Transcriptional Regulation of the Vanillate Utilization Genes (vanABK Operon) of Corynebacterium glutamicum by VanR, a PadR-Like Repressor

Kambiz Morabbi Heravi, a Julian Lange, a Hildegard Watzlawick, a Jörn Kalinowski, b Josef Altenbuchner a

Institut für Industrielle Genetik, Universität Stuttgart, Stuttgart, Germany; a Microbial Genomics and Biotechnology, Center for Biotechnology, Bielefeld University, Bielefeld, Germany

Corynebacterium glutamicum is able to utilize vanillate, the product of lignin degradation, as the sole carbon source. The vanillate utilization components are encoded by the vanABK operon. The vanA and vanB genes encode the subunits of vanillate O-demethylase, converting vanillate to protocatechuate, while VanK is the specific vanillate transporter. The vanABK operon is regulated by a PadR-type repressor, VanR. Heterologous gene expression and variations of the vanABK operon revealed that the functional VanR contains 192 residues (21 kDa) and forms a dimer, as analyzed by size exclusion chromatography. In vivo, ferulate, vanillin, and vanillate induced P vanABK in C. glutamicum, while only vanillate induced the activity of P vanABK in Escherichia coli lacking the ferulate catabolic system. Differential scanning fluorimetry verified that vanillate is the only effector of VanR. Interaction between the P vanABK DNA fragment and the VanR protein had an equilibrium dissociation constant (Kd) of 15.1 ± 1.7 nM. The VanR-DNA complex had a dissociation rate constant (k0) of (267 ± 23) × 10⁻⁶ s⁻¹, with a half-life of 43.5 ± 3.6 min. DNase I footprinting localized the VanR binding site at P vanABK extending from +9 to +45 on the coding strand. Deletion of the nucleotides +18 to +27 inside the VanR binding site rendered P vanABK constitutive. Fusion of the T7 promoter and the wild-type VanR operator, as well as its shortened versions, indicated that the inverted repeat AACTAACTAA(N4)TTAGGTATTT is the specific VanR binding site. It is proposed that the VanR-DNA complex contains two VanR dimers at the VanR operator.

Plant biomass is the main carbon supply in the soil. The plant cell wall is a lignocellulosic complex consisting of hydrophilic polymers, i.e., cellulose, hemicellulose, and pectin, along with the hydrophobic aromatic heteropolymer lignin (1, 2). The integrity of the plant cell wall depends on its lignin content, which is cross-linked to polysaccharides by hydroxycinnamic acids bridges, mainly ferulate (3–5). These feruloylated polysaccharides, especially the ferulate-arabinosylan complex, serve as initiation sites for lignification in the cell walls (6, 7). Lignin is degraded by lignolytic enzymes, including lignin peroxidase, manganese peroxidase, or laccase, into β-aryl ether, di-aryl ether, and biphenyl, which are further catabolized to other aromatic compounds, such as vanillin and vanillate. Likewise, ferulate bound through ester linkages to hemicellulose is released by esterases and degrades to vanillin and vanillate (for a review, see references 4, 5, 8, 9, and 10).

Phenolic acids released by degradation of lignin, e.g., ferulate or p-coumarate, are toxic for many Gram-positive bacteria, such as Bacillus subtilis, at low pH. Thus, there is a system for phenolic acid stress response which detoxifies phenolic acid by decarboxylation and generation of vinyl phenol derivatives (11). In contrast to B. subtilis, Corynebacterium glutamicum utilizes ferulate, vanillin, and vanillate derived from lignin degradation as a carbon source. Generally, aromatic compounds are channeled via gentisate, catechol, protocatechuate, 1,2,4-trihydroxybenzene, or phenylacetyl coenzyme A (phenylacetyl-CoA) intermediates into the central carbon metabolism of C. glutamicum (12). Ferulate is catabolized via vanillin and vanillate as the intermediate products to protocatechuate (3,4-dihydroxybenzoate) (see Fig. S1 in the supplemental material) (13). Protocatechuate is further metabolized in the aerobic β-ketoacidate pathway and finally flows into the carbon and energy cycle (14). So far, only the genes for degradation of vanillate are identified in C. glutamicum. Conversion of vanillate to protocatechuate is carried out by vanillate O-demethylase (see Fig. S1) (13). The vanillate O-demethylase enzyme has two subunits, which are encoded by vanA (NCgl2300) and vanB (NCgl2301) (13, 15). In addition to vanillate O-demethylase, the vanillate utilization system consists of a vanillate transporter encoded by vanK (NCgl2302), which forms the vanABK operon along with vanA and vanB (16). Transcription of the vanABK operon is regulated by VanR (NCgl2299), which forms a divergent with the vanABK operon (12).

VanR belongs to the PadR-like transcriptional regulator family (17). The PadR-like protein family (Pfam accession no. PF03551) contains 26 reported structures deposited in the Protein Data Bank (http://wwrcsb.org/). Generally, PadR-like regulators play an important role in their bacterial host, especially concerning virulence and stress. Structurally, PadR-type regulators contain a highly conserved N-terminal winged helix-turn-helix (wTH)
domain with about 80 to 90 residues which is responsible for the binding of these regulatory proteins to their target DNA. In addition to the wHTH domain, there is a variable C-terminal domain in PadR-like proteins which is involved in dimerization of these proteins. Depending on the length of this C-terminal domain, PadR-like proteins have been classified into two subfamilies. Subfamily 1 has a C-terminal domain of 80 to 90 residues, such as AphA from Vibrio cholerae (18), LadR from Listeria monocytogenes (19), and PadR from Pedicococcus pentosaceus, Lactobacillus plantarum, or Bacillus subtilis (11, 20, 21). The proteins of subfamily 2 have a shorter C-terminal domain than subfamily 1 and contain 20 to 30 residues, such as LmrR from Lactococcus lactis (22) as well as Bacillus cereus PadR1 (bcPadR1) and bcPadR2 (23). Apart from their structures, physiological characteristics of the PadR, AphA, and LmrR regulators were intensively studied (21, 24–26). Intriguingly, the very first member of the PFO3551 family protein, PadR, also deals with phenolic acids, albeit as a stress response regulator in B. subtilis (20, 21). PadR represses padC, encoding the phenolic acid decarboxylase in B. subtilis. Nevertheless, the exact deactivation mechanism of PadR is unknown, since phenolic acid does not directly interact with PadR (25).

So far, studies concerning the PadR-like protein family were focused mainly on transcriptional regulators which are not involved in the catabolic pathways. In this study, another PadR-like protein, VanR, was intensively studied in order to understand the physiological characteristics of a PadR-like regulator involved in the metabolic pathways of C. glutamicum. For the first time, we show the direct interaction of VanR, as a member of the PadR-like regulator family, with vanillate (effector), which is necessary for deactivation of the protein. The interaction of VanR and its target DNA at the promoter region of vanABK (P_vanABK) also was thoroughly studied.

MATERIALS AND METHODS

Strains, media, and growth conditions. All bacterial strains used in this study are listed in Table S1 in the supplemental material. Escherichia coli strain JM109 was used for plasmid propagation and gene expression studies, while strain BL21-Star(DE3) was used for gene expression by P_T7. E. coli strains were cultivated in lysogeny broth (LB) at 37°C (27). Another host for expression studies, Corynebacterium glutamicum ATCC 13032 and its derivatives were incubated at 30°C. Brain heart infusion complex medium (BHI) (Bacto brain heart infusion; Becton, Dickinson and Company, USA) was used for cultivation of C. glutamicum (28). For growth in minimal medium, a modified CGXII medium containing (NH4)2SO4 (5 g liter⁻¹), urea (5 g liter⁻¹), KH2PO4 (1 g liter⁻¹), K2HPO4 (1 g liter⁻¹), MgSO4 · 7 H2O (0.25 g liter⁻¹), 3-([N-morpholino]propanesulfonic acid (11, 20, 21). PadR represses padC, encoding the phenolic acid decarboxylase in B. subtilis. Nevertheless, the exact deactivation mechanism of PadR is unknown, since phenolic acid does not directly interact with PadR (25).

So far, studies concerning the PadR-like protein family were focused mainly on transcriptional regulators which are not involved in the catabolic pathways. In this study, another PadR-like protein, VanR, was intensively studied in order to understand the physiological characteristics of a PadR-like regulator involved in the metabolic pathways of C. glutamicum. For the first time, we show the direct interaction of VanR, as a member of the PadR-like regulator family, with vanillate (effector), which is necessary for deactivation of the protein. The interaction of VanR and its target DNA at the promoter region of vanABK (P_vanABK) also was thoroughly studied.

MATERIALS AND METHODS

Strains, media, and growth conditions. All bacterial strains used in this study are listed in Table S1 in the supplemental material. Escherichia coli strain JM109 was used for plasmid propagation and gene expression studies, while strain BL21-Star(DE3) was used for gene expression by P_T7. E. coli strains were cultivated in lysogeny broth (LB) at 37°C (27). Another host for expression studies, Corynebacterium glutamicum ATCC 13032 and its derivatives were incubated at 30°C. Brain heart infusion complex medium (BHI) (Bacto brain heart infusion; Becton, Dickinson and Company, USA) was used for cultivation of C. glutamicum (28). For growth in minimal medium, a modified CGXII medium containing (NH4)2SO4 (5 g liter⁻¹), urea (5 g liter⁻¹), KH2PO4 (1 g liter⁻¹), K2HPO4 (1 g liter⁻¹), MgSO4 · 7 H2O (0.25 g liter⁻¹), 3-([N-morpholino]propanesulfonic acid (11, 20, 21). PadR represses padC, encoding the phenolic acid decarboxylase in B. subtilis. Nevertheless, the exact deactivation mechanism of PadR is unknown, since phenolic acid does not directly interact with PadR (25).

So far, studies concerning the PadR-like protein family were focused mainly on transcriptional regulators which are not involved in the catabolic pathways. In this study, another PadR-like protein, VanR, was intensively studied in order to understand the physiological characteristics of a PadR-like regulator involved in the metabolic pathways of C. glutamicum. For the first time, we show the direct interaction of VanR, as a member of the PadR-like regulator family, with vanillate (effector), which is necessary for deactivation of the protein. The interaction of VanR and its target DNA at the promoter region of vanABK (P_vanABK) also was thoroughly studied.

MATERIALS AND METHODS

Strains, media, and growth conditions. All bacterial strains used in this study are listed in Table S1 in the supplemental material. Escherichia coli strain JM109 was used for plasmid propagation and gene expression studies, while strain BL21-Star(DE3) was used for gene expression by P_T7. E. coli strains were cultivated in lysogeny broth (LB) at 37°C (27). Another host for expression studies, Corynebacterium glutamicum ATCC 13032 and its derivatives were incubated at 30°C. Brain heart infusion complex medium (BHI) (Bacto brain heart infusion; Becton, Dickinson and Company, USA) was used for cultivation of C. glutamicum (28). For growth in minimal medium, a modified CGXII medium containing (NH4)2SO4 (5 g liter⁻¹), urea (5 g liter⁻¹), KH2PO4 (1 g liter⁻¹), K2HPO4 (1 g liter⁻¹), MgSO4 · 7 H2O (0.25 g liter⁻¹), 3-([N-morpholino]propanesulfonic acid (11, 20, 21). PadR represses padC, encoding the phenolic acid decarboxylase in B. subtilis. Nevertheless, the exact deactivation mechanism of PadR is unknown, since phenolic acid does not directly interact with PadR (25).

So far, studies concerning the PadR-like protein family were focused mainly on transcriptional regulators which are not involved in the catabolic pathways. In this study, another PadR-like protein, VanR, was intensively studied in order to understand the physiological characteristics of a PadR-like regulator involved in the metabolic pathways of C. glutamicum. For the first time, we show the direct interaction of VanR, as a member of the PadR-like regulator family, with vanillate (effector), which is necessary for deactivation of the protein. The interaction of VanR and its target DNA at the promoter region of vanABK (P_vanABK) also was thoroughly studied.
DNA as a template. Oligonucleotides s8522/s8523 (downstream flanking) and s8524/s8525 (upstream flanking) were used in PCRs. Both amplified DNA fragments were merged in an additional fusion PCR using oligonucleotides s8523 and s8524. The deletion cassette then was inserted into the pK19mobsacB plasmid via an XbaI restriction site, yielding pJUL20.198. Deletion of vanAB was carried out by transformation of C. glutamicum with pJUL20.198. The kanamycin-resistant transformants were selected on BHI plates supplemented with kanamycin (15 μg ml\(^{-1}\), 2 days of incubation). The kanamycin-resistant colonies were the result of the integration of whole pJUL20.198 into the C. glutamicum chromosome via a single crossover. A single colony containing the pUL20.198 plasmid integrated into its chromosome next was cultivated in BHI (without kanamycin) for 24 h at 30°C so that the second crossover takes place. Since the second crossover could result in the loss of complete plasmid (generating wild-type [wt] cells) or only the plasmid backbone (generating the vanAB mutant), the relevant dilutions of the cell suspension were plated on BHI plates with 10% (wt/vol) sucrose. Expression of saeR encoding levanucrase is lethal for C. glutamicum in the presence of sucrose; therefore, the growth on sucrose-containing media selected wild-type cells or cells with the deletion of vanAB. The deletion of vanAB finally was verified using oligonucleotides s8523 and s8524 in a PCR as well as by sensitivity of the strain to kanamycin.

**Overexpression of the vanR variants in E. coli** JM109. JM109 strains containing pJUL21.11 (vanR192), pJUL22.1 (vanR177), pJUL23.1 (vanR164), or pJUL24.1 (vanR146) were cultivated in LB at 37°C. When the bacterial culture reached an OD\(_{600}\) of 0.4, 0.2% (wt/vol) L-rhamnose, or pJUL24.1 (vanR192), pJUL23.1 (vanR177), kanamycin.

The bacterial lysate was prepared by disruption of the crude extracts were supplemented with 20 mM MgCl\(_2\) and Benzo- nase (2 μl per 10 mg crude extract proteins) and incubated for 15 min at room temperature, and the incubation was continued for another 15 h at 4°C. The cleared bacterial lysate, containing approximately 25 μg E. coli protein, was loaded onto a heparin column (5 ml Hi-Trap heparin; GE Healthcare) connected to an fast protein liquid chromatography (FPLC) system (Pharmacia Biotech, Uppsala, Sweden). The retained proteins were eluted by applying a linear gradient of 1 M NaCl in buffer A (to 100%). The VanR protein eluted with 20% buffer B as analyzed by SDS-PAGE and electrophoretic mobility shift assay. Fractions containing the VanR protein were combined, diluted with buffer A to approximately 50 mM NaCl, and applied to a MonoQ HR5/5 column. Here, the VanR protein was not retained and eluted with the flowthrough, whereas further protein impurities were retained on the column and eluted with a linear gradient. The VanR pool of the flowthrough was dialyzed and concentrated with Millipore devices (10-kDa cutoff) to 50 mM Tris-HCl, 150 mM NaCl, pH 7.0, and stored at 4°C. The purified VanR protein was stable for a week at 4°C. SDS-PAGE analysis of the purified VanR indicated a single protein band with a molecular mass of 21 kDa. The protein concentration was measured as described by Bradford (37).

The molecular size of the native purified VanR protein was determined by size exclusion chromatography. A high-performance liquid chromatography (HPLC) system (Merck Hitachi, Darmstadt, Germany), consisting of L-7100 pump, L-7000 interface module, Rhodyne sample injector 9725i with 100-μl sample loop, D-7000 HPLC system manager software, and an S3205 UV-visible light (UV-Vis) detector (Sakym GmbH, Gilching, Germany), were used for the size exclusion chromatography. Two TSK-GEL G3000SWXL columns (7.8 by 300 mm, 5 μm) (Tosoh Bioscience, Stuttgart, Germany) were connected to each other and used for chromatographic separation at room temperature. As the mobile phase, 100 mM Tris-HCl (pH 7.0) containing 150 NaCl with a flow rate of 0.5 ml min\(^{-1}\) was applied. The protein size standards were aprotinin (6.5 kDa), RNase A (13.7 kDa), carbonic anhydrase (29 kDa), ovalbumin (44 kDa), and blue dextran (2,000 kDa) (GE Healthcare, Uppsala, Sweden). Additionally, bovine serum albumin (BSA) (66 kDa) was utilized (Bio-Rad Laboratories GmbH, Munich, Germany). Twenty micrograms of each protein in a maximal volume of 50 μl was injected for analysis.

**EMSA.** 5'-end Cy5-labeled DNA fragments were synthesized in PCRs using Cy5-labeled oligonucleotides. Cy5-labeled P\(_{\text{vanAB}}\) DNA fragments containing either wild-type or modified sequences were amplified with oligonucleotides s9071 and s9177 from pJOE8077.1, pJOE8296.2, pJOE8297.6, pJOE8299.3, pJOE8300.2, and pJOE8298.4. Unless otherwise specified, electrophoretic mobility shift assays (EMSA) were carried out in a total volume of 20 μl containing 1 μl Cy5-labeled P\(_{\text{vanAB}}\) DNA fragment (100 fmol μl\(^{-1}\)), 4 μl of 5% pre-stained buffer (50 mM Tris-HCl, pH 7.5; 250 mM KCl; 10 mM dithiothreitol [DTT]; 25% [vol vol\(^{-1}\)] 1 glycerol; 250 μg ml\(^{-1}\) BSA; 25 μg ml\(^{-1}\) herring sperm DNA). To obtain a clear shifted band, different amounts of the purified VanR (or VanR-Strep tag II) were used. After addition of the components, the reaction mixture was incubated on ice for at least 15 min, and 10 μl of the reaction mix was loaded onto a 6% (wt/vol) native polyacrylamide gel. The gel was run at 20 mA for 40 min to separate the free DNA and DNA-protein complexes. The migration of the bands of free DNA and the DNA-protein complexes was visualized by a PhosphorImager (Storm 860 PhosphorImager; Molecular Dynamics).

**Determination of equilibrium dissociation constant and dissociation rate.** The DNA binding properties of VanR, including the equilibrium dissociation constant (K\(_D\)) and the dissociation rate constant (KD), were determined as described before (38, 39). Calculation of K\(_D\) was carried out in a total volume of 20 μl by mixing various amounts of purified VanR-Strep tag II (1,754, 175, 58.5, 17.5, 11.6, 8.7, 5.8, and 5 nM) with 2.5 nM Cy5-labeled P\(_{\text{vanAB}}\) DNA fragment amplified from pJOE8077.1. The reactions were carried out on ice for 15 min, and 10 μl of each reaction mixture was loaded onto a native polyacrylamide gel. After electrophoresis, the density of each Cy5-labeled DNA band was analyzed using ImageQuant software (version 5.0; Molecular Dynamics, Sunnyvale, CA). The ratio of free DNAs and protein-DNA complexes was calculated and plotted over the amount of VanR added to each reaction mixture. The K\(_D\) value was calculated from the plot as the amount of VanR required to shift 50% of the Cy5-labeled DNA band. The half-life of the VanR-DNA(P\(_{\text{vanAB}}\)) complex and the K\(_D\) were studied by mixing 2.5 nM Cy5-labeled P\(_{\text{vanAB}}\) DNA fragment amplified from pJOE8077.1 and 58.5 nM purified VanR-Strep tag II in a total volume of 100 μl. The reaction mixture was incubated on ice for 10 min, followed by the addition of a 50-fold molar excess of nonlabeled P\(_{\text{vanAB}}\) DNA fragment amplified from pJOE7658.2 with oligonucleotides s8754 and s8823. Samples were taken at 10-min intervals and loaded onto a
native polyacrylamide gel with the current switched on. The intensity of each VanR-DNA complex was measured and analyzed with the ImageQuant software package and plotted logarithmically over time. The $K_D$ value was calculated according to the common decay law from the best-fit straight line with the equation $\ln([DNA-VanR]_0 - [DNA-VanR]) = -K_D t$. [DNA-VanR]$_0$, shows the concentration of DNA-VanR complex at time 0, and [DNA-VanR]$_t$ represents the concentration of DNA-VanR complex at the beginning of the reaction. The half-life of the VanR-DNA complex was calculated according to the equation $t_{1/2} = \ln 2/K_D$. All experiments were repeated independently at least three times.

**RESULTS**

**Transcriptional analysis of the vanR-vanABK region in *C. glutamicum***. The whole *C. glutamicum* transcriptome was analyzed recently, describing promoters, transcripts, and operons in a mixed RNA sample harvested under different growth conditions (42). In this way, it was intended to be as comprehensive as possible, since many, if not all, transcripts should be represented by the data sets. The data sets were screened for the transcriptional organization of the vanR-vanABK gene cluster and revealed the following (Fig. 1A). The monocistronic vanR gene is transcribed leaderless, with the first G of the GUG translation start codon as the transcription start site. The vanR promoter has the sequence tggGACAAT-N6-G, including an extended −10 region (the −10 hexamer is underlined, the 5′ extension is in lowercase letters, and the transcription start is in boldface) and does not show a −35 motif (Fig. 1B).

The genes vanABK form an operon and can be transcribed by a promoter located upstream of vanA (Fig. 1A). This promoter has the sequence tggGACAAT-N6-G, providing a consensus −35 region (Fig. 1B). In the RNA-seq data, this promoter probably was only weakly induced in (one of) the chosen conditions, and the vanABK transcript has a leader sequence of 51 bases, including a ribosome-binding site (Fig. 1A).

**Structure of P_{vanABK} and enhancing its strength**. Analysis of the P_{vanABK} sequence indicated the TTGACA sequence as a perfect −35 box, whereas the −10 box (CAATAT) was less conserved. Although the CAATAT sequence was proposed previously as the −10 box of P_{vanABK} (44), TATAT or AATATA (a and b lines; Fig. 2A) also were predicted as the probable −10 boxes of P_{vanABK} (12, 13). Here, the spacer sequences in both cases (TATAT or AATATA) are members of the 17-bp consensus spacer sequence of the housekeeping promoters. Hence, to study the core elements and generally improve the P_{vanABK} activity, a P_{vanABK}-eGFP gene fusion was inserted into a pCG1 (*E. coli*) shuttle vector containing P_{vanR}-vanR in the opposite direction, similar to their genetic organization at the wild-type operon (pJOE7658.1) (see Fig. S2 in the supplemental material). The expression of the eGFP gene from the wild-type promoter was investigated in *C. glutamicum* harboring the plasmid pJOE7658.1. For cultivation, CGXII medium containing 0.5% (wt/vol) glucose, with and without valilliant (5 mM) as an inducer, was used. Comparison of the control (no valilliant) and induced bacterial culture indicated a 12.5-fold induction of eGFP production (Fig. 2A).

To verify that the CAATAT sequence is the real −10 box of P_{vanABK} (framed in Fig. 2A), the single C nucleotide was replaced by T (CAATAT→TATAT). This single-nucleotide exchange tremendously increased the maximal eGFP expression level without significant influence on the basal eGFP expression level (73-fold induction in *C. glutamicum* pJOE7747.1) (Fig. 2A). In addition to −10 modifications, mutation of the conserved −35 box drastically decreased the expression level of eGFP, as shown by *C. glutamicum* pJOE8054.9, although it is described that *C. glutamicum* promoters do not share a highly conserved −35 region in general (44). In addition to promoter core elements, a GlxR binding sequence has been found inside the spacer.
sequence of P\textsubscript{vanABK} (Fig. 2A, gray shading) (45). The binding site of GlxR also was mutated in order to render P\textsubscript{vanABK} independent of GlxR activity (pJOE7936.1) (Fig. 2A). The modified P\textsubscript{vanABK} promoter on pJOE7936.1 showed activity similar to that of P\textsubscript{vanABK} on pJOE7747.1, indicating that the mutation inside the spacer sequence did not significantly alter the strength of P\textsubscript{vanABK}, although its basal activity slightly increased (Fig. 2A). Overall, mutations of the \(10\) and \(35\) boxes confirmed the P\textsubscript{vanABK} core elements, and the \(10\) box mutation enhanced the P\textsubscript{vanABK} strength. Therefore, enhanced P\textsubscript{vanABK} on pJOE7936.1 was used for further studies.

To test whether VanR also can repress the P\textsubscript{vanABK} activity in a heterologous host, E. coli JM109 was transformed with pJOE7936.1. As mentioned before, the shuttle vector pJOE7936.1 contained P\textsubscript{vanABK}-VanR in the orientation opposite that of the P\textsubscript{vanABK}-eGFP cassette. As a result, an active VanR should repress the activity of P\textsubscript{vanABK} and reduce the expression level of the eGFP reporter gene. The production of eGFP then was measured after 24 h of cultivation of JM109 pJOE7936.1 in LB with or without 2 mM vanillate. Since the production of eGFP was not influenced by the addition of vanillate (Fig. 2B), we assumed that either P\textsubscript{vanR} was not active in E. coli or VanR had no functional conformation. In order to test the first possibility, two alternative strategies were tested to provide a sufficient amount of active VanR. The first strategy used a second pACYC184-based plasmid encoding a constitutively expressed VanR. Hence, plasmid pJOE8097.3 was constructed from which VanR was constitutively expressed by the tetA promoter (P\textsubscript{tetA}) and was introduced into the E. coli strain containing the P\textsubscript{vanABK}-eGFP reporter cassette on the first plasmid. A second strategy was to create an autoregulative system, where VanR was inserted downstream of the eGFP gene (P\textsubscript{vanABK}-eGFP gene-VanR on pJOE8077.1). eGFP expression in E. coli showed that both strategies were successful and the expression of eGFP was repressed in both cases, while the addition of vanillate induced eGFP production (Fig. 2B). Since VanR repressed its own promoter in JM109 pJOE8077.1, the eGFP level was significantly lower than that of JM109 with both pJOE7936.1 and pJOE8097.3. All in all, the latter experiment showed that VanR functions in E. coli, although its promoter, P\textsubscript{vanR}, was not recognized by this heterologous host.

Identification of the translation start site of vanR. The vanR open reading frame (ORF) is located upstream and in the opposite direction from the vanABK operon (see Fig. S2 in the supplemental material). A database search demonstrated four potential vanR gene
translation products with different translation start sites and the following amino acid lengths: 192 (21.6 kDa; NP_601583.2), 177 (20.0 kDa; WP_003859272.1), 164 (18.8 kDa; YP_008067014.1), and 146 (16.7 kDa; YP_001139170.1). These results, together with those from the RNA-seq study, pointed out that if the VanR protein contained 192 residues, the VanR protein was translated from a leaderless mRNA. To clarify the beginning of the vanR open reading frame, all four of these vanR variants were inserted into an E. coli expression plasmid, pJOE5751.1, where the genes were expressed by rhamnose-inducible rha-PBAD (34). These vanR-containing plasmids, namely, pJUL21.11 (VanR192), pJUL22.1 (VanR177), pJUL23.1 (VanR164), and pJUL24.1 (VanR146), were introduced into E. coli JM109. Preliminary studies showed that VanR146 could not be produced in significant soluble amounts, indicating an incomplete VanR product, which presumably is degraded in E. coli (see Fig. S3). VanR164 and VanR177 were produced almost as inclusion bodies, and no significant soluble protein was detected (see Fig. S3). Even the reduction of the production temperature could not increase their solubility (data not shown). Only strain JM109 pJUL21.11 could successfully produce soluble VanR192, proposing the possible correct ORF (see Fig. S3).

To verify our results from VanR production in E. coli, the potential start codon of vanR192 was mutated. In this experiment, the expression of eGFP in E. coli JM109 carrying plasmids with the autoregulatory P_{vanABK}-eGFP gene cassette was studied. P_{vanABK} was induced by the addition of 5 mM vanillate into CGXII medium supplemented with 0.5% (wt/vol) glucose, and the fluorescence intensity was measured after 16 h of cultivation. Mean values and SD (error bars) of the fluorescence intensity from three independent experiments using a cell suspension at an OD_{600} of 0.1 are indicated. (B) Activity of the P_{vanABK}-VanR regulatory system in E. coli. vanR was expressed by P_{vanR} on pJOE7936.1 (wt) or P_{tetA} on pOE08079.3, or it was expressed within the P_{vanABK}-eGFP gene-vanR autoregulatory cassette on pOE8077.1. The vanR-Strep tag II was expressed by P_{tetA} on pHWG1124.9. Strain JM109, containing the desired plasmid(s), was cultivated in LB medium with or without 2 mM vanillate, and the fluorescence intensity was measured after 24 h of incubation. Mean values and standard deviations (SD) (error bars) of the fluorescence intensity of the cell suspension at an OD_{600} of 0.1 from three independent experiments are shown.

FIG 2 (A) Activity of P_{vanABK} and derivatives in C. glutamicum. Sequence alignment of the wild-type (wt) P_{vanABK} (pJOE7658.1) and the constructs thereof (pJOE7747.1, pJOE7936.1, and pJOE8054.9) are indicated. Identical nucleotides are shown as dots, while the mutations are represented as letters. The transcription start site as reported before (13), as well as −10 and −35 boxes, are framed and shown by boldface letters. The lines a and b mark the −10 boxes proposed in previous studies (12, 13). The binding site of the GlxR global regulator is highlighted in gray (45). Production of eGFP in C. glutamicum containing pJOE7658.1 (wt), pJOE7747.1, or pJOE7936.1 with the vanR−P_{vanR−P_{vanABK}}−eGFP gene cassette was studied. P_{vanABK} was induced by the addition of 5 mM vanillate into CGXII medium supplemented with 0.5% (wt/vol) glucose, and the fluorescence intensity was measured after 16 h of cultivation. Mean values and SD (error bars) of the fluorescence intensity from three independent experiments using a cell suspension at an OD_{600} of 0.1 are indicated. (B) Activity of the P_{vanABK}-VanR regulatory system in E. coli. vanR was expressed by P_{vanR} on pJOE7936.1 (wt) or P_{tetA} on pOE08079.3, or it was expressed within the P_{vanABK}-eGFP gene-vanR autoregulatory cassette on pJOE8077.1. The vanR-Strep tag II was expressed by P_{tetA} on pHWG1124.9. Strain JM109, containing the desired plasmid(s), was cultivated in LB medium with or without 2 mM vanillate, and the fluorescence intensity was measured after 24 h of incubation. Mean values and standard deviations (SD) (error bars) of the fluorescence intensity of the cell suspension at an OD_{600} of 0.1 from three independent experiments are shown.
Determination of the effectors of VanR. Ferulate and vanillate previously were proposed as the VanR effectors (13). To identify the effector metabolite of VanR, ferulate, vanillate, vanillin, and vanillyl alcohol were tested. Vanillin is an intermediate metabolite formed during the catabolism of ferulate to vanillate (see Fig. S1 in the supplemental material). Unlike vanillin, vanillyl alcohol is not an intermediate compound of the proposed ferulate metabolic pathway in C. glutamicum (see Fig. S2). Nonetheless, it was used due to its structural resemblance to the natural metabolites of the ferulate pathway. Prior to testing these possible effectors, the vanAB genes encoding the vanillate O-demethylase subunits were deleted from the genome of C. glutamicum in order to inhibit the degradation of inducers. In this way, strain JL1 (ΔvanAB) was constructed in which the inducibility of P_{vanABK} by ferulate was significantly prolonged (data not shown). JL1 harboring the optimized P_{vanABK}-eGFP gene plasmid pJOE7936.1 was used to test all of the potential effectors during cultivation in CGXII minimal medium with 0.5% (wt/vol) glucose. While vanillyl alcohol only led to a negligible increase of eGFP expression, ferulate, vanillin, and vanillate remarkably induced the eGFP production in C. glutamicum compared with that of the uninduced control (Fig. 4A). Since ferulate and vanillate most likely were converted to vanillate inside the cell, we assumed that vanillate was the main effector of VanR.

To confirm this hypothesis, a similar experiment was conducted in E. coli, which naturally lacks the intrinsic ferulate metabolism. For this purpose, the eGFP production in strain JM109 pJOE7936.1 was compared to that of JM109 pJOE7936.1 containing the second plasmid pJOE8097.3, expressing vanR by P_{vanK}. The availability of VanR in E. coli practically offered an in vivo effector screening assay. As shown in Fig. 4B, only vanillate could restore the eGFP expression when JM109 carried both pJOE7936.1 and pJOE8097.3. Overall, the in vivo experiment pointed out that vanillate is the main effector of VanR, while ferulate or vanillin affects the VanR activity after their conversion to vanillate in C. glutamicum.

Furthermore, the differential scanning fluorimetry assay was used to investigate the interaction of VanR with effectors (41). The thermal unfolding of VanR in the presence of SYPRO Orange was monitored with and without effectors. Intriguingly, the melting temperature of VanR was increased by 5°C only in the presence of 1 mM vanillate (Fig. 4C). Reduction of the vanillate concentration to 0.1 mM decreased the melting temperature of VanR to the level of VanR without any effector. Other effectors had no significant influence on the melting temperature of VanR. In conclusion, all results verified that vanillate is the main effector and ligand of the VanR repressor.

In vitro binding of VanR to the P_{vanABK} DNA fragment and properties of their complex. Prior to identifying the binding site of VanR at P_{vanABK}, EMSA was conducted. For this purpose, VanR was overproduced in E. coli with and without Strept tag II, and the purified protein was used for EMSA. Both VanR variants, with or without Strept tag II, were able to repress the transcription of the eGFP gene by P_{vanABK} in vivo (Fig. 2B). Primary studies showed that both proteins shifted a Cy5-labeled P_{vanABK} DNA fragment in EMSA. Hence, VanR and VanR-Strept tag II were active in vitro and were able to form a complex with the Cy5-labeled P_{vanABK} DNA fragment (data not shown). Furthermore, the equilibrium dissociation constant (K_D) of the VanR-DNA(P_{vanABK}) complex was calculated using EMSA. K_D is the concentration of VanR necessary to shift 50% of the Cy5-P_{vanABK} DNA fragment. Hence, variable concentrations of purified VanR (5 nM to 35 nM) were mixed with 2.5 nM Cy5-P_{vanABK} DNA fragment amplified from pJOE8077.1 carrying the wild-type P_{vanABK} sequence (Fig. 5A). Intriguingly, reduction of the concentration of VanR in the binding reaction resulted in a second VanR-DNA(P_{vanABK}) complex which was lighter than the main VanR-DNA(P_{vanABK}) complex (Fig. 5A). Therefore, the K_D value in this experiment was calculated based on the disappearance of the free Cy5-DNA_PJOE8077.1 band (Fig. 5C). The corresponding K_D value for VanR-DNA_PJOE8077.1 amounted to 15.1 ± 1.7 nM (Fig. 5C). Likewise, the dissociation rate constant (K'_D) of the VanR-DNA(P_{vanABK}) complex was calculated using a binding competition assay, where a 50-fold molar excess of the nonlabeled P_{vanABK} DNA fragment competed with the Cy5-DNA_PJOE8077.1 fragment (Fig. 5B). The EMSA showed that K'_D of the VanR-DNA(P_{vanABK}) complex was 267 ± 23 × 10^-6 s^-1 (Fig. 5D). Accordingly, a half-life of 43.5 ± 3.6 min was obtained for the VanR-DNA(P_{vanABK}) heavy complex.

The two VanR-DNA_PJOE8077.1 complexes observed during EMSA could be due to (i) the presence of two VanR binding sites, (ii) the binding of monomer and dimer forms of VanR, or (iii) the cooperative binding of the VanR monomers to the P_{vanABK} DNA fragment. To better understand the structure of these VanR-DNA complexes, size exclusion chromatography was carried out in order to determine the conformation of the active form of VanR. Comparison of the purified VanR with the standard proteins revealed that purified VanR entirely formed dimers (approximately 40 kDa) (see Fig. S4 in the supplemental material). No fraction revealed that purified VanR entirely formed dimers (approximately 40 kDa) (see Fig. S4 in the supplemental material). Taken together, the two VanR-DNA complexes observed in the EMSA were caused by the binding of two VanR dimers to the DNA. Accordingly, the VanR binding site was further analyzed to find out the essential nucleotides located in the VanR operator, as well as the structure of VanR-DNA complex.

Identification and mutation of the VanR binding site at P_{vanABK}. The DNA binding site of VanR was identified using DNase I footprinting at both coding and noncoding strands of

FIG 3 Identification of the translation start site of vanR. The functional vanR open reading frame was determined by mutation of the possible start codon inside the P_{vanABK}-eGFP gene-vanR autoregulatory cassette. E. coli JM109 carrying pJOE8077.1 (wt), pJOE8197.2 (GTG→GT), pJOE8231.1 (GTG→GT), or pJOE9198.1 (GTG→ATG) was cultivated in LB with or without 2 mM vanillate. The fluorescence intensity of the cell suspension at an OD_600 of 0.1 was measured after 8 h of induction. Mean values and SD (error bars) are shown from three independent experiments.
The results indicated that VanR protects the coding strand of \( P_{\text{vanABK}} \) DNA from to 45 nucleotides with respect to the transcription start site, while the protected noncoding strand was a longer sequence of between 9 and 60 positions (Fig. 6C). Since the result of the \( P_{\text{vanABK}} \) DNA footprinting assay showed an asymmetric VanR operator, the VanR operator was systematically disrupted with deletion or mutation of nucleotide blocks to verify the VanR operator (Fig. 7A). The effect of these mutations on the repression of \( P_{\text{vanABK}} \) was investigated in \emph{E. coli} JM109 containing plasmids with the \( P_{\text{vanABK}} \)-eGFP gene fusion. In all plasmids, the \emph{vanR} gene was inserted downstream of the eGFP gene and expressed in an autoregulatory manner. In addition to \emph{in vivo} studies, the \( P_{\text{vanABK}} \) DNA fragment was amplified with Cy5-labeled oligonucleotides to analyze the interaction between VanR and the \( P_{\text{vanABK}} \) DNA fragment \emph{in vitro} by EMSA. Wild-type \( P_{\text{vanABK}} \) on pJOE8077.1 was highly inducible with vanillate (Fig. 7A). In \emph{in vitro}, VanR formed a heavy complex with the \( P_{\text{vanABK}} \) DNA fragment containing the complete VanR binding site (pJOE8077.1) (Fig. 7B). Surprisingly, eGFP was not produced when the VanR operator was completely deleted (Fig. 7A). This was due presumably to the shortened untranslated region of mRNA\textsubscript{eGFP}. Likewise, VanR could not bind to the Cy5-\( P_{\text{vanABK}} \) DNA fragment from pJOE8297.6 (Fig. 7B). Deletion of 7 bp from the 5’ end (positions 6 to 12 with respect to the transcription start site) of the VanR binding site did not change the repression of \( P_{\text{vanABK}} \) on pJOE8296.2 (Fig. 7A) compared with that of pJOE8077.1. In contrast to pJOE8296.2, further deletion of the VanR binding site on pJOE8297.6 (positions 7 to 18) (Fig. 7A) drastically reduced the repression of \( P_{\text{vanABK}} \). In \emph{in vitro}, VanR could form a heavy complex with the \( P_{\text{vanABK}} \) DNA fragment from both pJOE8296.2 and pJOE8297.6, although VanR-DNA\textsubscript{pJOE8297.6} formed a weaker complex than VanR-DNA\textsubscript{pJOE8296.2} (Fig. 7B). Deletion of the nucleotides from +18 to +27 positions on pJOE8299.3 rendered \( P_{\text{vanABK}} \) fully constitutive (Fig. 7A). No VanR-DNA\textsubscript{pJOE8299.3} complex was detected in the EMSA (Fig. 7B). Deletion of the nucleotides from +31 to +37 (pJOE8300.2) reduced the repression of \( P_{\text{vanABK}} \) only to a level similar to that of pJOE8297.6 \emph{in vivo} (Fig. 7A). Notwithstanding similarities \emph{in vivo}, a second VanR-DNA\textsubscript{pJOE8300.2} (light complex) appeared in the EMSA that was not seen with the wild-type DNA\textsubscript{pJOE8077.1} fragment or DNA\textsubscript{pJOE8297.6} fragment (Fig. 7B). Finally, \( P_{\text{vanABK}} \) with the mutation of 4 nucleotides (+38 to +41) (Fig. 7A) into its
complementary sequence (pJOE8239.1) was repressible to a level almost similar to the level for pJOE8077.1 (Fig. 7A); however, VanR formed both heavy and light complexes with $P_{\text{vanABK}}$ DNA fragment in vitro (Fig. 7B). Overall, the center of the VanR binding site ($/H11001_{18}$ and $/H11001_{27}$) has an essential role in the regulation of $P_{\text{vanABK}}$ (pJOE8299.3) (Fig. 7), whereas disruption of its flanking sites rendered $P_{\text{vanABK}}$ partially constitutive (pJOE8297.6 and pJOE8300.2) (Fig. 7). In the latter case, VanR was able to bind to the DNA; however, the migration pattern of the VanR-DNA complexes was different.

To better understand the VanR-DNA complexes formed with the $P_{\text{vanABK}}$ variants, the $K_d$ of VanR in the presence of $P_{\text{vanABK}}$ DNA fragment originating from pJOE8297.6 and pJOE8300.2 was measured. The $K_d$ of VanR mixed with the DNA$_{\text{pJOE8300.2}}$ fragment was $18.3 \pm 2.8$ nM (data not shown), which was almost similar to the $K_d$ of VanR-DNA$_{\text{pJOE8077.1}}$ complex (15.1 $\pm$ 1.7 nM). Unlike that for the VanR-DNA$_{\text{pJOE8077.1}}$ complex, the $K_d$ value of the VanR-DNA$_{\text{pJOE8297.6}}$ complex was significantly higher (29.8 $\pm$ 0.9 nM) than that for the VanR-DNA$_{\text{pJOE8077.1}}$ complex containing wild-type $P_{\text{vanABK}}$ showing that a larger amount of VanR is necessary to form VanR-DNA$_{\text{pJOE8077.1}}$. In other words, the affinity of VanR toward its target operator on pJOE8297.6 was reduced due to the nucleotide deletion. Afterwards, EMSA was conducted using the $K_d$ of VanR in order to analyze the migration pattern of DNA fragments from pJOE8077.1, pJOE8297.6, and pJOE8300.2 at their reaction equilibrium (Fig. 7C). When the wild-type $P_{\text{vanABK}}$ DNA fragment was used, the amount of the heavy VanR-DNA$_{\text{pJOE8077.1}}$ complex was triple the amount of the light VanR-DNA complex at the start point, while (VanR-DNA)$_{\text{pJOE8077.1}}$ shows the amount of heavy complex at the desired time (t). The Cy5-labeled DNA fragments for this experiment were generated from pJOE8077.1 by PCR using oligonucleotides s9177 and s9071, whereas nonlabeled DNA fragment was amplified from pJOE8077.1 using oligonucleotides s8754 and s8823. All experiments were carried out three times independently, and one of the independent repeats is exemplarily visualized here.

FIG 5 DNA-binding properties of VanR. (A) Determination of the equilibrium dissociation constant ($K_d$) of the VanR-DNA complex. The 2.5 nM Cy5-labeled $P_{\text{vanABK}}$ DNA fragment was incubated with 0 nM (lane 1), 5 nM (lane 2), 5.83 nM (lane 3), 7 nM (lane 4), 8.7 nM (lane 5), 16.6 nM (lane 6), 17.5 nM (lne 7), and 35 nM (lane 8) purified VanR on ice for 15 min. (B) Determination of the dissociation rate constant ($K_d$) and half-life of the VanR-DNA heavy complex. The 2.5 nM Cy5-labeled $P_{\text{vanABK}}$ DNA fragment was incubated with 0 nM (lane 1) or 35 nM purified VanR (lanes 2 to 9) on ice for 10 min. Upon the addition of a 50-fold molar excess of nonlabeled $P_{\text{vanABK}}$ DNA fragment, the stability of the complex was monitored at 10-min intervals, which were 0 min (lane 2), 10 min (lane 3), 20 min (lane 4), 30 min (lane 5), 40 min (lane 6), 50 min (lane 7), 60 min (lane 8), and 70 min (lane 9). (C) The amount of free DNA and the heavy and light VanR-DNA complexes is shown. The reduction of the fluorescence intensity of the free DNA band was used for calculations of the $K_d$. The $K_d$ value was equal to the amount of VanR necessary to shift 50% of the total Cy5-labeled DNA. (D) Calculation of the dissociation rate constant ($K_d$) of the VanR-DNA heavy complex by plotting the reduction of the VanR-DNA heavy complex during 70 min. (VanR-DNA)$_{\text{pJOE8077.1}}$ represents the amount of the VanR-DNA heavy complex at the start point, while (VanR-DNA)$_{\text{pJOE8077.1}}$ shows the amount of heavy complex at the desired time (t). The Cy5-labeled DNA fragments for this experiment were generated from pJOE8077.1 by PCR using oligonucleotides s9177 and s9071, whereas nonlabeled DNA fragment was amplified from pJOE8077.1 using oligonucleotides s8754 and s8823. All experiments were carried out three times independently, and one of the independent repeats is exemplarily visualized here.
Further analysis of the +9 to +36 region revealed two inverted repeats, shown by solid and dashed arrows as inverted repeat 1 (IR1) and 2 (IR2) (Fig. 7A).

**Studying the inverted repeat located inside the VanR binding site.** To test whether the inverted repeats IR1 and IR2 are vital for the binding of VanR, the VanR binding site was inserted downstream of the T7 promoter. The repression of the T7 promoter and, as a result, production of eGFP was studied in *E. coli* BL21Star(DE3) in the presence and absence of the second plasmid, pJOE8097.3, carrying P<sub>tetA</sub>-vanR. The first plasmid construct, pJOE8550.1, contained the complete inverted repeats (Fig. 8A). In the BL21Star(DE3) pJOE8550.1 strain, expression of vanR by P<sub>tetA</sub> reduced the production of eGFP by 2.8-fold (Fig. 8B). The 3′ end of the operator then was gradually shortened by 4 bp (pJOE8551.1), 6 bp (pJOE8520.1), and 9 bp (pJOE8554.1) (Fig. 8A). Only the deletion of 9 bp in pJOE8554.1 rendered P<sub>T7</sub> constitutive (Fig. 8B). EMSA results were in line with the in vivo results showing the loss of the VanR-DNA<sub>pJOE8554.1</sub> complex, whereas VanR slowed the migration of the DNA<sub>pJOE8551.1</sub> and DNA<sub>pJOE8520.1</sub> fragments, similar to the DNA<sub>pJOE8550.1</sub> fragment (Fig. 8C). This showed that deletion of 6 bp from the 3′ end of the VanR operator was tolerated, since the T7 promoter remained repressible. Further deletions were carried out by removing 3 bp (pJOE8574.2) and 6 bp (pJOE8552.1) from the 5′ end of the VanR operator (Fig. 8A). Deletion of 3 bp from the 5′ end of the VanR operator rendered the T7 promoter weakly repressible by 2-fold (pJOE8574.2), whereas by deletion of 6 bp from the 5′ end (pJOE8552.1), P<sub>T7</sub> became fully constitutive (Fig. 8B). In vitro, VanR formed a complex with both DNA<sub>pJOE8574.2</sub> and DNA<sub>pJOE8552.1</sub> fragments; however, the VanR-DNA<sub>pJOE8552.1</sub> complex was highly unstable; therefore, the VanR-DNA complex was dissociated during the migration of DNA fragments in the EMSA and formed a smeared band (Fig. 8C). Since deletion of 3 bp from the 5′ end and 6 bp from the 3′ end were tolerable for the VanR-DNA complex formation in vitro as well as the repression of T7 promoter in vivo, a shortened version of the VanR operator (pJOE8506.1) was generated containing a deletion of 9 bp from both sides (Fig. 8A). In this case, P<sub>T7</sub> was fully constitutive (Fig. 8B), while the VanR-DNA<sub>pJOE8506.1</sub> complex remained unstable, similar to VanR-DNA<sub>pJOE8552.1</sub> (Fig. 8C). Further deletion of 2 bp from both ends of the VanR operator on pJOE8506.1 rendered P<sub>T7</sub> fully constitutive on pJOE8508.1 (Fig. 8B). In this case, no VanR-DNA<sub>pJOE8508.1</sub> complex was detected in the EMSA (Fig. 8C). Altogether, the results indicated that the 5′ end of the VanR operator plays an important role in the interaction of VanR dimers with their target operator. The 5′ end of the VanR operator contained the sequence AACTAACTAA (demonstrated as IR1F), while the second repeat (IR1R) had two mismatches (AAATACCTAA) compared with the IR1F sequence (Fig. 8A).

To test the binding affinity of each inverted repeat for VanR, two versions of vanR operator were generated, each of which contained a single inverted repeat (IR1 or IR2). For this purpose, the spacer sequence of IR2 was mutated, and 4 bases from the 5′ end of the operator were removed (pJOE8553.1). In another construct, the spacer sequence of IR1 was mutated to its complementary nucleotides, and 4 bp from the 3′ end of the operator were removed (pJOE8524.1) (Fig. 8A). Interestingly, only the VanR operator of pJOE8524.1 was able to repress...
the T7 promoter to a level similar to that of the wild-type VanR operator on pJOE8550.1 (Fig. 8B). In vitro, the migration of the VanR-DNApJOE8524.1 complex was similar to that of VanR-DNApJOE8550.1, whereas no VanR-DNApJOE8553.1 was detected (Fig. 8C). By mutation of the nucleotides, including the spacer between IR1F and IR1F and the nucleotides inside IR1F (pJOE8528.1), PT7 became constitutive (Fig. 8B) and no VanR-DNApJOE8528.1 complex formed (Fig. 8C), showing that the latter VanR operator was defective. Altogether, these experiments verified the inverted repeats found inside the VanR operator as the cis elements of the vanillate regulation system in C. glutamicum. In addition, these results indicated that the presence of IR1 is a prerequisite for formation of the VanR-DNA complex.

DISCUSSION

In this study, the regulation of the vanillate catabolic system in C. glutamicum was investigated. The vanillate catabolic pathway is encoded by the vanABK operon. The promoter of the vanABK operon (PvanABK) is negatively regulated by its specific repressor, VanR. The exact locations of the promoters of vanR and the vanABK operon were deduced from transcriptome sequencing experiments (42). It was interesting that vanR is leaderless. Since its promoter motifs include only an extended −35 region and no visible −10 region, this arrangement seems to be the most compact. In contrast to this, the PvanABK promoter has a perfect −35 region, and the promoter is located well upstream of the coding region. This arrangement gives room for binding sites for two different transcriptional regulators. It is an interesting speculation that leaderless transcripts prefer such a compact transcriptional regulation and are less prone to being regulated by (multiple) transcriptional regulators. Principally, three factors directly influence the PvanABK activity: (i) the PvanABK core elements, including −35 and −10 boxes, (ii) the GlxR binding site, and (iii)
the VanR binding site. During the preliminary studies, the strength of P\text{vanABK} was optimized by altering the promoter core elements and removing the GlxR binding site. Improvement of the P\text{vanABK}/H11002 box tremendously enhanced the P\text{vanABK} activity, indicating that the recently published P\text{vanABK} core elements were correctly predicted (44). Since C. glutamicum does not show diauxic growth on glucose together with protocatechuate or vanillate, it is presumed that the vanillate metabolism does not underlie a glucose-mediated carbon catabolite repression (13). Nevertheless, the global regulator GlxR, which is a cyclic AMP (cAMP)-dependent global regulator, can bind P\text{vanABK} (45). It is known that GlxR is unable to bind the target DNA when the cAMP level is low, e.g., growth on acetate (45,46). Nevertheless, the effect of GlxR on P\text{vanABK} has not been thoroughly studied; therefore, the GlxR binding site located inside the P\text{vanABK} spacer was removed in order to prevent the possible P\text{vanABK} repression by GlxR in the complex media. In fact, changing the spacer of P\text{vanABK} did not affect its strength. In addition, the newly constructed P\text{vanABK} without the GlxR binding site showed robustness in complex media similar to that in minimal media supplemented with various carbon sources (data not shown).

The specific transcription repressor of the vanillate utilization system, VanR, is a PadR-type transcriptional regulator. Since determination of the translation start site revealed that VanR is a protein with 192 amino acids, VanR belongs to the first subfamily of the PadR-like proteins, including the thoroughly studied PadR, AphA, and LadR regulators. Structurally, size exclusion chromatography showed that the active VanR molecules only form dimers. Similarly, PadR molecules tend to form dimers in the presence of a cross-linking reagent, glutaraldehyde, at low concentrations (47). In addition, protein crystallography, fusion of AphA to the DNA binding domain of LexA, and the use of cross-linking reagents showed that AphA protein forms a dimer (18, 48). Obviously, members of the PadR-like proteins form dimers due to their C-terminal domain, which is a coiled-coil leucine zipper-like structure (23). Despite this structural resemblance, the already-known PadR-like regulators function in different regulatory pathways. Therefore, (de)activation of these proteins also is

![Image](http://jb.asm.org/)

**FIG 8** Characterization of the essential inverted repeat located inside the VanR binding site at P\text{vanABK}. (A) Schematic view of the P\text{γ}-operator\text{van}-eGFP gene. The proposed essential inverted repeats 1 (IR1) and 2 (IR2) are highlighted by arrows. The proposed VanR binding site is shown by capital letters, whereas flanking sequences of the VanR binding site are written in lowercase letters. The exchanged nucleotides are demonstrated with lowercase underlined letters. (B) Production of eGFP in *E. coli* BL21-Star (DE3) containing the P\text{γ}-operator\text{van}-eGFP gene on pJOE8550.1, pJOE8551.1, pJOE8520.1, pJOE8541.1, pJOE8574.2, pJOE8552.1, pJOE8506.1, pJOE8508.1, pJOE8553.1, pJOE8524.1, or pJOE8528.1 alone or together with pJOE8097.3 carrying P\text{tetA}-vanR. The fluorescence intensity of the cell suspension at an OD600 of 0.1 of each bacterial culture was measured after 6 h of induction. Mean values and SD (error bars) are shown from three independent experiments. (C) EMSA of the Cy5-labeled DNA fragments containing full-length, shortened, or mutant variants of the VanR operator. Cy5-labeled DNA (2.5 nM) was incubated with (+) or without (−) 73 nM purified VanR. The reaction mixture was incubated on ice for 15 min and loaded onto a native PAGE. Cy5-labeled DNA fragments were generated by PCR using oligonucleotides s8753 and T7.
therefore, we assumed that two VanR dimers bind the target DNA. Interestingly, the VanR appeared in the EMSA gel (Fig. 8C, pJOE8553.1), whereas disruption of IR1 region, all of the VanR-DNA complexes (one or two VanR dimers) (Fig. 7C, pJOE8524.1). It must be noted that VanR represses the RNA polymerase elongation activity (Fig. 9A). Characterization of the DNA binding site of VanR revealed two overlapping inverted repeats (IR1 and IR2) (Fig. 8A). Obviously, there is heterogeneity in the binding of VanR dimers to IR1 and IR2, and these two binding sites are not equivalent (Fig. 8, compare constructs pJOE8524.1 and pJOE8553.1). Likewise, calculation of the KD indicates the heterogeneity of IR1 and IR2, since mutation of IR2 had no effect on KD, whereas mutation of IR1 significantly increased the KD (Fig. 7). This heterogeneity caused a cooperative binding of VanR dimers in a way that formation of the complex 2 depends on the formation of complex 1 (shown as C1 and C2) (Fig. 9B). The conclusion of a cooperative binding mechanism is based on the EMSAs in which the VanR operator was systematically truncated from both sides (Fig. 7C), as well as the disruption of each inverted repeat alone (constructs pJOE8524.1 and pJOE8553.1) (Fig. 8). By disruption of the IR1 region, all of the VanR-DNA complexes (one or two VanR dimers) completely disappeared in the EMSA gel (Fig. 8C, pJOE8553.1), whereas disruption of IR2 resulted in the formation of a light VanR-DNA complex (VanR dimer) (Fig. 7C, pJOE8300.2) or heavy complex at high VanR concentration (Fig. 8C, pJOE8524.1). It must be noted that VanR represses the RNA polymerase elongation in vivo when both complexes 1 and 2 are formed. Accordingly, the sequence motif AACTAACTAA is likely the perfect repeat for binding VanR (Fig. 9B). As shown, there are only two mismatches in the first VanR-DNA complex (C1), compared with 7 mismatches in the second VanR-DNA complex (C2). Interestingly, the VanR-DNA binding model is highly similar to the PadR-DNA interaction in which two dimers of PadR bind to a 40-bp DNA region in a way that both binding sites overlap in the center (47). In addition, the cooperative DNA binding mechanism also is shown for LmrR, where two LmrR dimers repress the transcription of lmrCD genes encoding a multidrug ABC transporter in L. lactis (24).

In summary, this study delivered new insights into the regulation system of the vanillate utilization system in C. glutamicum, as well as the PadR-type transcriptional regulators. Further studies are being performed in order to clarify the structure of the VanR by protein crystallography. Determination of the VanR structure would show us how this protein interacts with its DNA target, its specific ligand, and clarifies the formation of VanR-DNA complex.

ACKNOWLEDGMENTS
We appreciate Bastian Blombach and Jung-Won Youn for their support in handling Corynebacterium glutamicum. We thank Jana Hoffmann for her support during size exclusion chromatography experiments and the technical assistance of Annette Schneck and Gisela Kwiatkowski throughout this study.

This study was partially supported by the EU FP7 grant 265992 AMYLOMICS.

REFERENCES

Morabbi Heravi et al.