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Minireview

Positively regulated bacterial expression systems

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Summary

Regulated promoters are useful tools for many aspects related to recombinant gene expression in bacteria, including for high-level expression of heterologous proteins and for expression at physiological levels in metabolic engineering applications. In general, it is common to express the genes of interest from an inducible promoter controlled either by a positive regulator or by a repressor protein. In this review, we discuss established and potentially useful positively regulated bacterial promoter systems, with a particular emphasis on those that are controlled by the AraC-XylS family of transcriptional activators. The systems function in a wide range of microorganisms, including enterobacteria, soil bacteria, lactic bacteria and streptomycetes. The available systems that have been applied to express heterologous genes are regulated either by sugars (L-arabinose, L-rhamnose, xylose and sucrose), substituted benzenes, cyclohexanone-related compounds, ε-caprolactam, propionate, thiostrepton, alkanes or peptides. It is of applied interest that some of the inducers require the presence of transport systems, some are more prone than others to become metabolized by the host and some have been applied mainly in one or a limited number of species. Based on bioinformatics analyses, the AraC-XylS family of regulators contains a large number of different members (currently over 300), but only a small fraction of these, the XylS/*Pm*, AraC/P_{BAD}, RhaR-RhaS/*rhaBAD*, NitR/*PnitA* and ChnR/*Pb* regulator/promoter systems, have so far been explored for biotechnological applications.

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Introduction

Expression of proteins in bacteria represents among the oldest techniques within recombinant DNA technology. The recombinant gene usually is placed under control of an inducible promoter on a multicopy vector and then transferred to a suitable bacterial host. For the most commonly used host organisms, a wide range of different expression systems are available, each with its specific characteristics. The goals when such tools are used may vary, ranging from high-level protein production at industrial scales to low (physiological) levels of expression to control metabolic pathways. Ideally, expression systems should allow tight control of expression at all relevant levels. They should be inducible by agents or conditions that are cheap, should not require particular inducer uptake transport systems and the inducers should be metabolically inert relative to the host. It may also be preferable if the expression system works across species barriers, and for industrial applications, it is crucial that the entire system is stable during scale-up under high-cell-density cultivation (HCDC). No such idealized system probably exists, and not all criteria are equally important for each specific application. On the other hand, knowledge of the characteristics of the many systems available may be very helpful for the researcher in order to be able to choose the system which is most likely best for each specific case.

Regulation of promoter activity is usually achieved by modulating environmental signals (pH, temperature, ligands) which are coupled to gene expression via transcriptional regulators that stimulate or repress transcription from specific promoters (Browning and Busby, 2004). The transcriptional regulators may function as activators (positive regulation), repressors (negative regulation) or both (positive and negative regulation). Inducer-activator complexes stimulate transcription by binding to DNA at specific sites upstream of promoters and often proximal to the sites of binding for the host RNA polymerase (RNAP). They then interact with the polymerase or interfere in the transcription initiation process in such a way that transcription becomes facilitated (Adhya and Garges, 1990). In contrast, in the absence of inducer, repressors bind to operator sequences overlapping with or adjacent to the RNAP binding site, thereby blocking RNAP binding or inhibiting other steps of the transcription initiation process (Rojo, 1999). Upon

activation, the inducer binds to the repressor, leading to a conformational change that makes the repressor unable to bind to the operator, allowing transcription by RNAP.

There is a continuous ongoing search for new expression systems that may either have more universally preferable properties, or are particularly useful for certain types of applications, often in specific hosts. In particular, there is a need for development of more gene expression systems that function in many different bacterial species and that can be used for fine-tuning the expression of genes at low and physiologically relevant levels (Keasling, 1999; Terpe, 2006).

In the last decade due to the massive genome sequencing projects, identification of new inducible expression systems has become easier. By bioinformatics analyses, it is possible to rapidly identify numerous candidate systems, although it may require substantial amounts of experimental work to characterize the regulators. It is also often far from trivial to find out what particular inducer is activating the system. The regulatory proteins (transcription factors, TFs) controlling the expression may be classified according to sequence similarity of their DNA binding motifs or by alignments of their amino acid sequences. Currently, about 50 families of bacterial TFs have been reported (Rodionov, 2007), and the largest of these is LysR, followed by AraC-XylS (see http//www.bactregulators.org). A common feature shared by many TFs is that they bind ligands with high specificity and this binding determines their activation state. The TFs may also be subjected to laboratory evolution strategies in order to change their ligand specificities (Calcagno Galvão and de Lorenzo, 2006). Interestingly, TFs have been depicted as molecular targets for drugs aiming at neutralizing or killing pathogenic bacteria (Bowser *et al.*, 2007).

In this review, we will focus on TFs and their cognate promoters that are utilized to generate positively regulated expression tools, with a particular emphasis on regulators belonging to the AraC-XylS family. Several positively regulated expression systems have already been developed for use in biotechnology, some are known but have not been developed into expression tools, and based on genome sequence analyses, numerous yet uncharacterized such systems certainly exist in nature. Some of the yet unexplored systems may potentially offer new and advantageous properties, and may be used in combination with existing systems to independently control the expression level of more than one gene in bacteria.

Overview of positively regulated expression systems that have been developed for biotechnological applications

The vast majority of all reported adjustable bacterial expression systems are negatively regulated. However, it has been proposed that positively regulated systems can confer certain advantages over those that are negatively regulated (Wilms *et al.*, 2001 and references therein), particularly when very tight control (i.e. low background expression under non-induced conditions) is needed (Altenbuchner and Mattes, 2005). Such properties are generally critical when expressing host-toxic proteins, when running the production process under HCDC, and also when using the system as a tool for physiological studies. Positively regulated bacterial expression tools reported in the scientific literature are listed in Table 1.

Peptide induced expression systems

The popular nisin-based system originating from *Lactococcus lactis* is somewhat atypical in that nisin (the inducer) is an antibacterial peptide (for a review, see Mierau and Kleerebezem, 2005) which can inhibit growth of gram-positive bacteria. Nisin is ribosomally synthesized as a 57-residue precursor peptide and then subjected to various modifications, eventually resulting in a secreted 34-residue mature peptide. This peptide is a food grade inducer that can be extracted and purified from milk and it is useful for production of heterologous proteins in *L. lactis*. The nisin biosynthetic gene cluster in *L. lactis* was found to be under positive control of the NisR regulator (Kuipers *et al.*, 1993). NisR, together with NisK, constitute a two-component signal transduction system that responds to nisin. By placing the nisin gene promoter, *PnisA*, together with the *nisR* gene, a set of shuttle vectors for nisin-inducible and tightly controlled heterologous protein expression in *L. lactis* bacteria have been constructed (De Ruyter *et al.*, 1996; Bryan *et al.*, 2000; Mierau and Kleerebezem, 2005; Monné *et al.*, 2005; Wu *et al.*, 2006). One important limitation by using lactic acid bacteria as hosts for heterologous protein production is their low metabolic capacity typically resulting in relative poor volumetric yields (Mierau and Kleerebezem, 2005). The NisR/*PnisA* system, including NisK, has been demonstrated to function also in other gram-positive bacteria, including streptococcal and enterococcal species (Eichenbaum *et al.*, 1998; Bryan *et al.*, 2000). Nisin acts as an inducer on the outside of the cells and is sensed by NisK. However, due to the antibacterial properties of nisin for a number of gram-positive bacterial species, the host range of this expression system may be limited.

The so-called pSIP vectors have been developed based on regulatory genes and promoters involved in the production of the bacteriocins sakacin A and sakacin P in *Lactobacillus sakei* (Axelsson *et al.*, 2003). The vectors contain a sakacin A or P promoter, *PsapA* and *PsapIP* respectively together with a two-component regulatory system (Sørvig *et al.*, 2003; 2005), encoded by the *sapRK* operon, analogous to the nisin-based system (see

Table 1. Positively regulated bacterial expression systems and examples of biotechnological applications.

Expression system	Regulator family	Representative characteristics and applications	Selected references
NisR/*PnisA*	Unknown	*L. lactis* expression vectors, food-grade and antibacterial inducer nisin, tight control, limited host range.	Kuipers *et al.* (1993); De Ruyter *et al.* (1996); Bryan *et al.* (2000); Monné *et al.* (2005)
SapR/*PsapA* and SapR/*PsapIP*	Unknown	pSIP vectors, induced by peptide sakacin A or P, tightly controlled and high-level expression in *L. sakei* and *L. plantarum*.	Sørvig *et al.* (2003; 2005); Axelsson *et al.* (2003)
StbR/*stbD*	Unknown	pTRKH2 vector, induced by peptide-pheromone (STP), regulated expression in *S. thermophilus*, relatively high background expression.	Blomqvist *et al.* (2006)
PrpR/*PprpB*	Unknown	Expression vector, propionate-inducible, regulated and high-level expression, *S. enterica* and other enteric bacteria.	Lee and Keasling (2005; 2006)
TipAL/*PtipA*	MerR-SoxR	Expression vectors, thiostrepton-inducible, regulated and high-level expression in *Streptomyces* spp.	Kuhstoss and Rao (1991); Takanao *et al.* (1995); Ali *et al.* (2002); Dong *et al.* (2004)
PxylA	Unknown	Expression vectors, xylose-inducible, function in low G + C gram-positive bacteria.	Qasi *et al.* (2001); Radha and Gunasekaran (2008)
PsacB	Unknown	Expression vectors, sucrose-inducible, adjustable and high-level expression in *Bacillus* spp	Ye *et al.* (1999); Biedendieck *et al.* (2007)
SnpR/*PsnpA*	LysR	*E. coli-Streptomyces* shuttle vectors, high level expression in *Streptomyces* spp, no inducer reported.	DeSanti and Strohl (2003); Nikodinovic and Priestly (2006)
AlkS/*PalkB*	LysR	*E. coli* expression vectors, alkane and DCPK inducible, high-level expression, HCDC.	Panke *et al.* (1999); Makart *et al.* (2007)
XylR/*Pu*	XylR-NtrC	Broad-host-range expression vectors, inducers are substituted benzenes, catabolite repressed, growth phase regulated.	Blatny *et al.* (1997a,b)
AraC/P_{BAD}	AraC-XylS	Expression vectors, L-arabinose-inducible, high-level expression phage display, HCDC, metabolic engineering, broad-host range.	Guzman *et al.* (1995); DeLisa *et al.* (1999); Sukchawalita *et al.* (1999); Huang *et al.* (2000); Lim *et al.* (2000); Loessner *et al.* (2007)
RhaR-RhaS/*rhaBAD*	AraC-XylS	*E. coli* expression vectors, L-rhamnose-inducible, tigh tregulation, high-level expression.	Haldimann *et al.* (1998); Wilms *et al.* (2001)
XylS/*Pm*	AraC-XylS	Broad host-range expression vectors, multiple inducers (substituted benzenes), tight control, high-level expression, metabolic engineering, HCDC.	HCDC.Mermod *et al.* (1986); Blatny *et al.* (1997a,b); Brautaset *et al.*, 1998; 2000); Winther-Larsen *et al.*, 2000a,b; Gimmestad *et al.* (2003); Sletta *et al.* (2004; 2007); Bakkevig *et al.* (2005)
NitR/*PnitA*	AraC-XylS	Expression vector, hyper-inducible (ε-caprolactam) high-level expression, functions in *Streptomyces* strains.	Komeda *et al.* (1996); Herai *et al.* (2004)
ChnR/*Pb*	AraC-XylS	Broad-host-range vectors, cyclohexanone-inducible, high-level and tightly controlled expression, metabolic engineering.	Steigedal and Valla (2008)

above). Recombinant expression can be induced by adding sakacin induction peptides to the growth medium, and the vectors were demonstrated useful for tightly regulated and high-level expression of heterologous proteins in *L. sakei* and *Lactobacillus plantarum* (Sørvig *et al.*, 2005).

Blomqvist and colleagues (2006) recently reported the construction of the vector pTRKH2, which carries the peptide pheromone-inducible StbR/*stbD* regulator/promoter elements from *Streptococcus thermophilus*. The vector was used for regulated expression of the *gusA* reporter gene in *S. thermophilus*, and one drawback observed was a relative high background expression level. To our knowledge, this expression system has not been tested in other bacterial species.

The PrpR/PprpB regulator/promoter system

Lee and Keasling (2005; 2006) described the construction of propionate-inducible expression vectors carrying the PrpR/*PprpB* regulator/promoter system. The *PprpB* promoter is responsible for transcription of the propionate catabolic genes and transcription is under positive control by the PrpR regulator. The authors demonstrated dose-dependent heterologous expression with up to 1500-fold induction ratios of various reporter genes in *Escherichia coli* and in *Salmonella enterica*. Propionate is cheap, it enters the host cell by passive diffusion (i.e. no particular transport system is required) and the background expression level in the absence of the inducer is very low. This system should be useful both for high-level protein

expression and for metabolic engineering studies. It should however be noticed that *PprpB* has CAP-dependent activation and accordingly the carbon source used in the growth medium can severely affect background expression. Moreover, the inducer propionate can likely be metabolised and modified by many bacterial species.

The TipAL/PtipA regulator/promoter system

The thiostrepton-inducible promoter *PtipA* from *Streptomyces lividans* was placed on a high-copy-number plasmid and used for adjustable expression of recombinant proteins in *Streptomyces* species (Kuhstoss and Rao, 1991; Takanao *et al*., 1995). Later, the MerR-SoxR family type positive *PtipA* regulator TipAL was identified and characterized, and placed together with *PtipA* on plasmid vectors useful for thiostrepton-induced recombinant expression in *Streptomyces* and other gram-positive bacteria (Ali *et al*., 2002; Dong *et al*., 2004). Interestingly, expression from *PtipA* is under osmotic regulation and adjustment of growth medium osmolarity led to increased and prolonged TipAL-dependent expression of heterologous genes in *S. lividans* (Ali *et al*., 2002). One important observation was that under conditions of high osmolarity, basal expression of this system increased considerably. It should also be pointed out that the inducer molecule, thiostrepton, is an antibiotic, and this may limit the host-range of this expression system.

The PxylA and the Psac promoters

Both the xylose-inducible *PxylA* promoter (Qasi *et al*., 2001; Radha and Gunasekaran, 2008) and the sucrose-inducible *Psac* promoter (Ye *et al*., 1999; Biedendieck *et al*., 2007) have proven to be useful for regulated and high-level recombinant gene expression of prokaryotic and eukaryotic proteins in *Bacillus megaterium* and other AT-rich gram-positive bacteria. The *Psac* promoter was shown to display an induction ratio of up to 350-fold in *B. megaterium*. The *PxylA* promoter was demonstrated to be useful for regulated high-level production of the protease keratinase in this organism. A common limitation with both these promoters is that the inducer molecules sucrose and xylose are both typically metabolized by host cells, and accordingly the availability of mutant host strains deficient in their utilization can be preferred when using these expression systems. To our knowledge, no TFs for any of these two promoters have to date been identified.

The SnpR/PsnpA regulator/promoter system

The LysR family represents the largest family of positively regulated TFs (for a review see Schell, 1993), yet few of its members have been used as tools for recombinant expression. However, the SnpR activated *PsnpA* promoter originating from *Streptomyces* sp. strain C5 was characterized (DeSanti and Strohl, 2003) and later applied for high-level heterologous expression in *Streptomyces* species (Nikodinovic and Priestley, 2006). To our knowledge, no inducer has been reported for this expression system, and it has been shown that SnpR-mediated expression from *PsnpA* is modulated by the host physiology and activated in the stationary phase. Accordingly, this system has certain limitations when controlled recombinant expression is needed (DeSanti and Strohl, 2003).

The AlkS/PalkB regulator/promoter system

The OCT plasmid of *Pseudomonas oleovorans* GPo1 carries two *alk* gene clusters encoding a set of enzymes for the degradation of alkanes (van Beilen *et al*., 1994). Expression of one of these clusters is controlled by the alkane-responsive AlkS/*PalkB* regulator/promoter system. AlkS is a LysR family positive regulator that, in addition to alkanes, can be activated by the inducer molecule dicyclopropylketone (DCPK) (Sticher *et al*., 1997). The activated AlkS then binds to its cognate promoter *PalkB* and stimulates transcription. Expression vectors carrying the AlkS/*PalkB* genetic elements have been constructed and demonstrated to posses several favourable properties. For example, the DCPK inducer is water-soluble and metabolically inert (Makart *et al*., 2007). Moreover, this inducer presumably requires no active uptake system to enter the cells (Sven Panke and Bernard Witholt, pers. comm.). The AlkS/*PalkB* expression system is not subject to catabolite repression in *E. coli* (Staijen *et al*., 1999). AlkS/*PalkB* has been used for tightly controlled and high-level expression of recombinant xylene oxidase for biotransformation of styrene into (S)-styrene oxide (Panke *et al*., 1999), and it has also been shown to function well for induced production of recombinant proteins under HCDC of *E. coli* (Makart *et al*., 2007).

The XylR/Pu regulator/promoter system

XylR/*Pu* controls expression of the upper-pathway genes of the *Pseudomonas putida* TOL plasmid pWW0, and this operon is positioned physically together with the meta-operon which is controlled by XylS/*Pm* (see *The XylS/Pm regulator/promoter system*). XylR belongs to the XylR-NtrC family of TFs (see Table 1) and it binds to toluene-based inducers and positively activates transcription from the *Pu* promoter. Interestingly, XylR also exerts positive control on the expression of the important TF XylS, which will be described in more detail below. The XylR/*Pu* genetic elements were fused to the RK2 broad-host-range

minimal replication elements, and the resulting expression vectors were found to be useful particularly for fine-tuning recombinant expression of heterologous genes at physiological levels (Blatny *et al.*, 1997a,b). It should be noticed that *Pu* is under catabolite repression control (Duetz *et al.*, 1996) and this expression system becomes leakier as the cells enter stationary phase (Blatny *et al.*, 1997a).

The remaining expression systems listed in Table 1 are all members of the AraC-XylS family of positive transcription regulators, and we will in the following pay particular attention to this important family.

The AraC-XylS family of positively regulated TFs

Members of the AraC-XylS family are among the most commonly applied positive regulators in experiments involving recombinant gene expression control in bacteria. Prior to the applied use, this family of regulators have attracted scientific interest for decades as novel model systems for basic research on bacterial gene regulation. The origin and biology of the AraC-XylS family, and in particular the 'mother' protein AraC, have been extensively reviewed previously (Gallegos *et al.*, 1997; Martin and Rosner, 2001; Egan, 2002; Tobes and Ramos, 2002; Schleif, 2003). Members of the group all share primary amino acid sequence homology to AraC, which was the first bacterial transcriptional activator described (Englesberg *et al.*, 1965; Greenblatt and Schleif, 1971). Some years after the discovery of AraC, the RhaR and RhaS proteins were reported (Tobin and Schleif, 1987; 1990). Since then the family has grown considerably and was recently reported to include above 300 different members (Tobes and Ramos, 2002; Ruiz *et al.*, 2003; Tropel and van der Meer, 2004), represented in a wide range of both gram-positive and gram-negative bacterial species. The vast majority of the family members are positive regulators, but some of them can also act as negative regulators. As a direct consequence of the massive ongoing bacterial genome sequencing (http://www.genomesonline.org/), the total number of putative AraC-XylS TFs identified is several thousands (Rodionov, 2007), and this number is likely to increase even more in the future. The members of the AraC-XylS family regulate diverse cellular functions, such as carbon metabolism, various stress responses including antibiotic biosynthesis, and also pathogenesis (Gallegos *et al.*, 1997). Interestingly, one member was recently demonstrated to be associated with cell survival following DNA damage in *Bacteroides fragilis* (Casanueva *et al.*, 2008). The AraC-XylS family sub-group that regulates carbon metabolism is characterized by being of similar molecular sizes (about 300 amino acids), and they stimulate transcription from cognate promoters in response to the presence of effectors. Below we discuss the well-characterized family members AraC and XylS as representative models to describe biology and structure/function characteristics of the AraC-XylS family regulators.

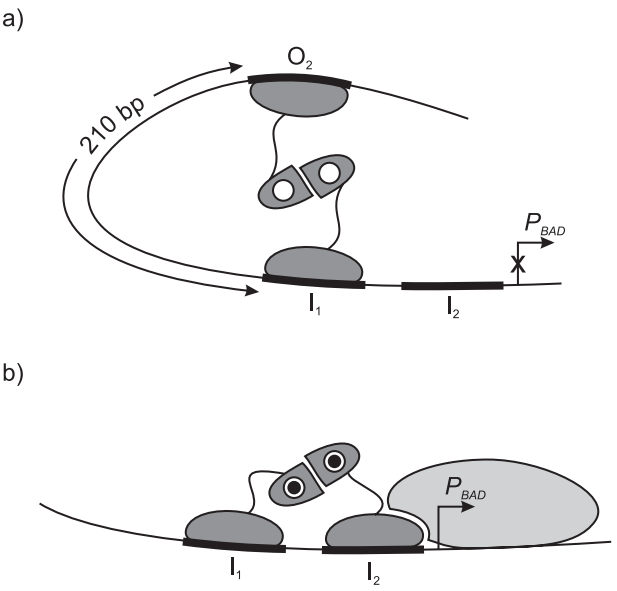
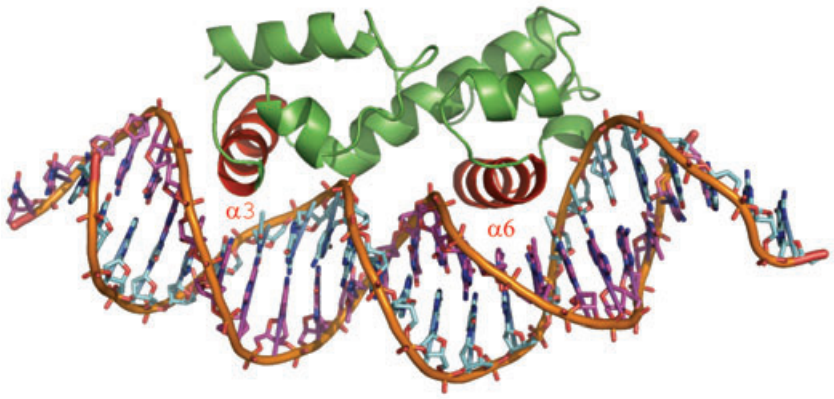


Fig. 1. Binding of AraC to P_{BAD} in the absence (A) and presence (B) of the inducer molecule L-arabinose (closed circle) (modified with permission from Schleif, 2003). O_2, I_1 and I_2 are DNA binding sites for AraC. The RNAP is depicted in light colour. For details see text section *The AraC/P_{BAD} regulator/promoter system*.

The AraC/P_{BAD} regulator/promoter system

AraC is the best-characterized member of the AraC-XylS family and it regulates transcription from promoters involved in catabolism of L-arabinose, which is also the inducer of the system (Englesberg *et al.*, 1965). When L-arabinose is present, it positively activates transcription from the *ara* promoters. Interestingly, when L-arabinose is not present, this protein also actively represses transcription from at least one of these promoters, P_{BAD} (Englesberg *et al.*, 1969). This promoter is catabolite-repressed, so uninduced levels of transcription is further reduced by cell growth in the presence of glucose (Miyada *et al.*, 1984). The AraC protein is a homodimeric protein, and each of its monomers contains a conserved C-terminal DNA binding domain made up of about 100 amino acids and an N-terminal non-conserved dimerization domain. The latter domain also contains an L-arabinose binding pocket (Wilcox and Meuris, 1976; Steffen and Schleif, 1977; Soisson *et al.*, 1997a,b). In the absence of L-arabinose, the AraC dimer binds to two DNA sites O_2 and I_1 separated by 210 base pairs (Fig. 1). This generates a characteristic DNA loop (Schleif, 1988) which in turn negatively interferes with the access of RNAP to the promoter in the looping region. When L-arabinose is present and bound to AraC, instead of



		α3		α6
XylS	241	PRSLYNLFEKH	294	LGRFAENYRSAF
MarA	41	KWHLQRMFKKE	91	QQTLTRTFKNYF
AraC	206	EKTIQRLFRKE	255	PSAFATMFKKQF
RhaS	201	LRTLHRQLKQQ	251	SNHFSTLFRREF
RhaR	236	ERVLRQQFRQQ	286	SNYFSVVFTRET
ChbR	203	QEYLTRATQRY	253	PSLFIKTFKKLT
		...L.....		...F...F....

Fig. 2. AraC-XylS family regulators' characteristic HTH DNA binding motif is shown by using the member MarA as a model (α-helixes 3 and 6 in red colour). Conserved amino acid residues are depicted in the bottom row. The alignment was derived from the full-length primary sequences of the given TFs by using the PROMALS3D web server (Pei *et al.*, 2008). Parameters were left at default values. The figure was prepared by using PyMOL (DeLano, 2003). Note that MarA binds DNA as a monomer.

looping the regulator binds to the I_1 and I_2 sites on the DNA, leading to stimulation of transcription from the P_{BAD} promoter via direct interaction with the host RNAP (Zhang *et al.*, 1996). AraC can also downregulate its own expression by binding to an operator close to the *araC* promoter P_C (see Schleif, 2003).

Experimental work involving functional expression, purification and biochemical characterization of AraC is technically very difficult, as reviewed by Schleif (2003). This property also holds for a large number of the AraC-XylS family members; they are extremely insoluble. The major reason is predicted to be due to the unusually long contact area between the protein DNA binding domain and its target DNA (almost 40 base pairs). Each monomer contacts two major groove regions of the DNA (see Fig. 1) using two helix–turn–helix motifs (HTH) (Ogden *et al.*, 1980; Hendricksen and Schleif, 1985; Brunelle and Schleif, 1989; Carra and Schleif, 1993). It has been proposed that the AraC protein, in particular the DNA binding domain, does not complete folding in the absence of DNA (Schleif, 2003). A partially folded DNA binding domain is sensitive to proteases and with an excessive number of hydrophobic residues exposed, this may also lead to aggregation. Due to all these problems, the AraC protein crystal structure was determined more than 20 years after its discovery (Soisson *et al.*, 1997a,b). The technical difficulties experienced with AraC are most likely one major reason why few of the large number of TFs of this family have not yet been characterized biochemically (Martin and Rosner, 2001). Currently, 3D structures have been experimentally solved for only three members; AraC, and the monomeric regulators RobA, and MarA (Rhee *et al.*, 1998; Kwon *et al.*, 2000). The structure of the MarA protein is shown in Fig. 2. The conserved C-terminal domain of the TF contains the characteristic HTH motif critical for DNA binding. In contrast, the N-terminal domains among AraC-XylS family TFs are structurally highly divergent, supporting the assumption that the insolubility properties of these proteins can be mainly assigned to the DNA binding domain.

The XylS/Pm regulator/promoter system

A handful of the known AraC-XylS family TFs, including XylS, control catabolic operons and they generally act as activators in the presence of a chemical effector molecule (for a review, see Tropel and van der Meer, 2004). The *P. putida* TOL plasmid encodes a pathway for catabolism of toluene and xylenes (Worsey and Williams, 1975), and the genes involved are grouped into the upper- and meta-pathway operons, positively regulated by TFs XylR and XylS respectively. The *xylS* gene can be transcribed from two alternative and individually regulated promoters, *Ps1* and *Ps2*; XylR can activate transcription from *Ps1* while transcription from *Ps2* is constitutive and low. Interestingly, translation of *xylS* mRNA generated from the *Ps1* promoter is 10 times more efficient than that generated from *Ps2* (González-Pérez *et al.*, 2004). Together, this ensures a tightly controlled and balanced expression level of XylS, and it gives rise to two modes of *Pm* activation according to the XylS concentrations in the cells. When overexpressed, XylS can bind to the operator sequence *Om* in the absence of inducer and activate transcription from the meta-pathway *Pm* promoter (Marqués *et al.*,

1999). In the presence of a benzoate-derived inducer, on the other hand, XylS activates transcription from *Pm* at low protein concentrations (for a review, see Ramos *et al.*, 1997). *Pm* is special in that the *in vivo* transcription from this promoter can be mediated by RNAP with different alternative σ factors, σ^{32} or σ^{38}, depending on the growth phase (Marqués *et al.*, 1999). Regardless of the growth phase, expression from *Pm* remains dependent on XylS (see above), and the transcription start point is the same (Marqués *et al.*, 1995; Domínguez-Cuevas *et al.*, 2006). A number of σ^{38}-dependent promoters have also been reported to be transcribed *in vivo* by RNAP with an alternative bound σ factor depending on the physiological conditions; however, in all cases so far reported (except for *Pm*), σ^{70} was responsible for the alternative transcription (Busby and Ebright, 1997).

As described for AraC (see above), XylS is also a modular protein with a conserved C-terminal domain for DNA binding and interactions with RNAP, and with a non-conserved N-terminal domain responsible for effector binding and protein dimerization. Interestingly, AraC and XylS show some degree of primary sequence homology also in the N-terminal region, and it has been proposed that these two proteins use similar mechanisms by which the effector controls the activity of the TF (Kaldalu *et al.*, 2000). The DNA binding motif and the effector binding pocket in XylS are not independent domains, as shown by intramolecular dominance of C-terminal mutations over N-terminal mutations and by the reversal of this dominance in double mutants tested *in vitro* (Mìchan *et al.*, 1992). Ruiz and Ramos (2001 and 2002) constructed XylS double mutants with substitutions in positions 137 and 153 of the C-terminal domain, and the mutant proteins displayed altered effector profile suggesting that these two residues are critical for effector recognition and regulator activation to stimulate transcription from *Pm*.

In the C-terminal end of XylS, a conserved stretch of about 100 amino acids is proposed to contain totally seven α-helices, including two HTH binding motifs (α2-T-α3 and α5-T-α6), connected by a linker α-helix (α4) and two flanking α-helices (α1 and α7). This structural organization has also been experimentally confirmed for the monomeric MarA protein (Rhee *et al.*, 1998) (see Fig. 2). The second HTH motif (α5-T-α6) is the most conserved in the proteins of the AraC-XylS family proteins (Gallegos *et al.*, 1997), suggesting a common role for this motif in all these regulators. Besides, mutational analyses have shown that the α5-T-α6 motif takes part in DNA binding by AraC and similar experiments have confirmed the role of this motif for XylS activity. In addition, conserved residues located outside of the HTH motifs were found to play important structural roles for XylS function (Manzanera *et al.*, 2000).

XylS (like AraC) binds to DNA as a dimer, to a 35-bp-long region of the promoter (Hendricksen and Schleif, 1985; Kaldalu *et al.*, 2000), and genetic analyses established that this TF recognizes two 15-bp direct repeats (TGCA-N6-GGNTA) (Kessler *et al.*, 1993; Gallegos *et al.*, 1997; González-Pérez *et al.*, 1999; 2002). It was demonstrated that XylS interacts with the C-terminal domain of the RNAP α subunit during activation of transcription from the *Pm* promoter, analogous to what has been found for the AraC-XylS family members AraC, MarA, SoxS, Rob, AlkA and RhaS (Ruiz *et al.*, 2001 and references therein). As reported for AraC, Rob and RhaS, the binding sites for XylS and RNAP overlap (Busby and Ebright, 1997; Dhiman and Schleif, 2000; González-Pérez *et al.*, 2002). It was suggested that this overlap may facilitate interactions between XylS and the α subunit of RNAP (González-Péres *et al.*, 2002). This may be different in other members of this family, e.g. for Ada (Landini *et al.*, 1998) and MarA (Martin *et al.*, 2000).

Recombinant expression systems based on AraC-XylS family TFs and their cognate promoters

To date, only a handful of AraC-XylS family regulator genes with their cognate promoters have been applied to construct expression vectors for various biotechnological applications (Table 1). A common trait observed with those that have been tested and used for such purposes is that they are tightly controlled and they are also demonstrated to generally be functioning in different bacterial species. Each of them is described below in some detail.

AraC/P_{BAD} can be used to achieve high-level and tightly controlled gene expression in different bacterial species

In general, genes cloned under the control of the P_{BAD} promoter can be efficiently expressed, and this system also allows tight regulation and inexpensive induction with L-arabinose (see above). In the presence of this sugar, the P_{BAD} promoter is rapidly turned on, while in the absence of L-arabinose and in the presence of glucose, very low or undetectable levels of transcription occurs (Lee *et al.*, 1991; Johnson and Schleif, 1995). Guzman and colleagues (1995) took advantage of the many favourable properties of AraC/P_{BAD} to construct the so-called pBAD expression vectors, carrying the ColE1 origin for replication in *E. coli*. In host strains deficient in *ara* genes (that cannot metabolize the effector molecule L-arabinose which in turn remains at a constant concentration in the cells), expression of cloned genes reaches maximal induction upon adding as little as 0.001% L-arabinose, while in L-arabinose fermenting strains, addition of about 1% inducer is necessary to achieve full induction (Mayer, 1995). The system has also been demonstrated to function for recombinant protein production under HCDC (DeLisa *et al.*, 1999). Huang and colleagues

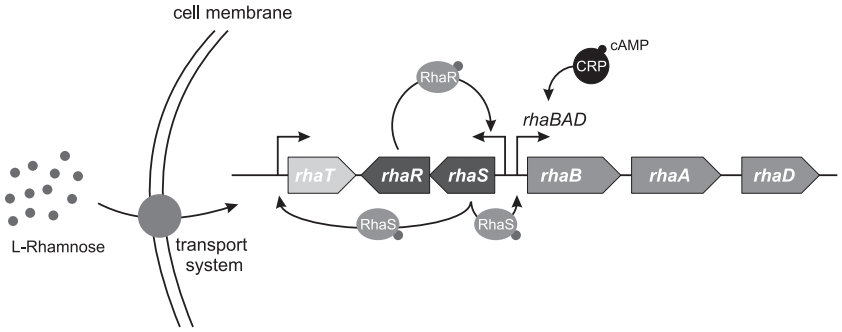


Fig. 3. Mechanism of induction of the RhaR-RhaS/*rhaBAD* system (modified with permission from Altenbuchner and Mattes, 2005). L-rhamnose is actively transported into the cells and it binds to RhaS which then becomes activated and stimulates transcription of *rhaT* (encoding L-rhamnose transporter protein) and the *rhaBAD* operon (encoding L-rhamnose catabolic enzymes). RhaR is also activated by L-rhamnose and stimulates transcription of the *rhaSR* operon (encoding RhaR and RhaS). The catabolite repression is indicated. For further details see text section *The RhaR-RhaS/rhaBAD system shares many expression properties with AraC/P_{BAD}*.

(2000) constructed phage display vectors using AraC together with P_{BAD}, and the vectors turned out to be useful to exhibit controlled expression of proteins on the surface of the bacteriophage M13. A typical challenge faced when using phage display is toxicity problems of the recombinant protein in the host bacterium, eventually leading to libraries dominated by deletion mutants (Fischer *et al.*, 1994; Huang *et al.*, 2000). One strategy to avoid such problems is to ensure tightly controlled expression of the heterologous protein, and the amount of protein displayed on the phage by using the AraC/P_{BAD} system could be modulated by the amount of L-arabinose inducer present in the growth medium during phage propagation. The active transport needed for L-arabinose to enter the cells may confer some application limitations with this system (see the next section).

Interestingly, by using alternative broad-host-range replicons, it has been demonstrated that AraC/P_{BAD} can function well in several bacterial species, including *Corynebacterium glutamicum* and *Xanthomonas* sp. (Ben–Saumoun *et al.*, 1999; Newman and Fuqua, 1999; Sukchawalita *et al.*, 1999). Lee and colleagues (2007) recently constructed novel *araC* mutants by directed enzyme evolution that displayed altered effector affinities, including 10 times higher sensitivity to L-arabinose, and improved tolerance to IPTG. The IPTG is a commonly used inducer molecule for the popular *Plac* promoter; however, this compound is also an inhibitor of the AraC/P_{BAD} system. This mutant AraC regulator can be useful in cases where *Plac* and P_{BAD} are applied simultaneously for controlled recombinant expression in the same host cell.

The RhaR-RhaS/rhaBAD system shares many expression properties with AraC/P_{BAD}

The L-rhamnose regulons of *E. coli* consist of the *rhaT* and the operons *rhaSR* and *rhaBAD*. The *E. coli rhaSR* operon encodes the L-rhamnose-responsive AraC-XylS type TFs RhaS and RhaR, while *rhaT* and *rhaBAD* encode L-rhamnose catabolic and transport enzymes respectively (Gallegos *et al.*, 1997). Both RhaS and RhaR are activated by L-rhamnose. L-rhamnose acts as an inducer which binds to the regulator RhaR which then activates transcription of the *rhaSR* operon leading to induced expression of RhaR and RhaS. The latter protein in turn acts as an activator in the positive control of *rhaT* and the *rhaBAD* operon (Fig. 3). The L-rhamnose regulons are, as described for the *ara* genes (see above), regulated by catabolite repression and full activation also requires the cyclic AMP receptor protein CRP in addition to RhaS and RhaR (Gallegos *et al.*, 1997; Wickstrum *et al.*, 2005). The *rhaBAD* promoter is, as shown for P_{BAD}, an interesting tool for regulated recombinant expression in *E. coli* (Haldimann *et al.*, 1998). This system has been stated to be even more tightly regulated than AraC/P_{BAD} and recombinant production of L-N-carbamoylase using *rhaBAD* has been demonstrated to function well under HCDC (Wilms *et al.*, 2001). As mentioned for P_{BAD}, it may be advantageous also for the *rhaBAD* promoter to use genetically engineered host strains that cannot metabolize the L-rhamnose inducer.

At this point, it should be mentioned that both L-rhamnose and L-arabinose require transport systems to enter the cells, and it has been stated that expression systems induced by such compounds display limitations with respect to dose-dependent induction (Keasling 1999). Typically, the genes encoding the respective transport systems are also controlled by the inducer, leading to an 'all-or-nothing' induction response; i.e. in a cell culture some cells are fully induced while some are not. Khlebnikov *and* colleagues (2002) demonstrated how this could be circumvented by using engineered *E. coli* host cells that overexpressed the L-arabinose transporter AraE.

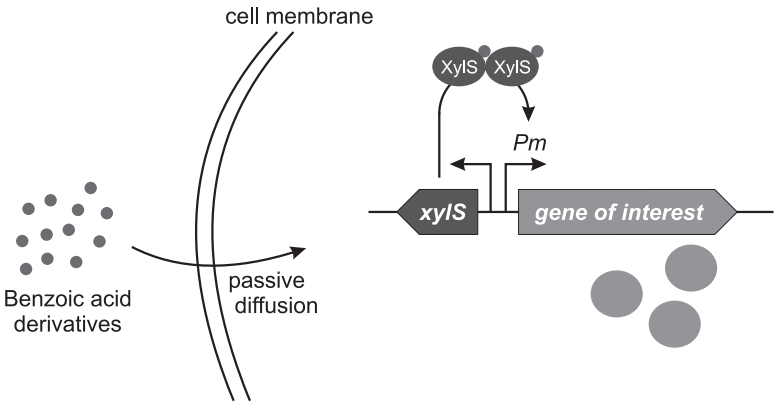


Fig. 4. The XylS/*Pm* expression system. Inducer molecules enter cells passively and bind to XylS which then becomes activated. The activated XylS stimulates transcription from *Pm*. For details see text section *Broad-host-range expression systems based on XylS/Pm.*

Broad-host-range expression systems based on XylS/Pm

The XylS/*Pm* regulator/promoter system has been used for the construction of multiple different expression systems which together represent a wide range of properties and application ranges. The first example of using this cassette to construct expression tools was described more than 20 years ago (Mermod *et al.*, 1986). The vectors were based on the broad-host-range RSF1010 replicon, and they were shown to be useful for various levels of regulated expression of the XylE reporter protein in a relatively large number of different gram-negative species. The vectors were later modified by using a mutant XylS protein, designated XylStr6, with altered effector affinity and which mediated up to eightfold higher expression levels from *Pm* compared with the wild-type XylS (Ramos *et al.*, 1988). These studies demonstrated that XylS/*Pm* can function well for regulated expression of heterologous proteins in a wide range of different gram-negative bacterial species.

The *xylS/Pm* expression cassette was later fused also to the broad-host-range minimal replication elements *oriV* and *trfA* from the RK2 plasmid (Blatny *et al.*, 1997a). The *trfA* gene encodes a replication initiation protein also exerting negative control of replication from *oriV*. Different versions of the plasmids were constructed with various antibiotic markers, multiple cloning sites and *oriT* for conjugal transfer, resulting in a set of broad-host-range cloning and expression vectors (Blatny *et al.*, 1997b). In all the vectors the *xylS* gene is constitutively transcribed from its native *Ps2* promoter, and the gene of interest is placed under transcriptional control by *Pm* (Fig. 4). The application potential of these vectors for both high- and low-level regulated gene expression in *E. coli*, *Pseudomonas aeruginosa* and *Xanthomonas campestris* was indicated (Blatny *et al.*, 1997a,b). In particular, the plasmid pJB658 which is a combined cloning and expression vector (Blatny *et al.*, 1997b) has proven to be very useful for many different aspects of recombinant expression. Relevant characteristics of this vector includes adjustable vector copy number (between 5 and 100 per chromosome), several cheap benzoic acid derivatives such as m-toluic acid can be used, they act in a dose-dependent manner and inducer uptake is presumably passive (no transport system required) (Lambert and Stratford, 1999 and references therein) which further simplifies the use of the system across species barriers. pJB658 was used to control sugar metabolism in *E. coli* by fine-tuning the expression levels of a number of phosphoglucomutase mutant enzymes genes with different kinetic properties (Brautaset *et al.*, 1998; 2000). The same plasmid was used to control xanthan biosynthesis in a *xanA*-deficient *X. campestris* mutant strain by regulated low-level expression of the *xanA* gene, encoding a bifunctional phosphomannomutase/phosphoglucomutase (Winther-Larsen *et al.*, 2000a). The unique ability of fine-tuning recombinant expression levels at low levels by using *Pm/xylS* has also been demonstrated in alginate producing *Pseudomonas fluorescens* (Gimmestad *et al.*, 2003; Bakkevig *et al.*, 2005). The phosphomannomutase gene *algC* and the mannuronan C-5-epimerase gene *algG* were individually put under transcriptional control of *Pm* and introduced into the bacterial chromosome by using transposons. The phosphomannomutase is involved in alginate biosynthesis while the mannuronan C-5-epimerase is involved in the concomitant alginate polymer modification. In this way, the alginate production level and the alginate composition in the resulting recombinant strains could be modulated by adding inducer at various concentrations.

By using firefly luciferase as a reporter protein, it was shown that the induced expression levels both in *E. coli* and *P. aeruginosa* could be varied over a wide range by using different types and different concentrations of inducers (Winther-Larsen *et al.*, 2000a). By integrating the *Pm/xylS* expression cassette into the *E. coli* chromosome, recombinant expression levels of the reporter protein could be controlled at very low levels. The −10 region of the *Pm* promoter sequence was also randomly

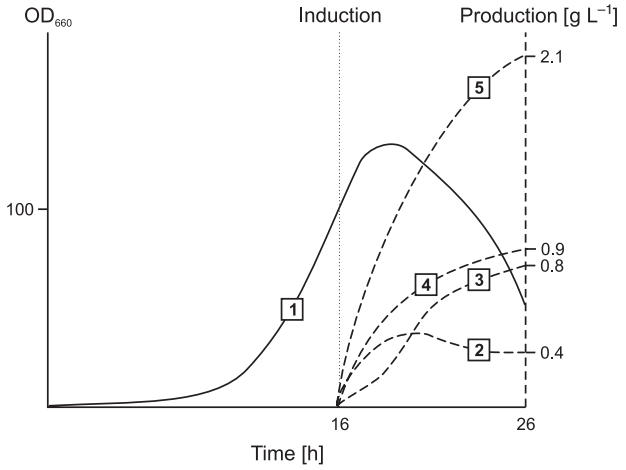


Fig. 5. Fermentation course of recombinant *E. coli* cells expressing totally 2.1 g L^{-1} of single-cell antibody fragment scFv-phOx (P) by using the XylS/*Pm* expression system. Lines: 1, growth curve; 2, soluble P in periplasm; 3, soluble P in growth medium; 4, insoluble P in cytoplasm; 5, total soluble + insoluble P (data imported from Sletta *et al*., 2004; 2007).

mutagenized by so-called doped oligonucleotides. *Pm* promoter mutants with enhanced induced expression levels were identified, as well as mutants with lower uninduced levels compared with the wild-type *Pm* (Winther-Larsen *et al*., 2000b). One interesting finding was that expression from *Pm* can be modulated by the growth conditions of the recombinant cells, and in particular the pH of the growth medium was found to be critical. At a relatively high pH expression from *Pm* decreases drastically, and it was postulated to be related to the dissociation state of the inducer. By combining the effects of environmental and genetic parameters expression from *Pm* could be varied over an almost hundred-thousand-fold continuous range (Winther-Larsen *et al*., 2000a,b).

Plasmid pJB658 has also been demonstrated to be highly useful for industrial-level production of three secreted human medically relevant proteins; single-chain antibody fragment (scFv-phOx), interferon α2b and granulocyte macrophage colony-stimulating factor, under HCDC in *E. coli* (Sletta *et al*., 2007). The advantage of optimizing, and not maximizing, expression levels was demonstrated to be crucial to achieve high-level functional expression of antibody fragment scFv-phOx in *E. coli* (Sletta *et al*., 2004; 2007). Protein scFv-phOx is host toxic and must be translocated to the periplasm to fold into soluble and functional form, and this translocation was guided by using a signal sequence as fusion partner. A very low background expression prior to induction, together with the strong induced expression from *Pm*, was demonstrated to be crucial to maintain plasmid stability under HCDC and to achieve very high volumetric production yields (Fig. 5).

Hyper-inducible expression in streptomycetes using NitR/PnitA

Streptomycetes are popular production hosts for a wide range of useful compounds, such as antibiotics, pesticides, immunosuppressive compounds and also enzymes. However, tools for regulated recombinant gene expression in these bacteria are in general poorly developed. In a report by Herai and colleagues (2004), plasmid vectors carrying the *Rhodococcus rhodochrous* J1 NitR/*PnitA* system were described. The AraC-family activator NitR controls transcription from the *PnitA* promoter of the nitrilase gene *nitA* in *R. rhodochrous* (Komeda *et al*., 1996). Nitriles are generally cell-toxic compounds; however, some microorganisms can use such compounds as carbon and nitrogen sources. Nitrilases are enzymes that catalyse the cleavage of nitriles to the corresponding acids and ammonia, and in *R. rhodochrous*, nitrilase can constitute up to 35% of all soluble proteins under induced conditions, e.g. in the presence of isovaleronitrile (Komeda *et al*., 1996). However, this compound is both cell-toxic and expensive and accordingly not well suited as an inducer molecule for practical applications. It was recently demonstrated that NitR can be activated by ε-caprolactam which is an alternative inexpensive, water-soluble and safe inducer that has no obvious influence of the physiological conditions of streptomycetes (Herai *et al*., 2004). By using NitR/*PnitA* and ε-caprolactam, tightly controlled and high-level expression of reporter genes in several different streptomycete strains was demonstrated (Herai *et al*., 2004). Another important trait observed with this system was that ε-caprolactam induction is dose-dependent, a highly useful property when fine-tuning of expression levels are needed, e.g. to optimize conditions for host-toxic proteins and also for physiological studies. Considering the molecular size and structure, it is likely that ε-caprolactam does not need any transport system to enter the bacterial cells.

Regulated heterologous expression using ChnR/Pb

Steigedal and Valla (2008) recently searched the AraC-XylS database in order to identify new expression cassette candidates with similar properties as those of XylS/*Pm* (see sections *the XylS/Pm regulator/promoter system* and *broad-host-range expression systems based on XylS*/Pm), and the ChnR/*Pb* regulator/promoter system in *Acinetobacter* sp. strain NCIMB 9871 was further investigated. The *chnR* gene is part of a gene cluster responsible for cyclohexanol oxidation, suggesting that the system could be activated by several different inducers, in addition to cyclohexanone (Iwaki *et al*., 1999), which does not need any particular transport system, and also that the inducers are not metabolized by most heterologous hosts strains. The genetic elements were cloned

into a RK2-based plasmid vector and tested for recombinant expression using various induction conditions in both *E. coli* and *P. fluorescens*. Cyclohexanone was found to be the most efficient inducer of this system in *E. coli* using firefly luciferase as a reporter protein. The maximum expression level obtained with the ChnR/*Pb* system was lower compared with that of XylS/*Pm* (see above); however, both systems shared the ability to display an inducer concentration-dependent response, and they both also function well in different gram-negative species. ChnR/*Pb*, in combination with *Pm/xylS*, was used to control the monomer composition of alginate in *P. fluorescens* (Steigedal and Valla, 2008), and this system should therefore represent a valuable new broad-host-range expression tool.

The AraC-XylS family as a source of TFs for identification of novel expression systems

Along with the growing exploitation of not yet characterized bacterial species for various biotechnological applications, there is a need for new and flexible expression systems. The identification and application of the ChnR/*Pb* expression system from *Acinetobacter* sp. strain NCIMB 9871 (see above) successfully demonstrated an approach in which the AraC-XylS family was screened *in silico* to identify promoter/regulator elements useful to develop a new bacterial expression system. In this particular case, some biological knowledge of the system in question was available (Iwaki *et al.*, 1999). Considering the broad diversity of biological functions likely to be controlled by AraC-XylS type of TFs, we propose that additional candidates with a potential for development of novel expression tools for both gram-positive and gram-negative bacteria may be identified from this family in the future.

As we see it, two major challenges may be met in this approach: the first challenge is the identification of correct cognate promoter(s) that are under transcriptional control of the identified AraC-XylS regulator. It is well known that several AraC-XylS regulators have been found to control expression of multiple different promoters (Gallegos *et al.*, 1997; Rodionov, 2007). This is a common feature shared by many bacterial TFs; although they bind to their TF binding sites (TFBSs) in a sequence specific manner, the sequences of the binding sites of a particular TF located upstream of different genes in the same genome may vary significantly, allowing for a more flexible transcriptional control. Potentially, this may lead to unpredicted binding to chromosomal host sites when using such expression systems in a heterologous host, and the biological effects of such events cannot be easily foreseen. The size of a bacterial TFBS is typically 16–20 nucleotides, and for TFs that recognize and bind to DNA as homodimers, it follows that the TFBSs usually possess an intrinsic symmetry. Accordingly, inverted and direct repeats are the most common structures of TFBSs, while some homomultimeric TFs cooperatively bind more complex TFBSs composed of both inverted and direct repeats, e.g. AraC in *E. coli*. Consensus sequences for important types of TFs have been reported, including for AraC-XylS family regulators, and computational algorithms have been developed for searching genomes for TFBSs based on such consensus signatures (for review see Zhou and Yang, 2006; Rodionov, 2007). As such *in silico* methods constantly improve, they will most likely become powerful tools to rapidly identify and map new AraC-XylS TF-controlled promoters. It should also be mentioned that many AraC-XylS family TF genes are located in close vicinity to the genes or operons they control, which further simplifies the identification of the desired promoters and their cognate regulators.

The second major challenge in selecting new AraC-XylS family members for construction of expression vectors will likely be to identify the appropriate inducer compound(s) that modulates regulator activity. Interestingly, it was recently shown that the AraC-XylS family Rns regulator in *E. coli* presumably does not respond to any ligand (Basturea *et al.*, 2008), demonstrating that this information may not be straightforward to obtain. One could argue that the ongoing basic research on gene regulation in bacteria will eventually also generate new functional knowledge on so far poorly characterized AraC-XylS family members, useful for easy selection of promising candidates for constructing new expression tools. According to Ibarra and colleagues (2008), about 60 different AraC-XylS regulators have to date been experimentally characterized, and this number is likely to increase in the future. For example, the first example of a cellobiose-induced AraC-XylS regulator was recently reported (Joshi *et al.*, 2007). However, as we see it, a deep basic insight regarding regulator/promoter/RNAP interactions should be no prerequisite for using newly identified regulators as expression cassettes. Ibarra and colleagues (2008) defined a consensus DNA binding domain based on alignment of all experimentally characterized AraC-XylS proteins, and by using this domain, almost 2000 putative AraC-XylS protein sequences were discovered in 212 bacterial genomes. The domain was then used as a template to generate a phylogenetic tree and as a tool to predict the putative regulatory role of new members of this family based on their sequence proximity to a particular functional cluster in the tree. By this approach, functional regulatory roles of about 75% of all identified AraC-XylS regulators were assigned (Ibarra *et al.*, 2008). It should however be stated that the accuracy of these predictions remains, to our knowledge, yet to be experimentally verified.

An interesting structure-based drug design strategy was described to identify chemical ligands that could bind to and inactivate AraC-XylS regulators (Bowser *et al.*, 2007). In principle, a similar approach could be used to identify inducers for a given AraC-XylS type of activator as long as a candidate cognate promoter is available. By placing a suitable reporter under control of this promoter, high-throughput screening of thousands of relevant chemical compounds can be done in a laboratory, eventually leading to the identification of useful effector compounds.

Acknowledgements

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