for recent compounds (e.g. many environmental pollutants) are not too specific for their substrates, which may reflect an ongoing evolution not yet optimized for these unusual, sometimes xenobiotic, nutrients [55].

Although these facts are intrinsically interesting, they also become a nuisance for developing new regulators by traditional genetic means. Ideally (as discussed above with DntR), availability of the crystal structure of the regulator under scrutiny can lead to directed mutagenesis of the effector pocket to change its specificity. But what if the target chemical to be detected is structurally distant from any of the known natural effectors of existing transcriptional factors? One promising strategy to address this problem has been the exploitation of some periplasmic sugar-binding proteins of *E. coli* as versatile scaffolds for regulatory protein engineering [57]. The basis of this

approach is to precalculate computationally the optimal binding sites for the chemicals of choice at the interface of the two domains that form the sugar-binding site of a cognate periplasmic receptor of *E. coli* [56••]. The best binding pockets predicted *in silico* are then engineered in the corresponding gene and integrated into a synthetic bacterial signal transduction pathway fused to a reporter gene. This method was used to construct receptors that bind trinitrotoluene (TNT), L-lactate or serotonin with high selectivity and affinity, and subsequently to construct biosensors for the new ligands. Future efforts will doubtlessly include both experimental evolution approaches and computational design to generate transcriptional regulators à la carte for biosensing applications. Yet, it is worth noting that in the periplasmic sugar-binding proteins successfully employed for engineering regulatory proteins [57], the protein surfaces for effector binding are separate from the hinge region that levers the protein into an active form. However, in many regulatory proteins the effector-binding site overlaps with the very constellation of amino acids that elicit activation, making it difficult for independent mutation of these two features. Computer-directed design of novel effector-binding pockets is thus not a choice for every regulatory protein, and *in vivo* evolution combined with efficient screening procedures will remain the most realistic option — at least in the near future.

Conclusions and outlook

Genetic, biochemical, and structural analyses have revealed the close relationship between effector binding and signal transmission in the steps leading to transcriptional activation. The different regulator classes share, broadly, basic features through which allostery takes place between the different regulator modules. A combination of directed evolution and structural studies can help identify amino acid changes leading to regulators with novel, non-promiscuous, specificities. An avenue that remains to be explored for obtaining more specific effectors is related to the screening systems used to isolate them from mutant libraries. The design of potent screening set ups may allow researchers to hone in on variants with the very desired features (effector specificity and affinity).

The regulators discussed here are induced by a limited range of compounds (aromatics and tetracycline), whereas the range of inducers employed by bacterial regulators is very wide. Thus, the approaches described need not be limited to the detection of compounds with environmental relevance. Biosensors, transgene expression systems, and signal amplification cascades can be constructed that detect compounds as varied as those related to quorum sensing, linear hydrocarbons, amino acid precursors and derivatives, toxins, hormones, and so on. In one example, protein-autoinducer binding mutants of TraR, a LuxR-type quorum sensing transcription factor, have been isolated that although fully functional in inducer-free repression, were found to be less responsive to effectors [58]. Engineered regulators can also be coupled to regulatory cascades, thus using circuit design for biodetection (Figure 1b) [59]. The range of applications of transcriptional regulators à la carte is by no means limited to biosensing of environmental pollutants. Regulatory factors with predefined effector specificities and delineated DNA-binding abilities are bound to multiply the inventory of biological parts for the rational design of regulatory networks in synthetic biology [59]. From a different angle, bacterial activators that respond to predetermined chemical species will be paramount for the setting of genetic traps to survey new enzymatic activities in metagenomic libraries [60]. Thus, the characterization of new types of regulators, combined with the computational predesign of new effector-binding sites, will pave the way for the generalization of bacterial biosensors for diverse biotechnological uses.

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Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
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