

# A comprehensive set of plasmids for vanillate- and xylose-inducible gene expression in *Caulobacter crescentus*

Martin Thanbichler<sup>1,\*</sup>, Antonio A. Iniesta<sup>2</sup> and Lucy Shapiro<sup>2</sup>

<sup>1</sup>Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Straße, 35043 Marburg, Germany and

<sup>2</sup>Department of Developmental Biology, Stanford University School of Medicine, Beckman Center B300, 279 Campus Drive, Stanford, CA 94305, USA

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## ABSTRACT

*Caulobacter crescentus* is widely used as a powerful model system for the study of prokaryotic cell biology and development. Analysis of this organism is complicated by a limited selection of tools for genetic manipulation and inducible gene expression. This study reports the identification and functional characterization of a vanillate-regulated promoter ( $P_{van}$ ) which meets all requirements for application as a multi-purpose expression system in *Caulobacter*, thus complementing the established xylose-inducible system ( $P_{xyl}$ ). Furthermore, we introduce a newly constructed set of integrating and replicating shuttle vectors that considerably facilitate cell biological and physiological studies in *Caulobacter*. Based on different narrow and broad-host range replicons, they offer a wide choice of promoters, resistance genes, and fusion partners for the construction of fluorescently or affinity-tagged proteins. Since many of these constructs are also suitable for use in other bacteria, this work provides a comprehensive collection of tools that will enrich many areas of microbiological research.

## INTRODUCTION

*Caulobacter crescentus* has emerged as a powerful model system to study bacterial cell cycle control, chromosome dynamics and cell differentiation. Its hallmark is an asymmetric cell division that gives rise to both a sessile stalked cell and a motile swarmer cell. A straightforward synchronization protocol and the existence of many well-defined developmental markers have allowed the identification of various molecular circuits that control and

execute the underlying differentiation process, acting at the level of transcription, translation, proteolysis and dynamic protein localization (1–4).

Cell biological studies are critically dependent on the application of fluorescent protein fusions to follow the subcellular distribution of proteins *in vivo* (5). They also rely heavily on the availability of inducible promoters that allow overproduction and depletion of proteins in a controlled manner, thus facilitating functional analyses and the characterization of essential genes. However, despite the excellent genetic amenability of *Caulobacter* (6) and years of intensive research, the molecular biological toolset for this organism is still poorly developed. So far, only a small number of plasmids is dedicated to the construction of protein fusions, offering a very limited choice of cloning sites, resistance markers and fusion partners. Moreover, regulatable gene expression in *Caulobacter* is currently based on a single, xylose-inducible promoter system (7), which has not yet been implemented into a standard set of broadly applicable vectors.

As the number of proteins identified as mediators of *Caulobacter*'s developmental program rises, unraveling the interactions between the different factors has become increasingly complex and frequently requires the construction of strains bearing multiple genetic modifications. To respond to this new technical challenge, it is essential to improve the tools available for genetic manipulation of *Caulobacter*. Here, we report the identification and characterization of a vanillate-inducible promoter, which is tightly regulated and can be used independently of the existing xylose-regulated system. Moreover, we present a comprehensive set of integrating and replicating shuttle vectors that are designed for the straightforward construction of protein fusions and the convenient expression of genes under control of the vanillate- and xylose-inducible promoters.

\*To whom correspondence should be addressed. Tel: +49 6421 178330, Fax: +49 6421 178209; Email: thanbichler@mpi-marburg.mpg.de

## MATERIALS AND METHODS

### Chemicals and enzymes

Vanillic acid ( $\geq 97\%$ ) was purchased from Fluka. Vanillin (99%), vanillyl alcohol ( $\geq 98\%$ ) and D-(+)-xylose ( $>99\%$ ) were obtained from Sigma-Aldrich. The following stock solutions were prepared: 50 mM vanillic acid/NaOH (pH 7.5 in  $H_2O$ ), 50 mM vanillin/NaOH (pH 7.0 in  $H_2O$ ), 50 mM vanillyl alcohol (in 70% ethanol) and 20% xylose (in  $H_2O$ ). For long-term storage, the vanillate and vanillin stocks were kept at  $-20^\circ C$ . Restriction and DNA-modifying enzymes were purchased from New England Biolabs and MBI Fermentas. PCR reactions were performed using KOD polymerase (Novagen). Oligonucleotides were synthesized by the Stanford PAN Facility.

### Bacterial strains and growth conditions

*Caulobacter crescentus* CB15N (8) and its derivatives were grown in PYE rich or M2G minimal medium (6) at  $28^\circ C$ . For cloning purposes, plasmids were propagated in *Escherichia coli* TOP10 (Invitrogen), which was cultivated in Luria-Bertani medium at  $37^\circ C$ . When appropriate, media were supplemented with antibiotics at the following concentrations (liquid/solid media for *C. crescentus*; liquid/solid media for *E. coli*; in  $\mu g/ml$ ): spectinomycin (25/50; 50/100), kanamycin (5/25; 30/50), rifampicin (2.5/5; 25/50), gentamicin (0.5/5; 15/20), oxytetracycline (1/1; 12/12), chloramphenicol (2/1; 20/30), apramycin (10/60; 30/30). Plasmid transfer into *C. crescentus* was achieved by electroporation (6). *Escherichia coli* was transformed using a chemical method (9). The CB15N derivatives MT219 ( $\Delta vanR$ ) and MT231 ( $\Delta vanA$ ) were generated with the help of plasmids pMT422 and pMT487, respectively, following a previously described gene replacement protocol (10). Strains MT232, MT236 and MT240 were created by transforming strain CB15N with integration plasmids pMT627, pMT704 or pMT760, respectively, and selecting for homologous recombination of the constructs into the chromosomal *vanA* or *xylX* locus.

### Vanillate degradation assay

Cells were grown in M2G minimal medium containing 0.5 mM vanillate. At the indicated timepoints, aliquots were withdrawn from the culture. The cells were pelleted by centrifugation for 5 min at 16 000 g, and the concentration of vanillate left in the supernatant was determined spectrophotometrically using a wavelength of 286 nm (the absorption maximum of vanillate).

### Microscopy

Cells were grown to exponential phase in M2G medium, induced for 1–2 h with vanillate or xylose at the indicated concentrations, immobilized on pads of 1% agarose in M2 salts (6) and visualized using a Nikon Eclipse E800 microscope equipped with a Nikon Plan Apo 100 $\times$ /1.40 differential interference contrast objective and a 5 MHz MicroMax cooled CCD camera (Princeton Instruments). Images were processed with Metamorph 4.5 (Universal Imaging Group) and Photoshop 6.0 (Adobe Systems).

### $\beta$ -Galactosidase activity assays

After growth of the cells to exponential phase, synthesis of  $\beta$ -galactosidase was induced by addition of sodium vanillate, vanillin or vanillyl alcohol to the indicated final concentrations. Unless indicated otherwise, cultivation was continued for 2 h (cultures in PYE medium) or 3 h (cultures in M2G medium), respectively, and  $\beta$ -galactosidase activities were determined according to Miller (11), with 0.01% SDS included in the reaction mixtures.

### RNA extraction

*Caulobacter crescentus* CB15N was grown in M2G medium to an  $OD_{660}$  of 0.15. Subsequently, two 25 ml aliquots were withdrawn and incubated for 3 h in the absence or presence of 0.5 mM vanillate, respectively. The cells were harvested by centrifugation, resuspended in 1 ml of Trizol (Invitrogen), and incubated for 10 min at  $65^\circ C$ . After addition of 0.2 ml chloroform, the samples were shaken by hand for 15 s and incubated for 5 min at  $4^\circ C$ . Following centrifugation, the aqueous phase was collected and transferred to a fresh tube. RNA was then precipitated by addition of 0.5 ml isopropanol and incubation at  $-80^\circ C$  for 1 h, and collected by centrifugation for 30 min at  $4^\circ C$  and 16 000 g. The pellets were washed with 75% ethanol and dried for 10 min at room temperature. The isolated RNA was dissolved in 50  $\mu l$  RNase-free water, incubated for 10 min at  $55^\circ C$ , and stored at  $-80^\circ C$  until further use.

### Primer extension analysis

Extension of the oligonucleotide 5'-CATACCAGGCGT CAAGCGGC-3', which hybridizes to the coding region (bases 36–55) of the *vanA* gene, was performed as described (12), using SuperScript II Reverse Transcriptase (Invitrogen) and an annealing temperature of  $45^\circ C$ . The Thermo Sequenase Cycle Sequencing Kit (USB) was used for DNA sequencing, with plasmid pMT437 as the DNA template.

### Plasmid construction

*Reporter plasmids and primer extension templates.* To construct the  $P_{vanA}$  reporter plasmid pMT122, a chromosomal fragment comprising the *vanR* gene, the *vanR-vanA* intergenic region, and the first four codons of *vanA* was PCR-amplified, with a HindIII site added to its 5' end and a PstI site added to its 3' end. The reaction product was cleaved with HindIII and PstI and ligated into the equally treated *lacZ* reporter plasmid pPR9TT (13). The reporter plasmid pMT129 was created by restriction of pMT122 with HindIII and SphI, blunting the newly generated ends with T4 DNA polymerase, and self-ligation of the plasmid backbone, thereby deleting bases 136–735 of the *vanR* gene. For the construction of pMT437, a chromosomal fragment comprising *vanR*, the *vanR-vanA* intergenic region, and the first 355 bases of *vanA* was PCR-amplified. The reaction product was then ligated into pCR4Blunt-TOPO using the ZERO Blunt TOPO PCR Cloning Kit (Invitrogen).

*Plasmids for generating in-frame deletions in vanR and vanA.* To construct plasmid pMT422, the upstream flanking region and first twelve codons of *vanR* were PCR-amplified, using primers that add a HindIII site to the 5' end and a SacI site to the 3' end of the reaction product. In parallel, a fragment comprising the last 12 codons and the downstream flanking region of *vanR* was PCR-amplified, with a SacI site added to its 5' end and an EcoRI site added to its 3' end. The PCR products were treated with HindIII/SacI or SacI/EcoRI, respectively, and ligated into allele exchange vector pNPTS138 (M. R. Alley, unpublished), which had been cut with HindIII and EcoRI.

The knockout construct pMT487 was generated in an analogous way. First, a fragment comprising the upstream flanking region and first twelve codons of *vanA* was PCR-amplified, with a HindIII and an NheI site added to its 5' and 3' end, respectively. In parallel, the last twelve codons plus the downstream flanking region of *vanA* were PCR amplified using primers that add an NheI site to the 5' end and an EcoRI site to the 3' end of the reaction product. The two fragments were digested with HindIII/NheI or NheI/EcoRI, respectively, and then ligated into plasmid pNPTS138 which had been treated with HindIII and EcoRI.

*Plasmids for the inducible expression of fluorescent protein fusions.* In order to create plasmid pMT627, the *mipZ* gene (10) was PCR-amplified, replacing its start codon with an NdeI restriction site and the stop codon with a VspI restriction site. The reaction product was cut with the corresponding enzymes and ligated into the NdeI/VspI-treated integration vector pVYFPC-1. Plasmid pMT704 was generated as follows: the *mipZ* gene was PCR-amplified, using primers that add a SacI restriction site to its 5' end and an EcoRI restriction site its 3' end. The reaction product was cut with the corresponding enzymes and then ligated into equally treated pXGFPN-2. For the construction of pMT760, a fragment carrying the *ftsZ* gene was isolated from plasmid pMT217 (10) by restriction with NdeI and SmaI, and ligated into NdeI/Ecl136II-treated pVCHYC-3.

*High-copy vectors for vanillate- and xylose-inducible gene expression.* To generate the high-copy number expression vector pBVMCS-4, the *vanR* gene and the *vanR-vanA* intergenic region were PCR-amplified, introducing an NdeI restriction site at the *vanA* start codon. The product was phosphorylated and ligated into the KpnI/SphI-treated and blunted broad-host-range vector pBBR1MCS-5 (14) such that  $P_{van}$  was adjacent to the plasmid-borne multiple cloning site (MCS). To generate pBVMCS-2 and pBVMCS-6, a fragment containing *vanR*,  $P_{van}$ , and the MCS was isolated from pBVMCS-4 by restriction with SspI and ligated into SspI-treated pBBR1MCS-2 (14) or PflFI/VspI-treated and blunted pBBR1MCS (15), respectively, in an orientation analogous to that in pBVMCS-4.

For the construction of pBXMCS-2, a fragment comprising the *E. coli rrnB* T1T2 transcriptional terminator (*ter*) and  $P_{xyI}$  was isolated from pMT27 (10) by

restriction with BamHI, blunting of the overhang, and subsequent treatment with NdeI. It was then ligated into pBVMCS-2, which had been cut with AscI, blunted, and treated with NdeI. Plasmid pBXMCS-4 was created analogously, using pBVMCS-4 as the target vector. To generate pBXMCS-6, a fragment comprising *ter* and  $P_{xyI}$  was released from pBXMCS-2 by restriction with SspI and ligated into PflFI/VspI-treated and blunted pBBR1MCS (14).

*Low-copy vectors for vanillate- and xylose-inducible gene expression.* To construct the low-copy number expression vector pRVMCS-5, an SspI-fragment from pBVMCS-4 containing *vanR* and  $P_{van}$  was ligated into the PstI/SphI-digested and blunted plasmid pMT288 (10) such that *vanR* was placed next to the plasmid-borne *rrnB* T1T2 terminators (16). A fragment containing *ter*, *vanR* and  $P_{van}$  was released from the resulting plasmid (pMT355) by restriction with NotI. It was blunted and ligated into pJB3Tc20 (17) whose NdeI site had been eliminated by a silent mutation in the *trfA* gene. The resulting plasmid, which carried *vanR* adjacent to the plasmid-borne *tetR* gene, was then treated with NdeI and PstI and ligated with a synthetic DNA duplex bearing an MCS. To create plasmid pRVMCS-6, a fragment comprising *ter*, *vanR*,  $P_{vanA}$  and the MCS was released from pRVMCS-5 by restriction with NotI, blunting of the overhang, and subsequent restriction with SfiI. It was then ligated into pJB3Cm6 (17), which had been cut with SapI, blunted, and subsequently cleaved with SfiI. Plasmid pRVMCS-2 was generated by ligating BstBI/Bpu10I-treated and blunted pRVMCS-6 with a blunted PagI-fragment from pMT27 (10) containing the *neo* gene.

For the construction of pRXMCS-2, -5 and -6, a fragment containing *ter* and  $P_{xyI}$  was isolated from pMT27 by restriction with NotI and NdeI and ligated into NotI/NdeI-treated pRVMCS-2, -5 and -6, respectively.

*Integration vectors for the one-step generation of depletion strains.* A fragment comprising *ter*, *vanR*,  $P_{van}$  and the MCS was isolated from pRVMCS-5 by restriction with NotI and PvuII and ligated into NotI/BsrBI-treated pMT27 (10). The resulting plasmid (pMT384) was then cut with NotI and ligated with a PCR-fragment which carried the RK2 origin of transfer (*oriT*) (18) and possessed engineered NotI and EagI sites at the 5' and 3' end, respectively. Colony PCR was used to identify a construct (pMT434) with *oriT* positioned immediately upstream of the plasmid-borne *neo* gene. A Spc/Str<sup>R</sup>-derivative of pMT434 (named pMT510) was created by restriction of the plasmid with PagI, blunting of the ends, and ligation with a blunted BstBI/StuI-fragment from pHPV465 (19) containing the *aadA* gene of plasmid R100.1.

Deletion of *vanR* from pMT510 by restriction with HindIII and NcoI, blunting of the ends, and self-ligation of the plasmid backbone resulted in pVMCS-1. Plasmids pVMCS-3 and -6 were created by ligating PagI/AvrII-treated pVMCS-1 with PCR fragments that comprised (i) the *parr-2* gene from plasmid pMW1409 (20) or (ii) the Tn9 *cat* gene from p34S-Cm (21), respectively, and carried an engineered PagI site followed by a ribosomal binding



site at the 5' end and an engineered *AvrII* site at the 3' end. To generate pVMCS-2, a fragment comprising *oriT*, *ter* and  $P_{van}$  was isolated from pVMCS-1 by restriction with *NotI* and *PstI* and ligated into equally treated pMT434. Plasmids pVMCS-4, -5 and -7 were subsequently generated by ligating *PagI*-treated and blunted pVMCS-2 with (i) a blunted *SacI*-fragment from pUCGm (22) containing the *Tn1696 aacC1* gene, (ii) a blunted *BssSI*-fragment from pJB3Tc20 (17) carrying the *tetR* and *tetA* genes of pFL129, and (iii) a *DraI/PmlI*-fragment from pHP45 $\Omega$ aac (23) containing the *E. coli aacC4* gene, respectively.

To construct pXMCS-1, the  $P_{xyl}$  fragment (including the *NdeI* restriction site) of pMT27 was PCR amplified, adding a *HindIII* restriction site to the 5' end. The reaction product was cut with *HindIII* and *NdeI* and ligated into equally treated pMT510. Ligation of a *NotI/PstI*-fragment from pXMCS-1 containing *oriT*, *ter*, and  $P_{xyl}$  into equally cut pVMCS-2, -3, -4, -5, -6 and -7 then resulted in plasmids pXMCS-2, -3, -4, -5, -6 and -7, respectively.

*Vectors for generating N- and C-terminal protein fusions encoded at the vanA or xylX locus.* In order to extend the  $P_{van}$  fragment carried by pMT510 to a length of 1586 bp, the 3' region of *vanR* (containing an *AscI* site) and ~0.7kb of the chromosomal *vanR* downstream region were PCR amplified, adding a *HindIII* restriction site to the 5' end. The reaction product was cut with *HindIII* and *AscI* and ligated into equally cut pMT510, resulting in pMT517. To create an integration plasmid with a 2316 bp  $P_{xyl}$  promoter region, a fragment containing  $P_{xyl}$  and its upstream region was isolated from pXGFP4 (M. R. Alley, unpublished) by restriction with *NheI*, blunting of the overhang, and subsequent restriction with *NdeI*. It was then ligated into pMT510 which had been cut with *HindIII*, blunted, and subsequently treated with *NdeI*, giving rise to pMT519. Synthetic MCS were inserted between the *NdeI* and *AgeI* restriction sites of pMT517 and pMT519, respectively, resulting in pMT517<sub>MCS-N</sub>, pMT519<sub>MCS-N</sub>, pMT517<sub>MCS-C</sub> and pMT519<sub>MCS-C</sub>.

For the construction of C-terminal fusion vectors, the *egfp*, *eyfp*, *ecfp* (Clontech), *venus* (24), *cerulean* (25) and *mCherry* (26) genes were PCR-amplified, adding an *AgeI* restriction site followed by a short linker to the 5' end and an *NheI* restriction site to the 3' end, respectively. The *AgeI/NheI*-treated fragments were then ligated into pMT517<sub>MCS-N</sub>, resulting in pVGFP-1, pVYFP-1, pVCFP-1, pVVENC-1, pVCERC-1 and pVCHYC-1. Ligation of the same fragments into pMT519<sub>MCS-N</sub>, on the other hand, gave rise to the plasmids pXGFP-1, pXYFP-1, pXCFP-1, pXVENC-1, pXCERC-1 and pXCHYC-1. Plasmid pXTCTC-1 was obtained by restriction of pXGFP-1 with *AgeI* and *NheI* and ligation of the plasmid backbone with a synthetic double strand encoding the tetracycline tag (27).

To create N-terminal fusion vectors, *egfp*, *eyfp*, *ecfp* (Clontech), *venus* (24), *cerulean* (25) and *mCherry* (26) were PCR-amplified, with an *NdeI* restriction site engineered at the respective start codon. After digestion with *NdeI* and *BsrGI*, the reaction products were ligated into equally treated pMT517<sub>MCS-C</sub>, yielding plasmids

pVGFP-1, pVYFP-1, pVCFP-1, pVVENC-1, pVCERN-1 and pVCHYN-1. Ligation of the same restriction fragments into pMT519<sub>MCS-C</sub>, by contrast, gave rise to plasmids pXGFP-1, pXYFP-1, pXCFP-1, pXVENC-1, pXCERN-1 and pXCHYN-1.

In order to extend the range of resistance markers, the plasmids described above were cut with *NheI* and *NotI*, releasing a fragment that contained the *aadA* *Spec/Str<sup>R</sup>* resistance gene. Subsequently, they were ligated with *NheI/NotI*-fragments from plasmids pVMCS-2, -3, -4, -5 or -6 carrying the *neo*, *parr-2*, *aacC1*, *tetRA* and *cat* genes, respectively.

Plasmid pXFLGC-2 was obtained by restriction of pXGFP-2 with *AgeI* and *NheI*, and ligation of the plasmid backbone with a synthetic double strand encoding the FLAG-tag (28).

*Vectors for generating C-terminal protein fusions encoded at a site of interest.* To generate the integration vectors pGFPC-1, pYFPC-1, pCFPC-1, pVENC-1, pCERC-1 and pCHYC-1, the  $P_{xyl}$  promoter fragment was released from pXGFP-1, pXYFPC-1, pXCFPC-1, pXVENC-1, pXCERC-1 and pXCHYC-1 by restriction with *HindIII* and *NdeI*, and replaced by a synthetic double-stranded linker. Plasmids pFLGC-1, pSTRC-1 and pTCYC-1 were obtained by restriction of pGFPC-1 with *AgeI* and *NheI*, and ligation of the plasmid backbone with synthetic double strands encoding the FLAG-tag (28), the Strep-tag II (29) or the tetracycline tag (27) peptides.

Derivatives of these plasmids bearing alternative resistance markers were constructed by replacement of the original *NheI/NotI*-fragment carrying the *aadA* gene with *NheI/NotI*-fragments from pVMCS-2, -3 and -4 carrying the *neo*, *parr-2*, and *aacC1* genes, respectively.

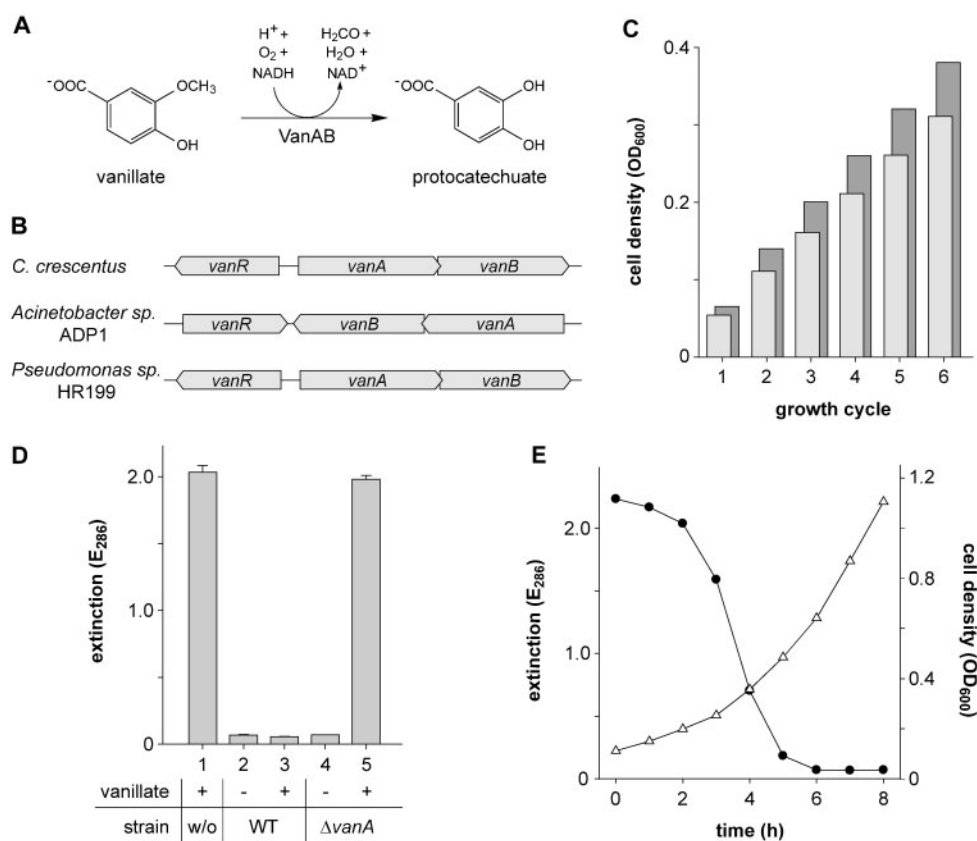
*Vectors for integration at a site of interest.* For the construction of pMCS-1, the  $P_{van}$  promoter fragment of plasmid pMT517<sub>MCS-C</sub> was released by restriction with *HindIII* and *NdeI*, and replaced with a synthetic linker. To create derivatives carrying alternative resistance markers, the *aadA* gene was excised from pMCS-1 by restriction with *NheI* and *NotI*. The plasmid backbone was then ligated with *NheI/NotI*-fragments from pVMCS-2, -3, -4, -5, -6 and -7 carrying the *neo*, *parr-2*, *aacC1*, *tetRA*, *cat* or *aacC4* genes, respectively.

*Low-copy vectors for generating N- and C-terminal protein fusions under the control of P<sub>vanA</sub>.* Fragments carrying the MCS and the fluorescent protein gene were released from pVGFP-1, pVYFP-1, pVCFP-1, pVGFP-1, pVYFP-1, pVCFP-1, pVVENC-1 and pVCHYC-1 by restriction with *NdeI* and *NheI*. They were then ligated with *NdeI/NheI*-treated pRVMCS-2, -4 and -5, respectively, giving rise to the pRV-series of gene fusion vectors.

## RESULTS

### Identification of a vanillate-inducible promoter in *Caulobacter*

*Caulobacter crescentus* thrives in oligotrophic freshwater habitats (30), where structural polymers derived from



**Figure 1.** Vanillate degradation by *Caulobacter*. (A) Conversion of vanillate into protocatechuate, as catalyzed by the vanillate demethylase (VanAB) complex. (B) Arrangement of the *vanR*, *vanA* and *vanB* genes in *C. crescentus*, *Acinetobacter sp. ADP1* and *Pseudomonas sp. HR199*. (C) Cell densities achieved with vanillate or glucose as the sole carbon source. Minimal media composed of 0.5 mM vanillate (light bars) or glucose (dark bars), respectively, in M2 salts (6) were inoculated with washed cells of wild-type strain CB15N. The cultures were grown until the carbon source was depleted, and their optical densities were determined (growth cycle 1). Subsequently, the cells were subjected to another five growth cycles, each of which started with replenishment of the carbon source (0.5 mM final concentration), followed by growth to stationary phase and determination of the respective cell densities. Note: Stepwise addition of the carbon source was necessary due to a negative effect of vanillate on growth at concentrations higher than 0.5 mM. (D) Involvement of CC2393 (VanA) in vanillate degradation. M2G minimal medium containing no (–) or 0.5 mM (+) vanillate, respectively, was inoculated with wild-type strain CB15N (WT) or its  $\Delta$ *vanA*-derivative MT231 and incubated for 24 h. Subsequently, the cells were pelleted, and the supernatant was analyzed spectrophotometrically at 286 nm (the absorption maximum of vanillate) to determine the amount of vanillate left in the medium. Sterile medium (w/o) was used as a control. Data represent the mean of three independent experiments ( $\pm$ SD). (E) Induction kinetics of vanillate degradation. Cells of wild-type strain CB15N were grown to exponential phase in M2G minimal medium, washed, and resuspended in M2G medium containing 0.5 mM vanillate to an OD<sub>600</sub> of 0.11. Samples were taken at one-hour intervals and analyzed for cell density (OD<sub>600</sub>, open triangle) and vanillate content (E<sub>286</sub>, filled circle).

decaying vascular plant material represent the predominant source of organic carbon (31). Its genome indeed encodes a number of hydrolases with the potential to degrade cellulose and hemicellulose (32), and it can grow on xylose, an abundant constituent of hemicellulose, as the sole carbon source (30). The presence of xylose serves as a general signal for the availability of plant polysaccharides and induces synthesis of a suite of proteins that mediate polymer degradation and funneling of the hydrolysis products into the general catabolic pathways (33). One of the most highly activated members of the xylose regulon is the *xyiX* gene, encoding a protein of unknown function, whose promoter ( $P_{xyi}$ ) has been widely used for inducible gene expression in *Caulobacter* (7).

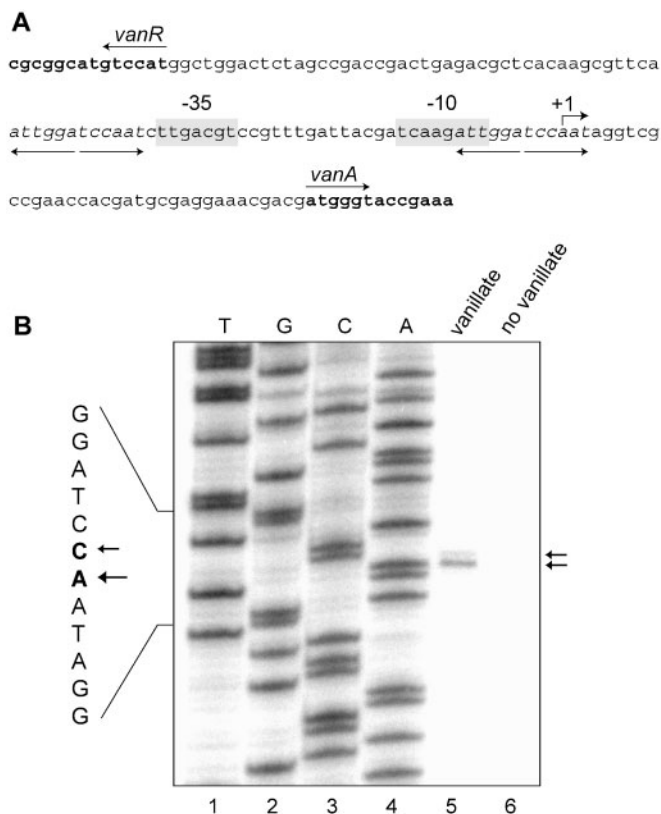
Apart from polysaccharides, lignin represents the second most abundant constituent of the vascular plant cell wall. It is a heterogeneous, highly crosslinked polymer derived from the aromatic monolignols coniferyl,

coumaryl and sinapyl alcohol (34). Due to its complex chemical structure, its initial, oxidative cleavage can only be performed by a small group of organisms, most importantly fungi (35). The soluble phenolic intermediates, however, released during this process are subsequently metabolized by a variety of different bacterial species. One of the compounds commonly produced from lignin is vanillate (Figure 1A). To allow its utilization as a carbon source, it first has to be converted into protocatechuate by removal of its O-methyl group. Cleavage of the aromatic ring system and further degradation via the  $\beta$ -ketoacid pathway then yield succinyl-CoA and acetyl-CoA, which can directly enter the citric acid cycle (36). In many bacteria, O-demethylation of vanillate is carried out by a two-component monooxygenase, composed of a terminal oxygenase (VanA) and a ferredoxin-like reductase (VanB) subunit. Usually, these two proteins are encoded in an operon, with their synthesis being

regulated by a transcriptional repressor, VanR, whose gene is transcribed together with or divergently from the *vanAB* transcriptional unit (37–40) (Figure 1B).

Motivated by the ability of *Caulobacter* to utilize plant material for growth and to degrade a variety of different aromatic compounds (41), we analyzed the *Caulobacter* genome (32) for homologs of the *vanAB* genes. This search identified two overlapping open reading frames, CC2393 and CC2394, whose deduced products show 67% and 48% identity with the well-characterized VanA and VanB proteins, respectively, from *Acinetobacter* sp. ADP1 (38). The polypeptide encoded by CC2393 contains sequence motifs (42) that are characteristic of the mononuclear and the Rieske-type iron-binding sites of class IA terminal oxygenases. CC2394, on the other hand, gives rise to a protein with the NAD-ribose-, flavin mononucleotide-, and chloroplast-type ferredoxin [2Fe-2S]-binding domains typically found in class IA reductases (42). This domain composition indicates that the *Caulobacter* VanA and VanB homologs could indeed constitute a two-component monooxygenase with vanillate demethylase activity. Encoding all components of the  $\beta$ -ketoadipate pathway (CC2405–CC2411) (32), *Caulobacter* might thus possess the full complement of enzymes necessary for the degradation of vanillate. Growth experiments indeed showed that it can utilize this aromatic compound as the sole carbon source, reaching cell densities comparable to those obtained in glucose minimal medium (Figure 1C). Metabolism of vanillate still occurred in the presence of excess glucose (Figure 1D, bars 2 and 3), which indicates that the degradative pathways involved are not subject to catabolite repression. By contrast, vanillate metabolism was completely abolished in a mutant strain carrying an in-frame deletion in the *vanA* homologue (MT231), thus strongly supporting a function of *Caulobacter* VanAB in vanillate demethylation (Figure 1D, bars 4 and 5). In order to investigate the kinetics of its degradation, vanillate was added to a culture of wild-type strain CB15N growing exponentially in glucose minimal medium and quantified in regular intervals (Figure 1E). Maximal degradation rates were reached after a short lag phase, leading to complete removal of vanillate from the growth medium within a few hours. The lag phase observed in these measurements suggested that the enzymatic pathway mediating vanillate utilization is not constitutively active but specifically induced in the presence of the substrate. Vanillate concentrations higher than 0.5 mM noticeably decreased the growth rate of *Caulobacter* (reduction at 1 mM: 25% in M2G medium and 2% in PYE medium). This effect might be attributable to a weak uncoupling activity, as observed for many other aromatic monocarboxylic acids (43).

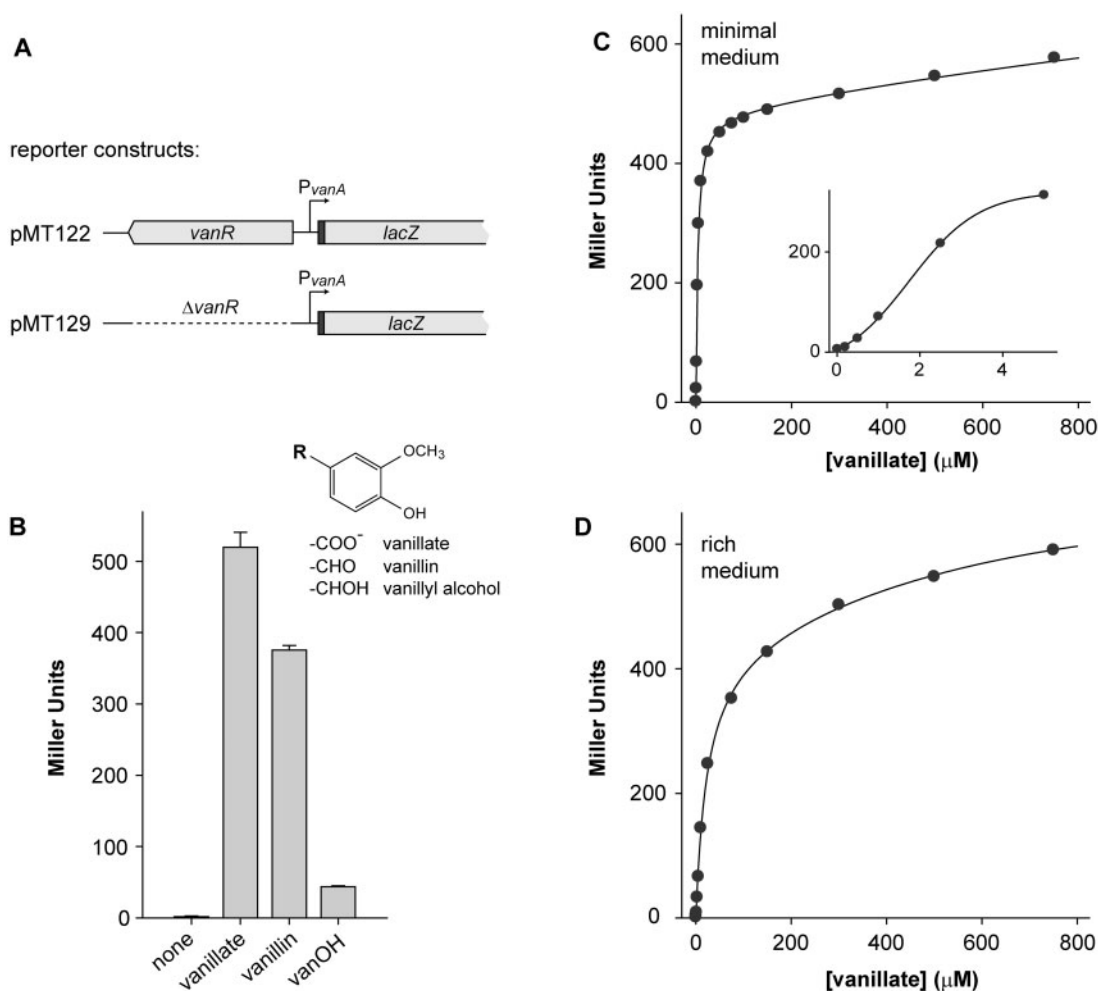
Having identified a putative vanillate degradation gene cluster, we determined if the promoter driving transcription of the *vanAB* operon ( $P_{van}$ ) could be exploited for inducible gene expression in *Caulobacter*. For this purpose, a detailed functional analysis of the 126 bp intergenic region shared by the *vanR* gene and *vanAB* genes (Figure 2A) was performed. Primer extension analysis of RNA isolated from wild-type strain CB15N grown in the presence of vanillate showed that



**Figure 2.** Characteristics of the *vanAB* promoter. (A) Overview of the *vanAB* promoter region. The *vanR*-*vanAB* intergenic region is shown in normal print, whereas the 5' ends of the flanking *vanR* and *vanA* genes are given in boldface, with their orientations indicated by arrows. The transcriptional start site of *vanA* (see Figure 2B) and the putative -10 and -35 motifs of the *vanA* promoter are labeled. Diverging arrows and italic letters mark two copies of a perfectly palindromic sequence, which is likely to represent the VanR target site. (B) Determination of the *vanAB* transcriptional start site. Primer extension analysis was conducted on RNA extracted from cells of wild-type strain CB15N which had been grown in M2G medium in the presence (lane 5) or absence (lane 6) of 0.5 mM vanillate. In parallel, sequencing reactions were performed (lanes 1–4). The two reaction products and the corresponding +1 sites are indicated by arrows.

transcription of *vanA* mostly initiates at an adenosine residue 35 bp upstream of the *vanAB* translational start site (Figure 2B, lane 5). In addition, a minor transcript species carrying an additional 5' cytosine residue could be detected. The primer extension reaction consistently failed to yield a product when performed on RNA from cells that had been grown in medium lacking vanillate (Figure 2B, lane 6), indicating that expression of *vanAB* is induced by vanillate or one of its degradation products. Upstream of the experimentally determined +1 site, sequences corresponding to the characteristic -35 and -10 boxes of a housekeeping,  $\sigma^{73}$ -dependent promoter can be identified (44,45) (Figure 2A). Their relative spacing and distance to the transcriptional start site suggests that they represent the core promoter elements responsible for *vanAB* transcription. The *vanAB* upstream region further contains two copies of a perfect inverted repeat (5'-ATTG GATCCAAT-3'), which cannot be found anywhere else in the *Caulobacter* genome (32). One of them immediately



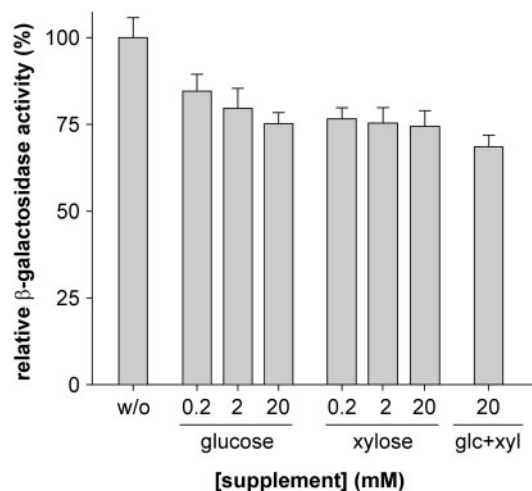


**Figure 3.** Response of the *vanAB* promoter to different inducers and inducer concentrations. (A) Reporter constructs used to determine  $P_{van}$  promoter activity. The 5' region of *vanA*, which was fused in frame to the *lacZ* gene, is shown in black. (B) Activity of  $P_{van}$  in the presence of different inducers. Wild-type strain CB15N was transformed with reporter plasmid pMT122 and grown in M2G minimal medium. In mid-exponential phase, vanillate, vanillin or vanillyl alcohol (vanOH) were added to a final concentration of 0.5 mM, respectively, and the activity of  $P_{van}$  was determined. The inset shows the structural formulas of the inducers used. (C and D) Response of  $P_{van}$  to different concentrations of vanillate. Cells of strain MT231 ( $\Delta vanA$ ) transformed with reporter plasmid pMT122 were grown in M2G minimal medium (C) or PYE rich medium (D), exposed for 3 h to different concentrations of vanillate, and used to determine the activity of  $P_{van}$ . The inset in (C) shows  $P_{van}$  promoter activity at vanillate concentrations of 0–5  $\mu$ M. Data depicted in panels (B), (C) and (D) represent the average of two independent experiments, each performed in triplicate. Standard deviations were smaller than 11% throughout.

precedes the  $-35$  box, whereas the other one overlaps the  $-10$  box and the  $+1$  site (Figure 2A). Palindromic sequences arranged in such a manner frequently reflect GntR-type transcription factor binding sites (46). The two repeats are, therefore, likely to interact with the *Caulobacter* VanR homolog, mediating repression of *vanAB* transcription in the absence of vanillate.

In order to investigate the regulation of *vanAB* expression in more detail, a fragment comprising the *vanR* gene, the *vanR-vanAB* intergenic region, and the 5' part of the *vanA* gene was cloned into the low-copy promoter probe vector pPR9TT (13), generating a translational fusion between *vanA* and the plasmid-borne *lacZ* reporter gene (Figure 3A). When wild-type strain CB15N was transformed with the resulting plasmid (pMT122) and grown in glucose minimal medium lacking any inducer, no transcriptional activity was detectable.

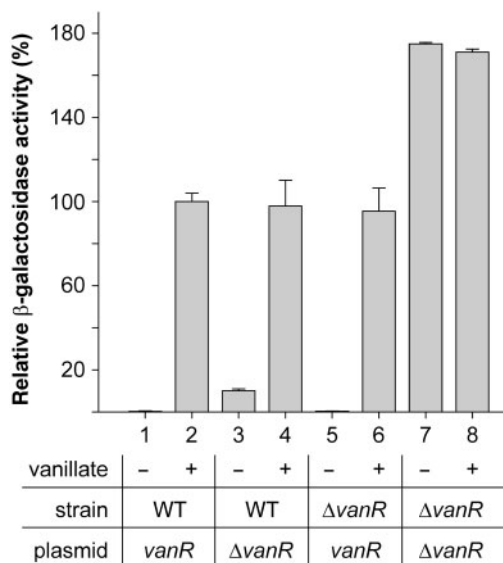
Addition of vanillate, by contrast, strongly activated the *vanAB* promoter, thus confirming the regulatory pattern suggested by primer extension analysis (Figure 3B). A similar response was elicited by vanillin, another common product of lignin biodegradation (35). As a structural relative of vanillate, vanillin might directly interact with the effector-binding site of VanR. Alternatively, since *Caulobacter* contains a gene (CC2402) whose product shows 58% identity with vanillin dehydrogenase from *Pseudomonas* sp. HR199 (47), the stimulating effect of vanillin could be indirect, resulting from its oxidation to vanillate, which then serves as the actual inducer. In contrast to the carboxy and aldehyde forms, vanillyl alcohol only slightly activated the *vanAB* promoter, possibly due to weak interaction with VanR or slow metabolic conversion into vanillate as observed in *Pseudomonas* sp. PN-1 (48).



**Figure 4.** Influence of sugars on the activity of the *vanAB* promoter. Cells of wild-type strain CB15N transformed with reporter plasmid pMT122 (Figure 3A) were grown in PYE medium and exposed to 0.5mM vanillate (w/o), a mixture of 0.5mM vanillate and glucose or xylose at the indicated concentrations, or a mixture of 0.5mM vanillate, 20mM glucose and 20mM xylose. Subsequently, the cell were subjected to  $\beta$ -galactosidase activity assays to determine the response of  $P_{van}$  under the different inducing conditions. Data represent the mean of two independent experiments ( $\pm$ SD), each performed in triplicate.

After having established vanillate as the most potent inducer, we analyzed *vanAB* promoter activity in response to different concentrations of this compound. For that purpose, cells of the *vanA*-deficient strain MT231 bearing reporter plasmid pMT122 were grown in either glucose minimal or rich medium and exposed to varying inducer concentrations (Figures 3C and D). The titration curves obtained show that vanillate concentrations in the nanomolar range are sufficient to activate  $P_{van}$ . While promoter responsiveness was higher in minimal medium, growth conditions had no effect on the maximal promoter activity reached at saturating vanillate concentrations. These results demonstrate that the *vanAB* promoter is tightly regulated and can assume a wide range of activities depending on the amount of inducer added to the cultures.

An important prerequisite for the general application of vanillate-inducible gene expression in *Caulobacter* is its compatibility with standard growth conditions. Many experimental setups require the use of rich media that are supplemented with glucose or xylose to repress or activate genes cloned under the control of the xylose-inducible promoter  $P_{xyb}$ , respectively (7). To assess the influence of sugars on the activity of the *vanAB* promoter at high nutrient levels, wild-type strain CB15N was transformed with reporter plasmid pMT122 (Figure 3A) and exposed to vanillate in the presence of varying concentrations of glucose, xylose or a combination thereof. In all cases, the availability of an additional carbon source reduced the promoter activity by  $\sim$ 25% (Figure 4). This effect was essentially independent of the nature or concentration of the supplement and was only slightly aggravated by the combined addition of both sugars. The same percentual reduction in transcription levels was observed over a wide range of vanillate concentrations (data not shown),



**Figure 5.** Involvement of VanR in the regulation of *vanAB* expression. Cells of wild-type strain CB15N (WT) and its mutant derivative MT219 ( $\Delta$ *vanR*) were transformed with reporter plasmids pMT122 (*vanR*) or pMT129 ( $\Delta$ *vanR*) and grown to exponential phase in PYE medium. Subsequently, they were exposed to 0mM (–) or 0.5mM (+) vanillate, and used to determine the activity of  $P_{van}$ . Data represent the mean of two independent experiments ( $\pm$ SD), each performed at least in triplicate.

indicating that the overall characteristics of the *vanAB* promoter are resistant to changes in the composition of the growth medium. On the other hand, we could not observe any effect of vanillate on the activity of  $P_{xyb}$  (data not shown). These results demonstrate that the  $P_{van}$ - and  $P_{xyb}$ -based expression systems are fully compatible and can be used in parallel and independently each other.

In order to improve the usability of the *vanAB* promoter, it was necessary to determine the identity of its cognate regulator. A gene (CC2392) encoding a GntR-type transcriptional repressor is found immediately upstream of the *Caulobacter vanAB* operon (Figure 1B), with its transcription oriented in the opposite direction. The corresponding protein shows 43% identity with VanR from *Acinetobacter* sp. ADP1 (49), suggesting a role in modulating *vanAB* expression in response to vanillate or structurally similar compounds. We, therefore, created a strain carrying an in-frame deletion in the chromosomal *vanR* gene (MT219) and analyzed the effect of this mutation on *vanAB* transcription, using either reporter plasmid pMT122 or its *vanR*-deficient derivative pMT129 (Figure 3A). When assayed in the presence of a plasmid-borne copy of *vanR*, promoter activities were indistinguishable from those observed in a wild-type background (Figure 5, bars 1, 2, 5 and 6). Without complementation, in contrast, transcription was fully activated irrespective of the inducer concentration, demonstrating that VanR indeed regulates expression of the *vanAB* operon in *Caulobacter* (Figure 5, bars 7 and 8). The reporter levels measured under these conditions were about 75% higher than in strains containing at least one functional copy of the *vanR* gene. This observation could indicate that the



concentration of vanillate used in these experiments still allows for residual binding of VanR to the *vanAB* promoter, thus resulting in sub-maximal transcription rates. Alternatively, the induction time used might not have been long enough for *vanR*-containing strains to reach the plateau established by constitutive expression of the reporter construct. When transcription was measured in wild-type strain CB15N in the absence of a plasmid-borne *vanR* allele, significant promoter activity was detectable in the uninduced state (Figure 5, bars 3 and 4). Thus, the number of repressor molecules synthesized from the chromosomal *vanR* gene alone is not sufficient to ensure tight regulation of multiple promoter copies provided *in trans* on a replicating plasmid.

### Construction of plasmids for inducible gene expression in *Caulobacter*

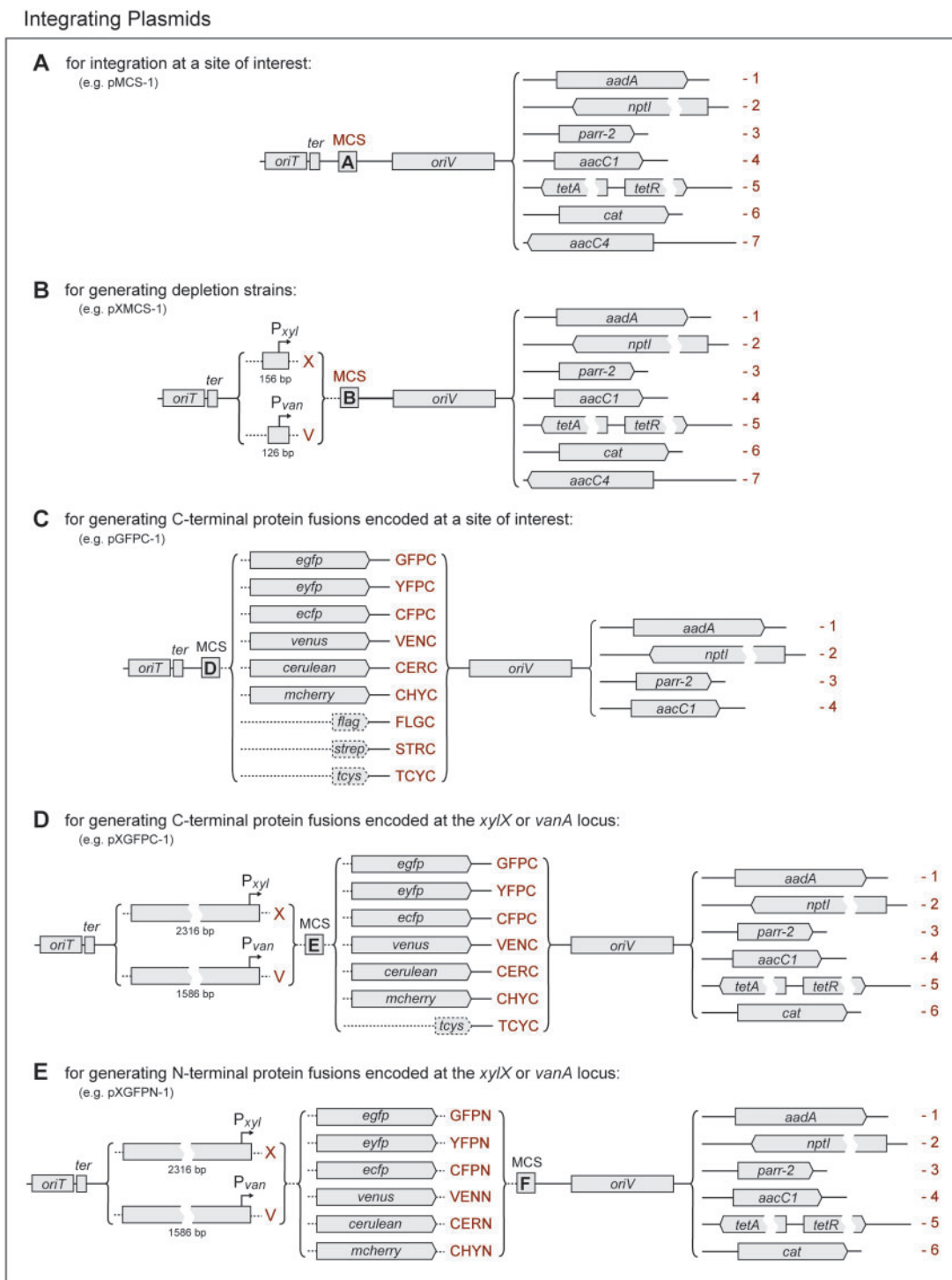
The results described above demonstrate that the *vanAB* promoter fulfills all criteria necessary for use as a multipurpose gene expression system. We, therefore, integrated this promoter into a set of vectors that facilitates its application to a variety of problems commonly encountered in cell biological research. In parallel, we created a comparable series of  $P_{xyI}$ -based plasmids as well as a number of promoterless constructs for general use, thereby establishing a comprehensive and standardized toolkit for genetic manipulation in *Caulobacter*.

**Integration vectors.** The most basic set of constructs comprises a number of general use integration vectors, which have been optimized for small size to maximize transformation yields (Figure 6A). They all have in common the RK2 origin of transfer, allowing conjugative transfer with the help of suitable host strain (18,50), the *E. coli* *rrnB* T1T2 transcriptional terminators, preventing leaky expression of cloned genes due to read-through from upstream regions (16), and a newly designed polylinker containing up to sixteen unique restriction sites (Figure 7, MCS A). Their replication is driven by the narrow host range pMB1 origin, which allows high copy numbers in *E. coli* but is inactive in *Caulobacter*. Together, these plasmids further offer a choice of seven different selective markers, thereby facilitating the generation of multiply modified strains. Five of these markers were derived from preexisting expression cassettes known to confer high-level resistance against spectinomycin, kanamycin, gentamicin, tetracycline or apramycin to *Caulobacter* (see Materials and Methods). In addition, novel rifampicin and chloramphenicol resistance determinants were created by combining the rickettsial *parr-2* (20) and Tn9 *cat* genes (21), respectively, with a more efficient ribosome binding site and the R100.1 *aadA* promoter (51).

A second group of plasmids (Figure 6B) essentially combines the features described above with a short chromosomal fragment comprising the minimal  $P_{van}$  or  $P_{xyI}$  promoter region and the corresponding downstream translation initiation signals. An NdeI restriction site (5'-CATATG-3') was engineered at the respective start codons to allow expression of cloned genes using the *xyIX*

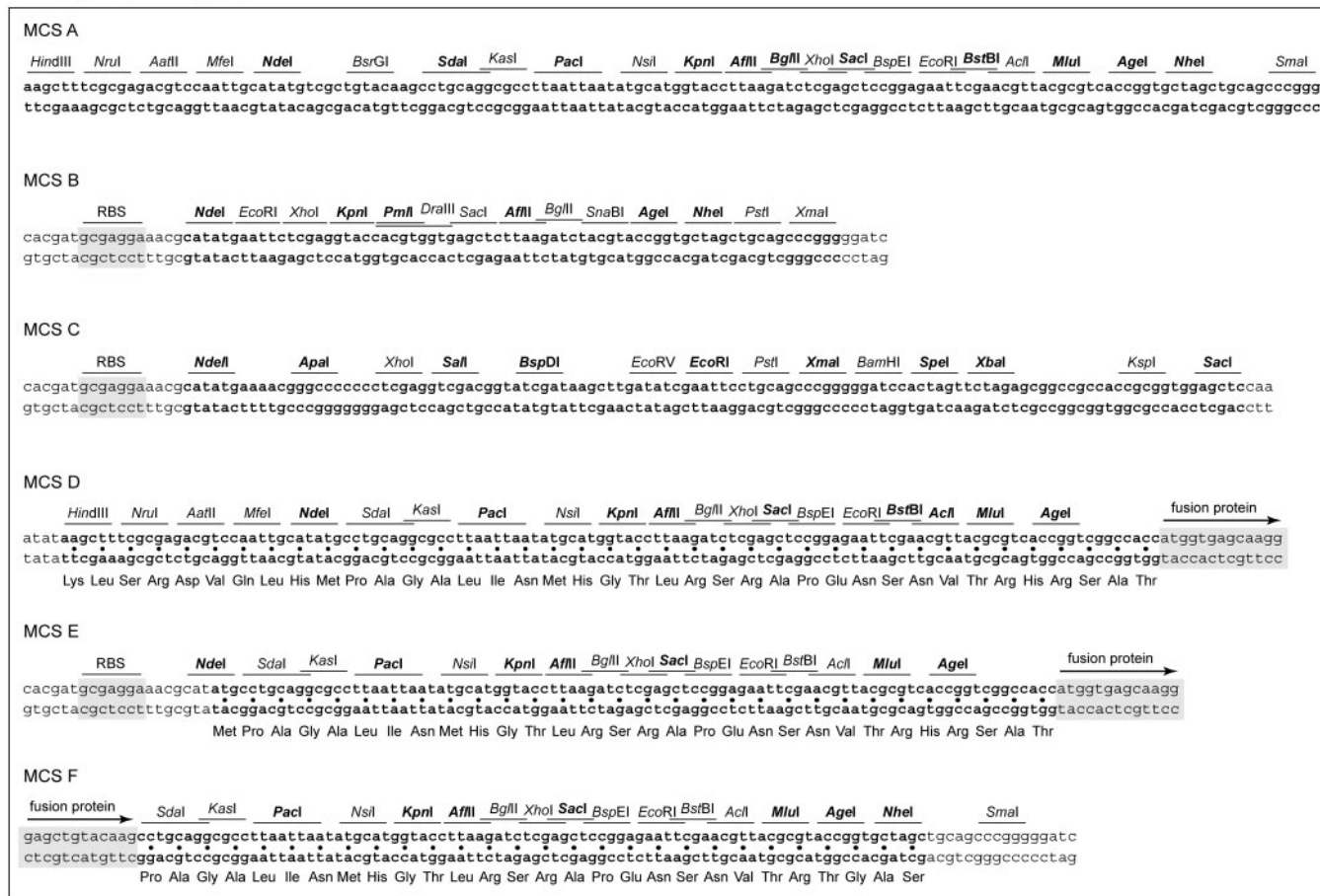
or *vanA* Shine-Dalgarno sequence (Figure 7, MCS B). Despite their small size, the promoter fragments used still contain all regulatory elements necessary to ensure tight transcriptional control. However, they are too short to undergo efficient homologous recombination, thus favoring integration of the plasmids at a chromosomal locus which matches the sequence inserted into the MCS. If the construct contains the 5' part of a gene only, its incorporation into the chromosome will generate a truncated version of the native gene and a second, full-length copy under control of the plasmid-borne inducible promoter, thus offering a straightforward method to create conditional alleles. Primers suitable for sequencing inserted genes are listed in Table 1.

A third set of integrations vectors (Figure 6C) is intended to facilitate the one-step generation of strains producing fluorescent and affinity-tagged hybrid proteins encoded on the chromosome under control of their native promoter. Based on a common basic architecture (Figure 6A), these constructs offer a choice of four different resistance determinants, each of which can be used in conjunction with a variety of carboxy-terminal fusion partners. The tag sequences are preceded by an extensive MCS, whose translation product has been optimized for low secondary structure content and the absence of bulky hydrophobic residues, thus ensuring high flexibility and minimal interference with protein function (Figure 7, MCS D). Moreover, they have all been inserted in the same reading frame, so that genes of interest can be shuffled between different plasmids without difficulty. Among the available fusion partners, the enhanced versions of green, yellow and cyan fluorescent protein (eGFP, eYFP and eCFP) have proved to be reliable tools for cell biological studies in *Caulobacter*. Recently, however, diverse mutagenic approaches have significantly improved the photophysical and folding parameters of these proteins (52). To make use of these advances, three of the newly developed variants were included in this vector set. Venus resembles eYFP in its excitation and emission spectra, but shows higher folding and maturation rates, leading to a large gain in overall fluorescence (24). Cerulean, by contrast, combines the spectral properties of eCFP with an increased quantum yield, extinction coefficient and fluorescence lifetime and thus outrivals its predecessor in net fluorescence intensity (25). The red fluorescent protein mCherry, finally, can be used complementary to eGFP in dual-color imaging experiments and is characterized by its high maturation rate and photostability (26). Furthermore, it is the only protein besides its precursor mRFP1 which has been shown to fluoresce after Sec-mediated export into the bacterial periplasm (53) and, therefore, represents an indispensable tool for studying the localization of cell envelope proteins. To account for the frequent use of affinity peptides in cell biological research, the selection of available fusion partners was extended by three short peptide tags. The FLAG-tag is specifically recognized by the M2 monoclonal antibody and can be employed for immunological detection and co-immunoprecipitation analyses (28). The same types of applications are supported by the Strep-tag II peptide (29). As a high-affinity ligand of streptavidin



**Figure 6.** Integrating Plasmids. This schematic shows different sets of vectors designed for (A) integrating constructs at a chromosomal site of interest, (B) inserting a minimal  $P_{xyl}$  or  $P_{van}$  fragment upstream of a defined chromosomal locus, (C) creating C-terminal protein fusions encoded at the native locus of the gene of interest, and generating inducible (D) C-terminal or (E) N-terminal protein fusions encoded at the *xylX* or *vanA* locus. Plasmid names are determined as follows: each construct carries the prefix 'p', followed by a string of terms that describes its characteristic features and resistance gene. The individual terms are shown in red print and are to be lined up from left to right to yield the correct designation. For each category of plasmids, the name of one construct is given as an example. The different MCS (MCS, A-E) are detailed in Figure 7. The following gene names were used: RK2 origin of transfer (*oriT*), *E. coli rrnB* T1T2 transcriptional terminator (*ter*), pMB1 origin of replication (*oriV*), aminoglycoside adenyltransferase (Spc/Str<sup>R</sup>, *aadA*), neomycin phosphotransferase I (Kan<sup>R</sup>, *nptI*), rifampicin ADP-ribosyltransferase (Rif<sup>R</sup>, *parr-2*), AAC(3)-I aminoglycoside acetyltransferase (Gent<sup>R</sup>, *aacC1*), tetracycline efflux permease (Tet<sup>R</sup>, *tetA*) and repressor (*tetR*), chloramphenicol acetyltransferase (Cam<sup>R</sup>, *cat*), AAC(3)-IV aminoglycoside acetyltransferase (Apr/Gent<sup>R</sup>, *aacC4*), enhanced green fluorescent protein (*egfp*), enhanced yellow fluorescent protein (*eyfp*), enhanced cyan fluorescent protein (*ecfp*), Venus (*venus*), Cerulean (*cerulean*), mCherry (*mcherry*), FLAG-tag (*flag*), Strep-tag II (*strep*), tetracycline tag (*tcys*). Plasmids are shown to scale, except where indicated by breaks or dotted lines.

Multiple Cloning Sites



**Figure 7.** Multiple cloning sites. The sequences of the different MCS (MCSA–E) are shown in boldface. Flanking Shine-Dalgarno motifs (RBS, as present in  $P_{van}$ -containing constructs) and fusion tags (exemplified by *egfp*) are indicated. Restriction sites that are unique in all plasmids carrying the respective MCS are highlighted in boldface, whereas those that are unique in a subset of constructs only are shown in normal print.

and its derivative StrepTactin® (54), it further allows direct immobilization of its fusion partner on surfaces coated with these proteins. Binding of the Strep-tag II to StrepTactin® is readily reversible by low concentrations of desthiobiotin, thereby offering a gentle and highly selective method for the affinity purification of proteins and protein complexes (54,55). The tetracysteine tag, by contrast, has primarily been developed for *in vivo* protein localization studies. It tightly interacts with the membrane-permeable biarsenical dyes FlaSH (Lumio Green®) and ReAsH, forming stable yellow and red fluorescent complexes that can be detected by light microscopy (27). However, it has also been used successfully to affinity-purify proteins with the help of agarose beads that were covalently modified with a FlaSH derivative (56).

To avoid polar effects, resulting from the integration of a plasmid at the native locus of a gene, or to allow tagging of essential proteins even if their function is compromised by the fusion partner, hybrid genes are frequently put under the control of a regulatable promoter and integrated ectopically at a neutral locus. We, therefore, created modified versions of the aforementioned gene fusion vectors which carry the *xyiX* or *vanAB* promoter and translation initiation signals inserted upstream of

their MCS (Figures 6D and 7, MCS E), thus allowing xylose- or vanillate-inducible expression of the fusions they bear (Figures 8A and C). Moreover, since the promoter fragments chosen for this purpose comprise more than 1.5 kb of the *xyiX* or *vanA* upstream region, respectively, they are on average significantly longer than the genes of interest fused to the tag sequences. Consequently, they represent the major targets of homologous recombination, favoring integration of the plasmids at the  $P_{xyi}$  or  $P_{van}$  locus.

The list of integration vectors is completed by a similar series of constructs which are intended for the generation of N-terminally tagged hybrid proteins (Figures 6E and 8B). In this case, the tag sequences were cloned immediately downstream of the *xyiX* or *vanA* promoter fragments, with the MCS fused to their 3' ends (Figure 7, MCS F). Since their start codons overlap with an engineered NdeI restriction site, they can easily be removed and replaced with any gene of interest, which makes these plasmids also suitable for ectopic expression of alleles lacking a fusion partner under the control of  $P_{xyi}$  or  $P_{van}$ .

*Replicating plasmids.* In order to facilitate inducible overproduction of proteins in *Caulobacter*, we inserted



**Table 1.** Primers for DNA sequencing and analytical PCR reactions

Primer <sup>a</sup>	Sequence	Application
Pvan-for	5'-GACGTCCGTTTGATTACGATCAAGATTGG-3'	Sequencing of the region downstream of P <sub>van</sub>
Pxyl-for	5'-CCCACATGTTAGCGCTACCAAGTGC-3'	Sequencing of the region downstream of P <sub>xyl</sub>
M13-for <sup>b</sup>	5'-GCCAGGGTTTTCCAGTCACGA-3'	Reverse sequencing primer
eGYC-1	5'-GTTTACGTCCCGTCCAGCTCGAC-3'	Sequencing of the region upstream of <i>egfp</i> , <i>eyfp</i> , <i>ecfp</i> , <i>venus</i> , or <i>cerulean</i>
eGYC-2	5'-ATGGTCTGCTGGAGTTCGTGACC-3'	Sequencing of the region downstream of <i>egfp</i> , <i>eyfp</i> , <i>ecfp</i> , <i>venus</i> , or <i>cerulean</i>
RFP-1	5'-CTGAAGCGCATGAACTCCTTGATG-3'	Sequencing of the region upstream of <i>mrfp1</i> or <i>mCherry</i>
RFP-2	5'-CGAGGACTACACCATCGTGGAACAGTAC-3'	Sequencing of the region downstream of <i>mrfp1</i> or <i>mCherry</i>
RecUni-1 <sup>c</sup>	5'-ATGCCGTTTGTGATGGCTTCCATGTCC-3'	Test of recombinants for integration of a plasmid into the <i>vanA</i> or <i>xylX</i> locus
RecVan-2 <sup>c</sup>	5'-CAGCCTTGGCCACGGTTTCGGTACC-3'	Test for integration into the <i>vanA</i> locus
RecXyl-2 <sup>c</sup>	5'-TCTCCGGCAGGAATCACTCACGCC-3'	Test for integration into the <i>xylX</i> locus

<sup>a</sup>All primers are suitable for DNA sequencing under standard conditions. They have been optimized for PCR reactions performed with an annealing temperature of 65°C in the presence of 5% DMSO.

<sup>b</sup>Primer M13-for is not applicable for sequencing plasmids bearing the *tetRA* resistance cassette and plasmids encoding C-terminal protein fusions.

<sup>c</sup>RecUni-1 anneals to a region upstream of the promoter fragment in all P<sub>van</sub>- and P<sub>xyl</sub>-bearing integration plasmids, whereas RecVan-2 and RecXyl-2 anneal to the 5' region of the chromosomal *vanA* and *xylX* genes, respectively. Insertion of a construct into the *vanA* and *xylX* locus can be tested by colony PCR using the primer pairs RecUni-1/RecVan-2 and RecUni-1/RecXyl-2, respectively.

the *xylX* and *vanAB* promoters including the respective translation initiation signals into vectors whose replication is driven by the broad host range and mid-copy number replication origin of plasmid pBBR1 (15) (Figure 9A). The corresponding start codons are positioned in the context of an NdeI recognition sequence and are followed by an extensive MCS (Figure 7, MCS C). Together, these constructs offer a selection of three different resistance markers. They further possess all functions necessary for mobilization by the RK2 conjugative transfer machinery (50,57). Since our results showed that the amount of VanR produced from a single, chromosomal allele is not sufficient to tightly regulate multiple copies of its cognate promoter (Figure 5), the *vanR* gene was included in all P<sub>van</sub>-bearing plasmids. The regulator responsible for xylose-dependent induction of *xylX* has been identified recently, but it was found to impair growth at elevated levels (B. Christen, C. Stephens and U. Jenal, unpublished). This observation precluded incorporation of its gene into the P<sub>xyl</sub>-bearing overexpression vectors. However, to keep leaky expression at a minimum, a transcriptional terminator was placed upstream of the promoter fragment in these constructs, thus reducing unwanted transcription originating in the plasmid backbone.

A comparable set of vectors was also created based on a minimal version of the low-copy number and broad host range plasmid RK2 (17) (Figure 9B). In addition to their specific resistance determinants, these constructs all carry a  $\beta$ -lactamase (*bla*) gene, allowing the use of standard ampicillin plates for cloning purposes. They are stably maintained under selective conditions. However, since they lack a partitioning system, the plasmids are easily lost in the absence of antibiotics (data not shown), which makes them a versatile tool for gene function and complementation analyses. All vectors contain the RK2 origin of transfer and can be mobilized into *Caulobacter* by conjugation using a suitable host strain (50).

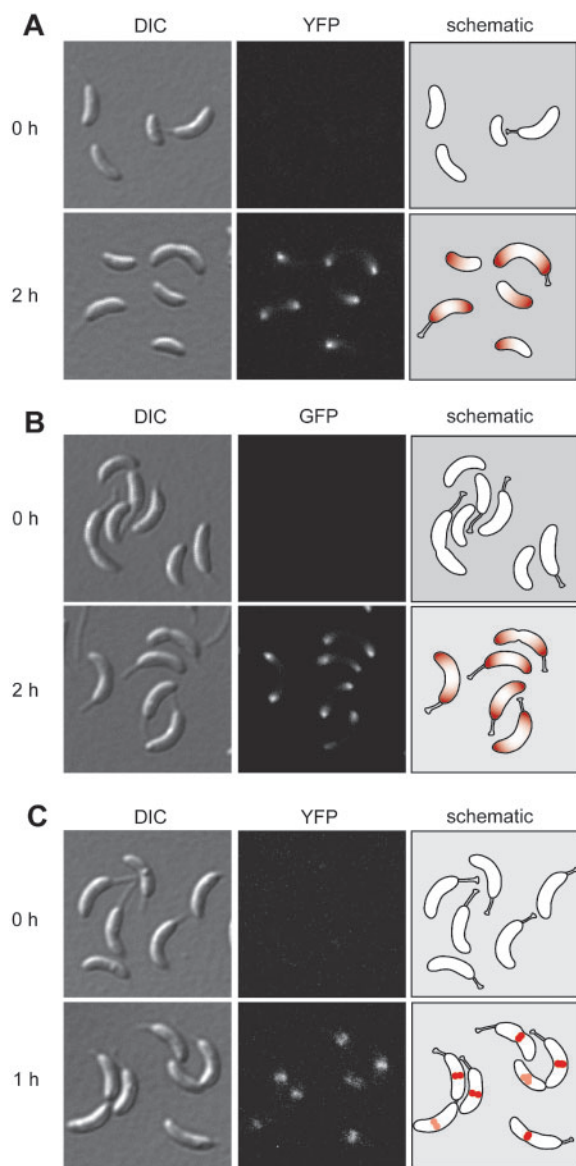
Finally, in order to cope with conditions that necessitate the synthesis of fluorescent protein fusions at levels higher than afforded by chromosomally integrated constructs,

we created two sets of replicating plasmids (Figures 9C and D) which allow the inducible expression of C- and N-terminally tagged proteins under control of the *vanAB* promoter and translation initiation signals. Based on the P<sub>van</sub>-bearing RK2 derivatives described above (Figure 9B), they offer a choice of three different resistance markers, an extensive MCS (Figure 7, MCS E and F), and a selection of fluorescent protein tags that covers all common cell biological applications.

## DISCUSSION

This work extends the molecular toolset for *Caulobacter* by a second inducible promoter and a variety of different gene fusion and expression vectors, thereby considerably facilitating the study of this model organism. Moreover, many of the constructs are also suitable for use as replicating or integrating vectors in other bacteria, complementing their genetic systems by adding additional resistance genes and a number of different protein fusion tags.

Our results demonstrate that *Caulobacter* is able to utilize the aromatic compound vanillate as the sole carbon source. The first step of vanillate degradation involves removal of the O-methyl group by a two-subunit vanillate demethylase complex, formed by the products of open reading frames CC2393 (*vanA*) and CC2394 (*vanB*). The reaction product, protocatechuate, is then funneled into the citric acid cycle via the  $\beta$ -keto adipate pathway. Expression of the *vanAB* operon is regulated by the GntR-type repressor CC2392 (*vanR*) and induced by vanillate as well as, to a smaller degree, by its reduced derivatives vanillin and vanillyl alcohol. Interestingly, while the enzymes constituting the  $\beta$ -keto adipate pathway are highly conserved among  $\alpha$ -proteobacteria, the closest homologs of CC2392, CC2393 and CC2394 are found in terrestrial and aquatic representatives of the  $\beta$ - (*Ralstonia*, *Burkholderia*) and  $\gamma$ -proteobacteria (*Pseudomonas*, *Acinetobacter*). This observation indicates that *Caulobacter* obtained the ability to degrade vanillate by horizontal



**Figure 8.** Application examples. (A) Expression of a *mipZ-eyfp* fusion, obtained by insertion of the *mipZ* gene into plasmid pVYFPC-1, under the control of  $P_{van}$ . Cells of strain MT232 ( $P_{van}$ -*mipZ-eyfp*) were examined by DIC and fluorescence microscopy before and 2h after induction with 0.5mM vanillate. Note: MipZ is a spatial regulator mediating proper positioning of the cell division apparatus in *Caulobacter* (10). It forms a complex with the DNA partitioning protein ParB at the chromosomal origin of replication. Before initiation of chromosome replication, the MipZ-ParB-origin complex localizes to the flagellated pole of the cell. After entry into S phase, MipZ and ParB re-associate with the newly synthesized origin regions, generating two nucleoprotein complexes which are positioned at the two opposite cell poles. (B) Expression of an *egfp-mipZ* fusion, created using plasmid pXGFPN-2, under the control of  $P_{xyt}$ . The images show cells of strain MT236 ( $P_{xyt}$ -*egfp-mipZ*) before and two hours after induction with 0.03% xylose. (C) Expression of an *ftsZ-eyfp* fusion, constructed with the help of plasmid pVCHYC-3, under the control of  $P_{van}$ . Cells were withdrawn from a culture of strain MT240 ( $P_{van}$ -*ftsZ-eyfp*) before and 1h after induction with 0.5mM vanillate and analyzed by microscopy. Note: The tubulin homologue FtsZ is a fundamental component of the bacterial cell division machinery. Upon initiation of chromosome replication and bipolar positioning of MipZ, it forms a ring-like structure at the cell center which recruits the other constituents of the cytokinetic ring and plays an essential role in the constriction process (10,60).

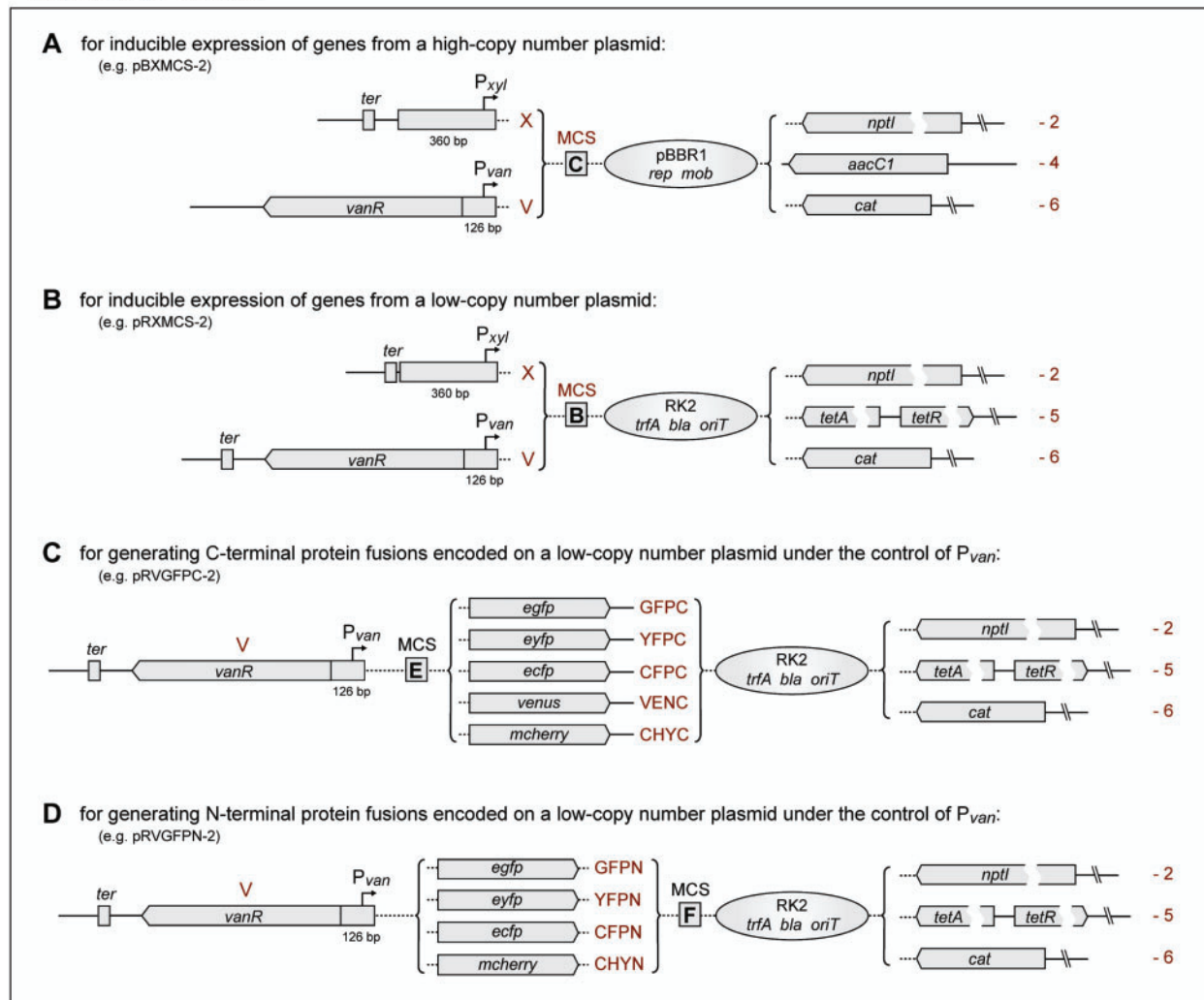
gene transfer from distantly related bacteria sharing the same environmental niche.  $P_{van}$  is tightly repressed in the absence of inducer, while vanillate concentrations in the nanomolar range are sufficient to stimulate its activity. In addition, expression of *vanAB*—like that of xylose-regulated genes (7)—is not subject to catabolite repression. These characteristics might reflect an adaptation of *Caulobacter* to the nutrient-poor environments it inhabits, facilitating the simultaneous utilization of multiple carbon sources, even if present in trace amounts only.

Due to its tight regulation, its titratability, and its low sensitivity to the presence of sugars,  $P_{van}$  is well suited as a general purpose gene expression system for *Caulobacter* which can be employed in parallel to the established xylose-inducible promoter (7). Its use is facilitated by a newly constructed series of integrating and replicating shuttle vectors, which cover all cell biological and physiological standard applications, such as the ectopic expression of native genes and gene fusions, the overproduction of proteins, and the construction of depletion strains. Generating a similar set of  $P_{xyt}$ -based plasmids, we created a standardized and versatile framework for inducible gene expression in *Caulobacter*, which considerably accelerates the process of plasmid construction and the swapping of promoters, resistance markers, and protein fusion tags. The performance of the new vectors is demonstrated by several application examples given in this (Figure 8) and previous (10,58,59) work. These studies indicate that, when used for the expression of fluorescent protein gene fusions,  $P_{van}$  generally yields results that are qualitatively similar to those achieved with  $P_{xyt}$  (Figures 8A and B). However, because of its lower transcriptional activity (data not shown), it might be superior for the control of weakly transcribed genes, avoiding negative side-effects resulting from overexpression. Due to its tight regulation, it is further particularly valuable for protein depletion studies. Our data show that vanillate is consumed within a few hours after addition to uninduced cells (Figure 1E). Thus, in cases where extended induction times or tightly controlled expression levels are required, the *vanA*-deficient CB15N derivative MT231 is recommended as a host strain to avoid exhaustion of the inducer pool. We further observed that elevated levels of vanillate reduce the growth rate of *Caulobacter* in a medium-dependent manner. Based on our results, we therefore suggest inducer concentrations lower than 0.5mM in minimal medium and 1mM in rich medium, which still allow near-maximal induction of  $P_{van}$  without adversely affecting the physiology of the cell.

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Annotated sequence files in GenBank format are available for all vectors and may be obtained upon request. The integrity of the constructs has been verified by DNA sequence analysis. Material containing patented components will only be distributed to individuals holding a valid user licence. We are grateful to Esteban Toro for help with the initial characterization of the *vanAB* promoter. We further thank Svein Valla, Atsushi Miyawaki,

## Replicating Plasmids



**Figure 9.** Replicating plasmids. Shown are four sets of replicating shuttle vectors designed for the inducible expression of genes from (A) a high-copy number or (B) a low-copy number plasmid and for the construction of (C) C-terminal and (D) N-terminal fluorescent protein fusions, expressed under the control of  $P_{van}$  from a low-copy number plasmid. The nomenclature of these plasmids follows the same rules as described in Figure 6, but the prefix is 'pB' for high-copy number plasmids and 'pR' for low-copy number plasmids. The gene names used are as follows: pBBR1 replication protein (*rep*), pBBR1 mobilization protein (*mob*), RK2 replication initiator protein (*trfA*),  $\beta$ -lactamase (Amp<sup>R</sup>, *bla*). All other gene designations are detailed in Figure 6.

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