Expanding the Boolean logic of the prokaryotic transcription factor XylR by functionalization of permissive sites with a protease-target sequence

by

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ABSTRACT

The $\sigma^{54}$-dependent prokaryotic regulator XylR implements a one-input / one-output actuator that transduces the presence of the aromatic effector $m$-xylene into transcriptional activation of the cognate promoter $Pu$. Such a signal conversion involves the effector-mediated release of the intramolecular repression of the N-terminal A domain on the central C module of XylR. On this background, we set out to endow this regulator with additional signal-sensing capabilities by inserting a target site of the viral protease Nla in permissive protein locations that once cleaved in vivo could either terminate XylR activity or generate an effector-independent, constitutive transcription factor. To find optimal protein positions to this end we saturated the $xylR$ gene DNA with a synthetic transposable element designed for randomly delivering in-frame polypeptides throughout the sequence of any given protein. This Tn5-based system supplies the target gene with insertions of a selectable marker that can later be excised leaving behind the desired (poly) peptides grafted into the protein structure. Implementation of such knock-in/leave behind (KILB) method to XylR was instrumental to produce a number of variants of this TF that could compute in vivo two inputs ($m$-xylene and protease) into a single output following a logic that was dependent on the site of the insertion of the Nla target sequence in the TF. Such Nla-sensitive XylR specimens afforded the design of novel regulatory nodes that entered protease expression as one of signals recognized in vivo for controlling $Pu$. This approach is bound to facilitate the functionalization of TFs and other proteins with new traits, especially when their forward engineering is made difficult by, for example, the absence of structural data.

INTRODUCTION

Promoters are the basic molecular devices that translate given physicochemical signals into decision to start transcription of specific DNA sequences into mRNA. Regulation of this process in bacteria is typically mediated by transcriptional factors that either trigger (activators) or inhibit (repressors) the action of RNA polymerase on DNA motifs that are bound on the basis of the sigma factor included in the enzyme. The many possibilities of interplay between different TFs, the RNAP, and the target DNA originate a considerable plasticity both in terms of the input/output logic of the regulatory nodes at stake and its kinetic properties. Both the logic structure and the parameters embodied in each singular
promoter often appear connected to other regulatory devices of the kind to form complex genetic
networks which ultimately rule the lifestyle of the bacteria that host them.

Virtually all known prokaryotic promoters can be described with Boolean formalisms under which each
regulatory event results from the action of one or more binary gates which compute up to two inputs into
a single output with a pre-fixed logic. Similarity of such logic circuits to electronic networks has
stimulated the design of gates artificially assembled with prokaryotic regulatory parts that can process
specific signals and can be combined with others for implementing simple computations. The repertoire
of such regulatory devices is typically limited to existing TFs and cognate promoters. The latter can be
easily engineered to contain binding sites in positions which make transcriptional output to follow
different outcomes depending on the signal-responsive properties of the transcription factors employed
in the design. Interestingly, most prokaryotic promoters compute signals on the mere basis of binding
(or lack of it) of cognate TFs to DNA. In contrast, extant TFs do not perform any binary computation by
themselves, but they simply transduce one signal (e.g. effector binding) into another (e.g. a
conformational change) that may result in productive attachment to the target promoter. Activators thus
intrinsically implement a YES gate while repressors execute a NOT gate. Dependency of such activities
on small effector molecules allows their connection for the sake of growingly complex gates and circuits.
Yet, the question at stake is whether one could artificially make single TFs not just to transduce single
signals but to compute two inputs with a predetermined logic – thus converting the TF itself (and not its
binding to DNA) in the executor of the desired logic operation. But what TF or TF family could be
optimal to this end? In this work, we advocate prokaryotic activators that depend on the alternative
sigma factor $\sigma^{54}$ as the platform of choice for artificially endowing new-to-nature possibilities to the
logic of bacterial promoters.

TFs that act in concert with $\sigma^{54}$ (also known as prokaryotic enhancer-binding proteins or NtrC-type
regulators) have a distinct modular structure that includes an amino-terminal, signal-reception region (A
domain), the hinge B domain which places the A domain in a position that allows or not transcriptional
activation, the central C domain responsible for binding and hydrolysis of the ATP and interactions with
$\sigma^{54}$ and the C-terminal D domain, which binds DNA. In a group of such TFs, the A domain represses
the ATPase activity of the TF in the absence of the activating signal (typically a small effector molecule).
TFs of this type are involved in different physiological processes, e.g. metabolism of aromatic
compounds (XylR, DmpR, HbpR, TbuT and PhhR), formate metabolism (FhlA), nitrogen fixation (NifA),
acetoin catabolism (AcoR), transport systems (DctD), and others. In the case of the XylR regulator of
the TOL pathway of Pseudomonas putida mt-2\textsuperscript{10,11}, the A domain interacts directly with the aromatic
effector m-xylene, an event that results in the release of the intramolecular repression (or anti-
activation) caused by the A domain itself on the rest of the protein. As a consequence, XylR variants
deleted of the A module (XylR\textsuperscript{ΔA}) are constitutively active\textsuperscript{12,13}. XylR plus m-xylene (or XylR\textsuperscript{ΔA}) then
activates the target $\sigma^{54}$-promoter $Pu$ in concert with a number of DNA binding proteins that endows the
regulatory node with a complex logic\textsuperscript{14}. However, XylR acts in this system only as a mere one-input/
one-output actuator that translates the presence of m-xylene into a protein form able to activate
transcription. Inspection of the XylR domain structure and its activation mechanism (Fig. 1) suggested
that it would be possible to produce TF variants with an expanded logic repertoire if the protein could be
conditionally cleaved in a fashion that either destroyed its activity altogether or deleted the A domain
and originated an effector-independent, constitutively active regulator.

The results below describe the design and implementation of a new molecular tool for functionalization
of target proteins (e.g. XylR) with novel properties brought about by insertion of purposeful polypeptides
at otherwise permissive sites of its primary sequence. The tool is based on the \textit{in vitro} saturation of the
TF-coding DNA with a synthetic transposon that, after insertion and selection, can be excised leaving
behind an in-frame functional sequence of choice (for example, a specific protease cleaving site), which
can be tested for permissiveness \textit{in vivo}. Application of this tool to XylR originated TF variants that
responded either positively or negatively to expression of such protease, which could then be entered
as one of the inputs of the system in live cells. The resulting TFs implemented by themselves a suite of
non-natural logic actions that have no precedents in extant prokaryotic regulators and thus expand the
repertoire of prokaryotic devices available for engineering logic circuits. Since XylR originates in a
system for catabolism of m-xylene, its functionalized variants have an especial value for programming
bacteria aimed at bioremediation of environmental pollutants.

RESULTS AND DISCUSSION

\textbf{Rationale for creating logic gates based on XylR.} The domain structure and the mechanism of action
of XylR on its cognate promoters $Pu$ and $Pr$ of the TOL plasmid pWW0 of \textit{P. putida} mt-2 are sketched in
Fig. 1. Three features of the process are worth considering for the sake of this work. First, unlike most prokaryotic TFs, this regulatory protein is clearly composed of 3 distinct domains: the N-terminus module, which interacts directly with the aromatic effector m-xylene (or some structural analogues), the central C domain contacts and activates the sigma factor $\sigma^{54}$ of RNAP for recognition and eventual formation of an open complex at the -12/-24 DNA motif that is typical of this type of promoters, and the C-terminal helix-turn-helix part (D domain) for binding upstream sequences$^{12,15}$. The A and C domains are connected by a small hinge B sequence. XylR is thus a complete actuator that transforms an input signal (m-xylene) into eventual motion of the RNAP. The other components necessary for transcription initiation (promoter DNA, ATP, IHF, and additional nucleoid-associated proteins) can be considered not to vary and thus can be abstracted with a default value$^{16}$. The second unique feature of XylR and other TFs of its class is that the mechanism of activation by m-xylene involves the release of an intramolecular occlusion exerted by the effector-binding A domain on the C domain$^{12,13}$. This makes deletion of the N-terminus of XylR to produce an effector-independent constitutive variant, which – for the sake of Pu activation is equivalent to the wild-type protein in the presence of m-xylene. Finally, XylR can also act as a repressor of its own synthesis, because it binds also sequences of the TOL plasmid that overlap the $\sigma^{70}$ promoter PR for transcription of the xylR gene$^{17}$. The logic structure of such a regulatory device of the TOL plasmid is shown in Fig. 1. Perusal of the primary sequence of XylR immediately suggested that it would be possible to enter an additional input to the system by inserting specific protease-cutting sites at strategically located spots of the protein structure, provided that they did not alter TF activity in the absence of cleavage. While many locations could be predicted to terminate XylR function upon proteolysis, those able to excise the A domain from the rest of the protein could in fact activate this TF with a different mechanism than that caused by exposure to m-xylene. These scenarios open the possibility of having the same TF responding to two entirely independent inputs (m-xylene and protease) and the output to have an opposite sign reliant on the site of the XylR structure subject to cleavage. This would expand considerably the number of logic gates that could be derived from XylR-targeted promoters and similar $\sigma^{54}$-dependent TFs. Yet, the technical bottleneck for this endeavor is the identification of such permissive sites for implantation of a functional target for a specific protease within protein structure. The sections below describe the design of a synthetic tool tailored precisely to this end and its application to generate XylR variants endowed with the desired signal-processing capacities.
Genetic grafting of protease-cleaving sites through the XylR structure. Since the permissiveness of protein structures to insertions of extra amino acid sequences is often difficult to predict upfront, we set out to develop a general molecular tool for searching such sites in any protein of interest to be grafted with any other functional polypeptide. To address this, we exploited the known mechanism of transposition of Tn5 for designing a high-efficiency mobile DNA segment that could first be delivered to the target DNA, selected for insertions and then excised to leave behind the grafted sequence. The organization of the synthetic mobile element engineered to this end, which we have termed mTn5 [GFP•Niα1], is sketched in Fig. 2. A detailed description of its functional parts and its performance in vivo and in vitro can be found in the Supporting Information. Once the method for in vitro transposition of mTn5 [GFP•Niα1] into any target sequence was in place we carried on to generate a large library of insertions of this element through the xylR gene born by plasmid pBCL4. This was then followed by excision of much of the transposon length to leave behind a sequence scar encoding the short amino acid sequence cleaveable by the viral protease Niα. The workflow for generating such knock-in-leave-behind (KILB) libraries is sketched in Fig. 3. The transposition reaction is predicted to introduce the mobile element throughout the whole plasmid i.e. both inside and outside the xylR sequence. Predictably, digestion of the transposition mix with enzymes BamHI and XbaI generated four restriction bands, which could be easily separated by means of electrophoresis in agarose gels (Supplementary Fig. S1). The product of the size of xylR plus one mTn5 [GFP•Niα1] insertion (3541 bp) was recovered and re-cloned in the same sites of the pUC18-SbfI plasmid pre-digested with BamHI and XbaI). This simple procedure allowed the recovery of the inserted xylR sequences only, as it discards transposition events occurring in vitro beyond the sequence of interest in the pBCL4 plasmid. The ligation pool was then transformed in E. coli, followed by selection on media with Ap R Km R. The whole of transformants were pooled again and the total plasmid contents extracted from the mixed population. The plasmidic material was then digested with either NotI or SbfI and the digestion products re-ligated. Owing to the design of the synthetic transposon (Fig. 2), such an excision of the internal NotI or SbfI segments of mTn5 [GFP•Niα1] followed by religation leaves xylR DNA with in-frame fit-in insertions of either GFP or the Niα target polypeptide, respectively. One out of 6 of these inserts was predicted to create sandwiched gene fusions between xylR and either GFP or the proteolyzable peptide. If the sites of start and end of such grafted polypeptides in XylR happen to be structurally permissive we would then expect to have this TF artificially added in its structure with a new trait i.e. either fluorescence (because of the
sandwiched GFP) or sensitivity to the Nla protease (due to the insertion of a cognate target site). XylR variants of both types were screened for functionality by transforming each pool in E. coli CC118 Pu-lacZ. This strain has a chromosomal insertion of a reporter β-galactosidase gene to the σ^54 promoter Pu that is activated by XylR in the presence of the aromatic inducer\(^19\). We in fact obtained a number of both XylR derivatives that were fluorescent and able to activate the cognate σ^54 promoter Pu and others that were responsive to the Nla protease. The sections below, however, focus exclusively on the last category, as they are the ones that change the input/output logic of the regulator, as pursued in this work (see above).

Analysis of Nla-tagged XylR variants. The negligible level of basal transcription of the Pu promoter under non-induced conditions (i.e. without XylR or with XylR but not m-xylene) made strain E. coli CC118 Pu-lacZ a phenomenal tool for examining the effect of the genetic grafts discussed above on XylR properties. The reference conditions for such functionality tests are shown in Supplementary Fig. S2. The lawns of plasmid-less E. coli CC118 Pu-lacZ (or the same strain transformed with insert-less vectors) are colorless when spotted on LB-Xgal plates. The same is true for E. coli CC118 Pu-lacZ transformed with the reference xylR+ plasmid pBCL4, which encodes the wild-type sequence of this TF —provided that the plates are not exposed to m-xylene. Exposure to this aromatic makes the lawns of E. coli CC118 Pu-lacZ (pBCL4) to turn intense blue. These visual phenotypes match exactly the levels of β-galactosidase that can be measured in liquid cultures of the same strains, as shown in Supplementary Fig. S2. Reporter readout in this system thus faithfully describes the functionality of XylR as an m-xylene responsive TF.

Once the conditions to measure XylR activity were standardized, the KILB library of Nla-target insertions born by plasmid pBCL4 was transformed in E. coli CC118 Pu-lacZ, plated on LB-Ap\(^R\) and the resulting colonies exposed to saturating vapors of m-xylene as described in Methods. Out of a whole library of 2.7x10\(^3\) clones, approximately 45 % turned blue under such conditions, suggesting that the extra in-frame polypeptide left in the protein structure by the KILB transposon had hit permissive sites of the protein structure. DNA sequencing of a randomly picked subset of ~ 50 clones indicated that not all permissive insertions had the proper orientation and/or the correct reading frame to generate productive Nla recognition sites within XylR. Finally, only four xylR clones inserted with Nla-sites were selected for further phenotypic analyses. Three of these Nla-site insertions were found at various places of the N-
terminal signal reception A module of the XylR protein (M75, G154 and D210) whereas a fourth one (E499) was located in the short linker that connects the central activation module C of the protein and the DNA-binding D domain. As shown in Fig. 4, insertions M75 and G154 were competent for induction of the Pu-lacZ fusion of the host, but originated lower β-galactosidase levels than the wild-type XylR when exposed to m-xylene. In contrast the Nla-target insertion at the very end of the A domain (D210) fashioned a XylR variant with a higher activity when induced with the same aromatic effector. A similar result was obtained with the Nla-targeted E499 XylR variant, which displayed a significantly higher Pu output when exposed to the protease in vivo (Fig. 4).

The wild type-like behavior of insertions D210 and E499 did however change when the host reporter strain was made to express the Nla protease by means of plasmids encoding the cognate PPV gene. In the first case, insertion of the Nla recognition site at the end of the A domain of XylR (D210) led to Pu induction irrespective of the presence or the absence of the XylR inducer (m-xylene) when it was expressed along with the protease. This phenotype is consistent with that expected of a XylRΔA protein, as previously described. That XylR\textsuperscript{D210} was cleaved by Nla in vivo could be visualized by means of a Western blot assay of protein extracts of the corresponding cells (Fig. 4b, lanes 7 and 8). Note that antibodies used to detect XylR were raised against the XylRΔA protein\textsuperscript{20} and therefore they do not recognize the A domain. Results equivalent to those of Fig. 4b were obtained when the Western blot test was made in the presence of m-xylene, i.e. the Nla protease appeared to proteolyze the XylR variants under examination with the same efficiency. These data thus accredited that XylR\textsuperscript{D210} can be converted into a TF form able to activate Pu by either exposure to m-xylene or by expression of the Nla protease or by both. This notion was further verified by reconstructing a XylR variant which had been deleted exactly of the same portion of the A domain that is predicted to be lost upon cleavage of XylR\textsuperscript{D210} with Nla (see below).

A quite different behavior was found in the XylR variant inserted with a Nla site in position E499. In that case, expression of the protease translated in a virtually inactive TF regardless of whether m-xylene was present in the medium (Fig. 4). Western blots of the protein extracts as before confirmed that Nla indeed cleaved XylR\textsuperscript{E499} in vivo (Fig. 4b, lines 9 and 10). Since such a cleavage must result in the deletion of the DNA binding domain of XylR, it makes sense that the TF factor loses activity altogether. This last experiment also provided a sidelight in the mechanism of activation of Pu by XylR, since it
makes clear that at least part of the D domain of the protein is essential not only for DNA binding but also for maintaining a form of the protein able to activate transcription from solution\textsuperscript{19, 21, 22}. Finally, Nla target insertions at sites M75 and G154 resulted in XylR variants that could be cleaved \textit{in vivo} as well (Fig. 4b, lines 3 to 6) but such site-specific proteolysis changed little the corresponding phenotypes regarding \textit{Pu} induction. It is possible that such variants that were identified in the first visual screening (see above) are in fact defective or only transiently active TFs.

\textbf{The novel Boolean logic of XylR\textsuperscript{D210} and XylR\textsuperscript{E499}.} As shown in Fig. 5a, insertion of Nla target sequences in D210 and E499 sites of XylR endowed this TF with the capacity to compute two signals (\textit{m}-xylene and protease) instead of the one-input/one-output observed in the naturally occurring regulator. In one case, XylR\textsuperscript{D210} brings about strong activation of the \textit{Pu} promoter whether cells are exposed to the aromatic inducer, to Nla or both. This state of affairs can be formalized as a Boolean OR gate (Fig. 5b). It is noteworthy that promoter activity caused by cleavage of XylR\textsuperscript{D210} is noticeably higher than that of \textit{m}-xylene and that the first overrides the second when the two are entered together (e.g., compare \(\beta\)-galactosidase levels of cognate assays in Fig. 4c). This makes sense in view of the mechanism of activation of XylR by aromatic inducers\textsuperscript{12}: the loss of the A domain leaves the TF unhindered for interacting with the \(\sigma^{54}\)-dependent transcription machinery. A different logic gate was created by the insertion of a Nla site in XylR\textsuperscript{E499}. In this instance, expression of the protease abolishes activation of the TF by \textit{m}-xylene (Fig. 5). For \textit{Pu} to be transcribed cells thus need to face the aromatic effector but must not be exposed to any proteolysis caused by Nla. The logic is therefore that of a Boolean AND gate\textsuperscript{1} in which one specific input must be present and the other absent to have a positive outcome of the computation. Note, however, that in the case discussed here, the inputs are not equivalent and their order of appearance makes a difference. In any case, the above manipulations of XylR expand the logic repertoire of this TF to additional signals that can result in either positive or negative outcomes.

\textbf{Pu promoter anti-activation: engineering a cleavable variant of XylR\textsuperscript{∆A}.} The inhibitory action of Nla on XylR\textsuperscript{E499} raised still one more possibility to develop a different logic gate based on this TF. Since the \textit{in vivo} deletion of the D domain leads to an entirely inactive regulator (Fig. 4), we wondered whether introducing directly the Nla site in the constitutively active protein XylR\textsuperscript{∆A} could reverse the action of this TF on \textit{Pu} upon expression on the protease \textit{in vivo}. To examine this possibility we produced a series
of XylR∆A variants that carry various sequences at their N and C termini as shown in Fig. 6 (see details on the protein ends in Supplementary Fig. S3). The collection included as controls the original XylR∆A2 protein of reference\textsuperscript{12,13} named SP1 in Fig. 6a) and a faithful reconstruction of the truncated product that is predicted to be released upon cleavage of XylR\textsuperscript{D210} with Nla (SP3 in Fig. 6). Each of these was then engineered with protease-cutting sites at position E499, originating cleavable protein variants SP2 and SP4 respectively (both designated as XylR∆A*). Finally, we recreated the polypeptide that could result from excision of the XylR protein at both D210 and E499 sites, which encompasses the whole C domain of the TF. Plasmids encoding each of these XylR variants were passed to \textit{E. coli} Pu-lacZ strains expressing or not Nla and the production of the regulator examined in each condition. As shown in Supplementary Fig. S3, control variants SP1, SP3 and SP5 were not affected by Nla, while SP2 and SP4 were cleaved as expected. When the same strains were patched on Xgal plates, the change of color of variants SP2 and SP4 in the cells producing Nla became evident. These visual phenotypes are consistently reflected in the actual levels of the reporter product displayed by each of the constructs with and without protease as shown in Fig. 6c. The most dramatic change was delivered by the SP4 variants, which passed from a high β-galactosidase level in the absence of protease (~2000 Miller units) to virtually undetectable in the strain that expressed Nla from plasmid pPPV1. Note that unlike full-length XylR, the default action of XylR∆A is activation of \textit{Pu} in the absence of any effector (a YES gate, Fig. 6d) and the effect of the protease is to defeat this event. The consequence of Nla expression is therefore to revert activation and thus suppress \textit{Pu} activity. If expression of the cleavable XylR∆A variant is given a digital value of 1 then proteolysis can be formalized as an inverter in which Nla is the sole input. But if expression of XylR∆A* is also variable, then the resulting regulatory device becomes an ANDN gate with both Nla and the engineered TF as inputs (Fig. 6d). To the best of our knowledge, this is the first case of either a naturally occurring or an engineered biological inverter that is implemented through an anti-activation mechanism. Although the logic of such NOT device is the same than that brought about by a repressor\textsuperscript{1}, the biological basis of the inversion is entirely different, what will surely be reflected in the parameters that govern the process \textit{in vivo}. While such parameterization of this and the other regulatory devices described above will be the subject of future work, we expect these new gates based on XylR to enrich the choices available for construction of complex genetic and metabolic circuitry.
Conclusion. The application of Boolean logic to a large number of biological phenomena has allowed both formalization of intricate occurrences in live systems and the engineering of genetic and metabolic devices for programming new-to-nature properties. The biological parts available for such engineering include transcriptional factors and cognate promoters, recombinases, metabolic reactions, small molecules, single cells, and even multicellular networks. The modularity of logic gates allows the buildup of a degree of multi-scale complexity that is limited only by the biological compatibility of the corresponding inputs and outputs. On this basis, contemporary Synthetic Biology claims a similarity between genetic networks and electronic circuits that include not only discrete decision-making modules, but also whole operating systems. Logic devices based on regulatory parts are typically implemented by combinations of transcriptional factors and small molecules that act as inputs in given promoters. DNA binding (or not) is, mechanistically, the event that mediates the corresponding computation. We show above that one family of prokaryotic TFs that act in concert with the $\sigma^{54}$-containing form of RNAP can be functionalized with protease-cleaving sites in a fashion that makes the TF itself—not its binding to DNA, the performer of the binary computation. Prokaryotic TFs that process two equally effective inputs are thus far unknown in the transcription literature. Some regulators may use intermediate metabolites as allosteric effectors but their effects are mild compared to the drastic change in $Pu$ promoter output caused by the XylR variants described above. Moreover, we have not overlooked that the genetic tools described above for implementing the KILB insertion saturation procedure (transposons mTn5 [GFP•Nla1], mTn5 [GFP•Nla2] and mTn5 [GFP•Nla3]) can be tailored à la carte for grafting functional sequences in permissive sites of virtually any other protein of interest. While the random insertion approach for sandwiching foreign polypeptides in existing proteins is not without precedents, the work reported here is the first time that the concept is applied to transcriptional factors with a view on changing its regulatory behavior. In this respect, although the data presented in this paper deal only with the ability of XylR to activate $Pu$, Fig. 1 shows also that the same TF represses its own promoter, $P_R$. It is thus conceivable that the logic of the new gates based on XylR$^{D210}$, XylR$^{E499}$ and XylRΔA* (Fig. 5b and Fig. 6d) is reverted when the target promoter is $P_R$ instead of $Pu$. Alas, the degree of repression of $P_R$ by XylR is not strong enough to grant a performance as stringent as the one observed with $Pu^{17}$. Still the binding of XylR to $P_R$ can be artificially improved, an issue that is currently under investigation. In sum, we argue the value of combining $\sigma^{54}$-dependent TFs, cognate promoters, small molecules and proteases as a way of...
increasing the toolbox of logic devices that are necessary to build genetic and metabolic circuits of growing complexity e.g. for *in situ* biodegradation of toxic pollutants.

**METHODS**

**Strains, plasmids, media and growth conditions.** The relevant properties of the strains and constructions used in this work are listed in Supplementary Table S1. *E. coli* DH10B, DH5α and CC118 strains were used for general procedures. The reporter strain *E. coli* CC118 Pu-lacZ was used for assessing XylR activity. Bacteria were grown routinely at 37 °C in LB (10 g l⁻¹ of tryptone, 5 g l⁻¹ of yeast extract and 5 g l⁻¹ of NaCl). When required, ampicillin (Ap, 150 µg/ml), kanamycin (Km, 75 µg/ml) or chloramphenicol (Cm, 30 µg/ml) was added to the culture media. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added where indicated to a final concentration of 0.1 mM. The Pu-lacZ fusion was induced by exposing cells either on plates or in liquid cultures to saturating vapors of m-xylene. When required, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) was added at 40 µg ml⁻¹ for visualization of β-galactosidase activity.

**DNA constructs.** General methods for DNA manipulation were performed as described. Oligonucleotides used in polymerase chain reaction experiments (PCR) are listed in Supplementary Table S2. Construction of a transposition target plasmid encoding xylR gene, involved two steps. First, the single SbfI site of pUC18 was eliminated by digestion with PstI followed by T4 DNA polymerase treatment and religation, resulting in vector pUC18-SbfI. Next, the DNA sequence of the xylR gene was amplified from strain *P. putida* mt-2 with oligos xylR-BamHI (containing an optimal RBS and a BamHI restriction site) and xylR-XbaI (which adds an XbaI site). The resulting fragment was cloned into a pGEM-T (Promega), excised with BamHI and XbaI and ligated into the corresponding sites of pUC18-SbfI. This produced plasmid pBCL4, which was subsequently used as the target DNA in transposition experiments. The DNA segments that compose the KILB transposon used in this work were synthesized (Life Technologies, Regensburg, Germany) and combined with a Km resistance gene amplified from plasmid pBAM1 cassette with primers Km-Swal-Fan dkM-PshAI-R, which generate terminal Swal and PshAI sites. The resulting segment, assembled in plasmid pGA-BCL1 (Supplementary Table S1) bears the mini-Tn5 transposon named mTn5 [GFP•Nla1], the structure of which is drawn in Fig. 2a. For *in vivo* transposition experiments, the DNA spanning the whole mobile
element was cloned as a PvuII fragment in the corresponding sites of plasmid pBAM1, thereby originating pBAM1-GFP (GenBank HQ908072). Two more versions of the same transposon were constructed -bearing either Ascl or Pmel restriction sites in lieu of the SbfI sequences, thereby generating mTn5 [GFP•Nla2] and mTn5 [GFP•Nla3]. Details of their DNA assembly steps are available upon request. Plasmids expressing different XylRΔA truncated variants were constructed as follows.

DNA segments encoding SP1 and SP2 -both deleted of their N-terminal domains as described for XylRΔA213 were amplified with primers DeltaA2F and M13 (-40) universal-F from plasmids pBCL4 (wt xylR gene) and pBCL4-E499 (xylR*E499 variant), respectively. The resulting DNAs were then digested with BamHI and Xbal and cloned into the corresponding sites of pUC18, giving rise to pBCL4-SP1 and pBCL4-SP2. Other XylRΔA variants were made with an N-terminus that mimics the result of the cleavage of XylR*D210 with the Nla protease. For SP3, the insert of plasmid pBCL4-D210 (encoding the xylR*D210 variant obtained by KILB) was amplified with primers D210F and M13 (-40) universal-F, the resulting DNA digested with BamHI and Xbal and ligated into the corresponding sites of pUC18, raising pBCL4-SP3. In the case of SP4 and SP5, two PCR reactions were run in each case to obtain separate 5’ and 3’ ends in each case, followed by a second overlapping reaction using products from the first PCR as templates. The 5´ region, which was common to both SP4 and SP5 was amplified from pBCL4-D210 with primers D210F and XylR-Sol.R. The 3´ portions were obtained by PCR of pBCL4-E499 (encoding the xylR*E499 variant obtained by KILB) with primers XylR-Sol.F and M13 (-40) universal-F -in the case of SP4 and XylR-Sol.F and E499stop-R in the case of SP5. Equivalent amounts of the 5´ DNA fragment together with each of the 3´ segments were used as templates for a second PCR reaction with primers D20F1 and M13 (-40) universal-F for full-length amplification of SP4 and D210F1 and E499stop-R for the same in SP5. The DNAs resulting from this reaction were then digested with BamHI and Xbal and ligated into the corresponding sites of pUC18, thereby originating pBCL4-SP4 and pBCL4-SP5.

**In vitro transposition and construction of knock-in-leave-behind (KILB) insertion libraries.** A hyperactive variant of the Tn5 transposase was purified from plasmid pGRTYB35 (kindly provided by W.S. Reznikoff) as described40. The donor DNA segment spanning the mTn5 [GFP•Nla1] transposon was amplified from pGA-BCL1 with primers Tn5ME-F and Tn5ME-R. The amplified fragment was then gel purified with NucleoSpin Extract II kit (Macherey-Nagel), and kept until use. In vitro transposition experiments were set up as described41. The reactions were assembled in a volume of 10 µl of
transposition buffer containing 0.1 µM purified transposase (0.1) and an equimolar amount of transposon and target DNA (ratio transposase:transposon:target DNA = 5:1:1). Reactions were incubated at 37°C for two hours and then halted with 1 ml of stop solution (1% SDS), mixed and heated at 70°C for 10 minutes. Next, the mixtures were dialyzed against MilliQ water and electroporated into E. coli DH10B. The transformation mixture was then plated on LB Km (75 µg/ml) to select cells with plasmids that had acquired the mTn5 [GFP•Nla1] transposon (Fig. 3). The efficiency of the transposition reaction was measured as CFUs per pMol of mTn5 [GFP•Nla1] DNA. Next, the KmR clones were pooled, the whole plasmid DNA extracted and digested with BamHI and XbaI. This generated four restriction products that were separated with electrophoresis in agarose gels (Supplementary Fig. S1a).

The band corresponding to the xylR gene with transposon insertions was recovered, re-cloned in pUC18-SbfI and retransformed in E. coli DH10B. Clones were pooled again, plasmid DNA extracted and separately digested with either NotI or SbfI and then religated (Supplementary Fig. S1b). As explained in Fig. 2, NotI digestion/religation creates in-frame sandwich GFP fusions, while the same with SbfI leaves the target gene sequence densely punctuated with in-frame insertions of the Nla protease target peptide (plus adjacent sequences inherited from the Tn5 ends, Fig. 2). The corresponding plasmid pool was recovered and transformed in reporter strain E. coli CC118 Pu-lacZ for XylR activity assays as explained next.

**Monitoring promoter activity in vivo.** The ability of XylR and its variants to activate transcription from the σ54 promoter Pu was measured by quantifying the β−galactosidase accumulation driven by a Pu-lacZ fusion engineered in the chromosome of E. coli CC11818. This reporter strain was transformed with the plasmids encoding xylR variants described above along, where indicated with plasmid pPPV142 or pPPVs2043 encoding the Nla protease. For the assays, cultures were pregrown overnight at 37°C in LB medium with appropriate antibiotics, then diluted in fresh medium to an OD600 = 0.1 and grown with vigorous shaking up to mid-exponential phase (OD600 = 0.4-0.5). At that point flasks were added 0.1 mM IPTG and the incubation continued up to OD600 ~ 1.0. Cultures under scrutiny were then exposed to saturating vapors of the XylR effector (m-xylene) in airtight flasks and incubated further for 3 hours. β-galactosidase levels were then determined in cells permeabilized with chloroform and SDS as described by44. The results shown represent a minimum of 3 experiments per each condition.
Western blot analyses of XylR expression. The performance of the Nla protease to cleave XylR variants \textit{in vivo} was diagnosed in bacteria from the cultures grown as described in the previous section. To this end, cells recovered by centrifugation were directly disrupted by boiling them for 7 min in a denaturing sample buffer containing 2% SDS and 5% β-mercaptoethanol. Samples were then run through 10% SDS-PAGE gels. Purified full-length \textit{6xhis}XylR and XylRΔA proteins kindly provided by C.A. Carreño and\textsuperscript{17}, respectively, were used as controls. Polyacrylamide gels were subsequently blotted onto a polyvinylidene difluoride (PVDF) Immobilon-P membrane (Millipore) and probed with 1:2000 dilutions of an anti-XylR recombinant phage antibody PhaB B\textsuperscript{20}. XylR bands were detected with anti-M13 peroxidase conjugates as described and their location revealed by reaction with BM Chemiluminiscence Blotting Substrate (POD) from Roche (Mannheim, Germany).

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Author Contributions: BC performed experiments and drafted the manuscript. VdL directed the project and wrote the paper.

Notes: The authors declare no conflict of interest

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SUPPORTING INFORMATION AVAILABLE

This information is available free of charge via the Internet at http://pubs.acs.org/.

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**Figure 1.** Functional organization and mode of action of the *m*-xylene responsive $\sigma^{54}$-dependent regulator XylR.

**Figure 1:**

(a) Functional domains of XylR. The organization of the modules that compose this TF is shown with indication of the amino acid residues that define the limits between the functional domains and the localization of the relevant functions within the protein sequence: A (signal reception and inducer binding), B (interdomain linker region), C (binding and hydrolysis of ATP and contacts with the $\sigma^{54}$-dependent RNA polymerase (R), and D (HTH motif).

(b) Model of XylR activation. The A domain interacts with the inducer (open circle) to recruit the B domain, which then binds to the promoter region (Pu) to activate transcription. The C domain interacts with the IHF protein to further enhance transcription.

(c) Schematic representation of the XylR binding to the promoter region. The UASd and UASp elements are shown, with the IHF protein binding to the -12/-24 region.

(d) Diagram of the promoter region showing the transcription start site (+1) and the position of the $P_{R1}$ and $P_{R2}$ promoters.

*Pu* indicates the presence of the promoter region, and PoPS represents the production of PoPS.
RNAP) and D (including a helix turn helix motif, for binding to the UAS of the target promoter DNA). (b) Activation of XylR by m-xylene. The drawing sketches how the TF folds such that the N-terminal A domain hinders an activation surface of the regulator. Effector binding to the A domain releases such a intramolecular repression and XylR becomes then competent for interacting with the $\sigma^{54}$-RNAP bound further downstream in $Pu$ and activating transcription. The same XylR surface can be presented to the $\sigma^{54}$-RNAP by deleting the whole A domain, thereby creating a effector-independent and constitutively active variant XylR$\Delta$A. (c) The $Pu$ promoter region. The DNA segment of interest is expanded, showing the location of relevant sequences, including distal and proximal upstream binding sites for the XylR oligomer (UASd and UASp, respectively), the -12/-24 motif recognized by $\sigma^{54}$-RNAP, and one integration host factor (IHF) binding site located within the intervening region. The logic of such an arrangement is an AND gate (inputs m-xylene and XylR) followed by a YES operator. If XylR has a default value of 1, then the regulatory node becomes a factual YES gate with m-xylene as input and transcription initiation as output (polymerase per second or PoPS). (d) The $P_R$ promoter region. XylR auto-regulates activity of this $\sigma^{70}$-promoter which includes two overlapping initiation sites ($P_{R1}$ and $P_{R2}$). $P_R$ is repressed by XylR because the UAS of a second divergent $\sigma^{70}$ promoter ($Ps$) overlap the two -10/-35 sequences that drive divergent transcription of the xylR gene. The logic is thus the opposite of that of $Pu$: an AND gate followed by an inverter. As before, if XylR is present throughout, the node becomes a NOT gate with m-xylene as input and PoPS as output.
**Figure 2.** Design and properties of synthetic transposon mTn5 [GFP•Nia1].

(a) Physical and functional organization. This mobile element is composed by an array of DNA segments which are bracketed by the so-called Tn5 mosaic ends (ME), i.e. 19 terminal inverted repeats, optimized for hyperactive transposition and both concluding in half PvuII sites. The sequences (left to right in the sketch) between the two MEs ends include [i] a *gfp* gene (GFP) devoid of start and stop codons and bound by restriction sites for the 8-bp cutters SbfI and NotI, [ii] a kanamycin resistance cassette (KmR) flanked by unique restriction sites SwaI and PshAI (not shown) plus another SbfI and [iii] a 39 pb sequence encoding the peptide that is specifically recognized by the viral Nia protease followed...
by one more NotI site. Note the correlation between the two alternate SbfI and Not sites. (b) Generation of sandwiched in-frame GFP fusions. Digestion/religation of the transposon-inserted DNA with NotI deletes the Km resistance gene and the Nla target sequence, thereby generating a fusion with both the 5' and the 3' ends of the \textit{gfp} sequence, the boundaries of which are blown up in the sketch. (c) Knocking-in target peptides for the Nla protease. Digestion/religation of the same transposon-inserted DNA with SbfI excises the internal GFP/Km segment of mTn5 [GFP•Nla1] and leaves behind an in-frame addition of the extended amino acid sequence recognized by Nla (in yellow).
Figure 3. Generation of knock-in-leave-behind (KILB) libraries.

(a) In vitro mutagenesis. The target gene is cloned in a plasmid as a BamHI-XbaI insert (in this example, sequences corresponding to the functional domains of xylR are indicated) and the DNA is used as the substrate of an in vitro mutagenesis reaction with mTn5 [GFP•Nla1] as detailed in the Materials and Methods section. (b) Recovery of inserted target sequences. The products of the transposition reaction are transformed in E. coli, KmR clones selected, pooled, their plasmids extracted...
and the DNA digested with BamHI and XbaI, what allows recovery of a pool of DNA segments with the

*xy/R* gene inserted randomly with mTn5 [GFP•Nla1]. This pool (see Supplementary Fig. S1) is then
recloned in the BamHI / XbaI sites of the same vector, so that only inserts in the gene of interest are
retained. **(c)** Generation of in-frame gene fusions. The ligation mixture is re-transformed and processed
in *E. coli* as before (Supplementary Fig. S1b) and the plasmid pool digested and religated with either
NotI (thereby creating in-frame sandwich GFP fusions) or with SbfI, that leaves a sequence scar that
can be cleaved by the Nla protease. The successful production of such knocked-in protein variants can
then be tested by transforming the plasmid pool in either plain *E. coli* CC118 and examining the plates
with blue light (for GFP expression) or the reporter strain *E. coli* CC118 *Pu-lacZ*, the colonies of which
turn blue upon exposure to vapors of the XylR effector *m*-xylene.
**Figure 4.** XylR variants knocked-in with Nla protease target sites.

(a) Insertion points of the Nla tag through the protein sequence. The modular organization of XylR is sketched with indication of the permissive locations where the peptide containing the Nla cleavage sites was delivered by the KILB procedure. (b) Expression and sensitivity to Nla protease of XylR variants in vivo. Equivalent amounts of crude protein extracts from *E. coli* cultures expressing the XylR types indicated along with Nla (or without protease, as specified) were run in a denaturing gel, blotted and
developed with anti-XylRΔA antibodies. (c) Quantification of the activity of Nla-cleavable XylR variants. Cultures of *E. coli* CC118 *Pu-lacZ* strain with plasmids encoding each of the XylR types and the Nla protease were grown and exposed to *m*-xylene as explained in the Materials and Methods section. The diagram plots the accumulation of β-galactosidase after 3 hours of induction with or without the protease as indicated.
Figure 5. The logic of protease-cleavable XylR variants.

(a) The two inputs of proteolyzable XylR. The drawing represents how cleavage of XylR in alternative sites of the regulator’s structure is propagated into the transcriptional activity of the reporter Pu-lacZ fusion. (b) Visual display of Pu activation by Nla-cleavable XylR variants. The left part shows the growth of E. coli CC118 Pu-lacZ expressing the XylR types labeled to the side and Nla, spotted on LB plates with Xgal and exposed to saturating vapors of m-xylene as indicated. The logic gates brought about by XylR versions D210 (cleavage in position 210 of the amino acid sequence, deleting the A domain) and E499 (split by Nla in 499 and excising the D domain) are shown to the right. Wild-type XylR operates as a YES (buffer) gate with m-xylene (X) as the only input. XylR<sup>D210</sup> produces an OR gate with both m-
xylene and Nla protease (N) as inputs. Finally, XylR generates an ANDN device, where Pu activity is on only when one of the inputs is present (X) and the other is absent (N).
Figure 6. The logic or anti-activation of Pu by XylRΔA* variants.

(a) Organization of the ΔA versions of XylR in respect to the full-length protein and its Nla-cleavable forms. The upper sketch shows a reference with the sites of the two Nla cutting sites at positions D210 and E499. The synthetic ΔA proteins (SP) below are aligned in respect to such reference with indication of the amino acids that lead the N-terminus and the presence or not of an engineered E499 site. The amino acid sequence of the C-terminus of the SP5 protein variant (XylRΔAΔC) is blown up as well (see Supplementary Fig. S3 for more details on the amino acid termini of each protein). (b) E. coli CC118 Pu-lacZ expressing each of the ΔA XylR types plus minus Nla as indicated and spotted on LB plates with Xgal. (c) Quantification of the activity of ΔA XylR variants. E. coli CC118 Pu-lacZ with plasmids encoding each of the XylR types were grown and Nla expression induced with IPTG. The protease was expressed through two alternative plasmids (pPPV1 and pPPSV20, Supplementary Table S1) as indicated. The graph shows accumulation of β-galactosidase after 3 hours of induction (see expression
and cleavage of each of the XylRΔA variants in vivo in Supplementary Fig. S3). (d) Formalization of the regulatory behavior of Nla-cleavable XylRΔA variants as a digital gate. The logic of Pu activation by XylRΔA is a YES gate where the TF is the input and PoPS the output. In contrasts, SP3 and SP3 versions of the same regulator generate an ANDN device, where Pu activity is on when the ΔA protein is present and the protease is absent. If such ΔA TFs are given a default value of 1, the same device becomes an inverter in which the only input is Nla.
SUPPORTING INFORMATION TO THE ARTICLE

Expanding the Boolean logic of the prokaryotic transcription factor XylR by functionalization of permissive sites with a protease-target sequence

by

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SUPPLEMENTARY INFORMATION

Structure and modus operandi of the synthetic KILB transposon mTn5 [GFP•Nla1]

The mobile element employed in this work for the knock-in-leave-behind (KILB) procedure adopted in this work has a total size of 1774 bp and allows generation of comprehensive libraries of either in-frame, sandwiched fusions to the green fluorescent protein (GFP) or specific cleavage sites recognized by the plant viral protease Nla (García et al, 1989a). Note that such Nla target sequence (NVVVHQA) is absent from the proteome of E. coli and therefore the duo Nla protease-Nla tagging peptide can be considered an orthogonal device. The engineering of the two cargoes in the same DNA segment allows an estimation of the efficiency of the transposition process as discussed below. The salient features of mTn5 [GFP•Nla1] include the following characteristics. First, the mobile element is flanked by optimized 19 bp inverted repeats (mosaic ends or ME) that are recognized by the Tn5 transposase (Goryshin et al, 1998). Such recognition catalyzes random insertion of mTn5 [GFP•Nla1] into target DNA through a process that results of duplication of the 9 bp adjacent to the transposition site (Reznikoff, 2008). Next to the ME left sequence (ME-L), mTn5 [GFP•Nla1] contains a leaderless gfp gene variant that is optimized for prokaryotic gene fusions to GFP (Miller and Lindow, 1997). Since the gfp sequence lacks a start codon, it should only be translated when inserted within another protein coding sequence in the right orientation and reading frame. mTn5 [GFP•Nla1] insertions that fulfill these conditions can be easily identified for fluorescence emission with the naked eye under blue light. Note also that gfp gene of mTn5 [GFP•Nla1] is flanked by an upstream SbfI site and a downstream NotI site. This is followed by a kanamycin resistance (KmR) gene (aminoglycoside phosphotransferase, aphA), which is instrumental for selecting transposition events in a wide range of Gram-negative bacteria. The DNA sequence of aphA (the expression of which is driven by its own promoter) was edited to improve codon usage and to eliminate naturally occurring SmaI and HindIII sites that could interfere with subsequent cloning procedures (Martinez-Garcia et al, 2011a). Furthermore, aphA was also flanked by restriction sites that follow the Standard European Vector Architecture (SEVA) format (Silva-Rocha et al, 2013) in such a way that this module can be easily exchanged by any other available marker of the collection (e.g. bla, cat, aadA, tet, aacC1). Finally, the aphA gene born by mTn5 [GFP•Nla1] is followed by a short DNA sequence that encodes the core peptide NVVVHQA that is specifically recognized by the Nla protease of a plant potyvirus (Garcia et al, 1989b) added with three flanking residues at each side (Lain et al,
This was arranged in such a way that the Nla site was delimited by an upstream SbfI site and a downstream NotI site, next to which the mTn5 [GFP•Nla1] ends with the right ME sequence (ME-R) of the mobile element. The alternative tandem arrangement of framed SbfI and NotI sites in mTn5 [GFP•Nla1] allows later excision of much of the inserted transposon after delivery of the mobile element to the target DNA sequence. This leaves in-frame, fit-in GFP or Nla- sequences sandwiched throughout the substrate DNA as described below. Finally, note that either boundary of mTn5 [GFP•Nla1] ends with a half PvuII site within the most external ME sequences. These are intended to ease the cloning and cutting out of the mobile segment in/from the cloning vectors (Supplementary Table S1) as needed for in vivo or in vitro transposition reactions. Two variants of the GFP•Nla- containing transposon were also added to the toolbox (see Materials and Methods section in main text) in which both SbfI sites were replaced by either AscI (resulting in mTn5 [GFP•Nla2]) or by PmeI (same, mTn5 [GFP•Nla3]). The validation of these tools and their exploitation for creating conditionally proteolizable variants of XylR is explained next.

Testing mTn5 [GFP•Nla1] transposition

The transposon described above was first verified for the functionality of all parts embedded in its design. To this end, the DNA sequence spanning the whole mTn5 [GFP•Nla1] segment was assembled as a PvuII fragment in the backbone of the suicide transposon delivery plasmid pBAM1 (Martinez-Garcia et al, 2011a) that encodes both conjugal transfer functions and the Tn5 transposase protein TnpA. In vivo mobilization and transposition assays (Martinez-Garcia et al, 2011a) followed by selection of KmR exconjugants and inspection of green fluorescent colonies suggested the synthetic transposon to work at a frequency of 2.6 ± 0.1 x 10^3, creating productive translational GFP fusions at a rate of 1.17 ± 0.1 x 10^-3 (not shown). Delivering of the same mTn5 [GFP•Nla1] segment to E. coli by transformation of a preassembled transposome (Goryshin et al, 2000) increased the frequency of both events (insertion and production of chromosomal GFP fusions) by 10-fold. While these results accredited the performance of the engineered transposon, the relatively low numbers discouraged its use in vivo and advised instead adoption of an all-in vitro, high insertional density alternative. For this we set up a method with three purified components i.e. the mTn5 [GFP•Nla1], a hyperactive variant of the TnpA transposase (purified from expression plasmid pGRTYB35, see Materials and Methods) and the target DNA. The last is plasmid pBCL4, consisting of plasmid vector pUC18 deleted of the SbfI site and
carrying the *xylR* sequence as a BamHI-XbaI restriction fragment. *In vitro* reactions were optimized for maximizing the efficiency of transposon insertions. For this, the mTn5 [GFP•Nla1] element was entered in the reaction mix either as DNA fragment spanning exclusively the sequences bound by the Tn5 ends or as part of the supercoiled or else linearized pBAM1-GFP plasmid (Martinez-Garcia et al, 2011a). Similarly, the target DNA was brought into the system either as a supercoiled or a linearized plasmid (in this last case, followed by a ligation of the transposition products, see below). The efficiency of each of the combinations could be easily quantified by transforming the transposition mixture in *E. coli* and selecting for Ap^R^ Km^R^. These tests revealed that the best insertion rates were brought about by using the very mTn5 [GFP•Nla1] DNA segment as the transposon donor to the *xylR*-encoding supercoiled plasmid (5 x 10^5^ Ap^R^ Km^R^ c.f.u / pmol DNA mix), much above using pBAM1-GFP (4.3 x 10^4^ c.f.u / pmol DNA) or its linearized version (8.2x10^4^ c.f.u / pmol DNA). The scenario was thus set for the generation of high-density knock-in / leave behind (KILB) libraries of protease-target sites through the sequence of *xylR* along the lines explained in the rationale above.
### Supplementary Table S1. Strains and plasmids used in this study

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<th>Strain or plasmid</th>
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<td>de Lorenzo et al, 1991</td>
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<tr>
<td>pBCL4-SP3</td>
<td>pUC18 derivative containing a BamHI/XbaI fragment encoding SP3 XylR variant</td>
<td>This work</td>
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<tr>
<td>pBCL4-SP4</td>
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<tr>
<td>Plasmid</td>
<td>Description</td>
<td>Reference</td>
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<tr>
<td>pBCL4-SP5</td>
<td>pUC18 derivative containing a BamHI/XbaI fragment encoding SP5 xylR variant</td>
<td>This work</td>
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<tr>
<td>pPPVs20</td>
<td>pSU8 derivative containing the SalI-PstI fragment of PPV cDNA consisting of the 3’ terminal region from nt 3627</td>
<td>Garcia et al, 1989</td>
</tr>
<tr>
<td>pPPV1</td>
<td>pVTR-B plasmid containing 0.6 Kb Stul-HindIII fragment encoding PPV NIa protease from pPPVs20 plasmid</td>
<td>Perez-Martin et al, 1997</td>
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</table>
Supplementary Table S2: primers used in the PCR reactions a)

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
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<tr>
<td>Tn5ME-F</td>
<td>CTGTCTTTATACACATCTCCTG</td>
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<tr>
<td>Tn5ME-R</td>
<td>CTGTCTTTATACACATCTGCGG</td>
</tr>
<tr>
<td>xylR-BamHI</td>
<td>ATGGATCCAAAGAGGAAAAAACAAATGTGC</td>
</tr>
<tr>
<td>xylR-XbaI</td>
<td>GTTCTAGACTATCGCCATTTGCTTTC</td>
</tr>
<tr>
<td>Km-Swal-F</td>
<td>CGCGCGATTTTTATTTGTCTCTAAAAATCTGATGTATGA</td>
</tr>
<tr>
<td>Km-PshAI-R</td>
<td>CGCGCGACCAGCGGTCAATATTATTAGAAAAATT</td>
</tr>
<tr>
<td>KpnI-Ascl(1)-F</td>
<td>CGCATGGTACGCTGTTTATACATCTGGCCCGCAGTAAAGAGGA</td>
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<td>AGAACCCTTCAC</td>
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<tr>
<td>KpnI-Pmel(1)-F</td>
<td>CGCATGGTACGCTGTTTATACATCTGGTTAAACAGTAAAGAGAA</td>
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<td>GAACCTTCAC</td>
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<tr>
<td>Ascl(2)-F</td>
<td>CTAATAATTAATTGGACCGCGGTCCGGCGCGGCGGGTGAAGACAGT</td>
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<td>GGTGGTG</td>
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<tr>
<td>Pmel(2)-F</td>
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<td>GGTGGTG</td>
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<tr>
<td>ApaLI-R</td>
<td>GGGTTCTGTGACACAGCGCCATTTGGAGCGGACC</td>
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<tr>
<td>DeltaA2-F</td>
<td>CCCGGGGATCCAAGGAAAACAAATGGAATCTGTAAGCAGTACATTGGGCAG</td>
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<td>GATAGGATCCAAAGGAAAAACAAATGGCGGATGAACGCGCGCGCAG</td>
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<td>D210F1</td>
<td>GATAGGATCCAAAGGAAAAACAAATG</td>
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<td>XylR-Sol.R</td>
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<tr>
<td>E499stop-R</td>
<td>GATATCTAGACCGCTGATGCACCACCCACGTGGCTTTC</td>
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</table>

a) Sequences of oligonucleotides employed in this study. Restriction sites entered for cloning purposes are underlined. Sequences corresponding to mutations are indicated in italics.
**Supplementary Figure S1.** Agarose gel analysis of DNA intermediates during KILB insertion saturation of a target gene with mTn5 [GFP•Nla1].

The figure shows two key steps of the process. Panel **(a)** illustrates the pattern of the 4 DNA products that result from digesting a plasmid with the gene of interest (xylR in this case) with XbaI and BamH (the sites that flank the cloned gene) following saturation insertions with mTn5 [GFP•Nla1] in vitro and recovery of plasmids (see main text for explanation). The pool of same-size DNA fragments with the xylR gene densely punctuated with transposon insertions appears clearly separated from the rest of the DNA segments: inserted plasmid backbone, non-inserted counterpart and non-inserted xylR. Panel **(b)** shows the excision of most of the mTn5 [GFP•Nla1] insert from the xylR sequences by digestion of the cognate plasmids with SbfI. Religation of the DNA of the upper DNA band of the gel generates an in-frame library of XylR variants inserted with cutting sites for protease Nla. Alternative digestion with NotI would have similarly created a library of GFP sandwiched in-frame fusions (not shown).
**Supplementary Figure S2.** Regulatory phenotypes of XylR-encoding and Nla protease-encoding plasmids and cognate insertless vectors.

(a) *E. coli* CC118 *Pu-lacZ* expressing XylR and Nla through the plasmids indicated (Supplementary Table S1) and spotted on LB plates with Xgal and exposed or not to saturating vapors of *m*-xylene. (b) Reference values of *Pu* activity. Liquid cultures of *E. coli* CC118 *Pu-lacZ* transformed with plasmids encoding XylR and Nla protease and their vectors were grown and exposed to *m*-xylene as explained in the Materials and Methods section. The diagram shows the levels of β-galactosidase detected after 3 hours of induction.
**Supplementary Figure S3.** Organization and *in vivo* expression of Nla-cleavable XylR and XylRΔA variants.

(a) Detail of the amino acid sequences delivered to permissive sites of the XylR structure upon insertion saturation of the *xylR* gene with the KILB procedure. The scheme shows the location of the various Nla cutting sites with an indication of the new sequences introduced in each case: native XylR amino acids in bold, transposition scar sequences entered by the trace of the Tn5 termini in plain (capital) letters and the peptide targeted by the Nla protease in red. (b) Blowup of the C- and N-termini of synthetic ΔA proteins (SP) engineered or not with a Nla cleavage site. The amino acid sequence of the C-terminus of the SP5 protein variant (XylRΔAAΔC) is blown up as well (color and letter codes same than before). (c) Expression and sensitivity to Nla protease of SP XylR variants in vivo. Equivalent amounts of crude
protein extracts from E. coli cultures expressing the proteins indicated along with Nla (or without protease, as specified) were run in a denaturing gel, blotted and developed with anti-XylRΔA antibodies.

SUPPLEMENTARY REFERENCES


