Testosterone-inducible Regulator Is a Kinase That Drives Steroid Sensing and Metabolism in Comamonas testosteroni*

Received for publication, December 13, 2007, and in revised form, March 6, 2008 Published, JBC Papers in Press, April 17, 2008, DOI 10.1074/jbc.M710166200

André Göhler, Guangming Xiong, Simone Paulsen, Gabriele Trentmann, and Edmund Maser

From the Institute of Toxicology and Pharmacology for Natural Scientists, University Medical School Schleswig-Holstein, Campus Kiel, Brunswiker Strasse 10, 24105 Kiel, Germany

The mechanism of gene regulation by steroids in bacteria is still a mystery. We use steroid-inducible 3α-hydroxysteroid dehydrogenase/carbonyl reductase (3α-HSD/CR) as a reporter system to study steroid signaling in Comamonas testosteroni. In previous investigations we cloned and characterized the 3α-HSD/CR-encoding gene, hsdA. In addition, we identified two negative regulator genes (repA and repB) in the vicinity of hsdA, the protein products which repress hsdA expression on the level of transcription and translation, respectively. Recently, a positive regulator of hsdA expression, TeiR (testosterone-inducible regulator), was found by transposon mutagenesis, but the mode of its action remained obscure. In the present work we produced a TeiR-green fluorescent fusion protein and showed that TeiR is a membrane protein with asymmetrical localization at one of the cell poles of C. testosteroni. Knock-out mutants of the teiR gene revealed that TeiR provides swimming and twitching motility of C. testosteroni to the steroid substrate source. TeiR also mediated an induced expression of 3α-HSD/CR which was paralleled by an enhanced catabolism of testosterone. We also found that TeiR responds to a variety of different steroids other than testosterone. Biochemical analysis with several deletion mutants of the teiR gene revealed TeiR to consist of three different functional domains, an N-terminal domain important for membrane association, a central steroid binding site, and a C-terminal part mediating TeiR function. Finally, we could demonstrate that TeiR works as a kinase in the steroid signaling chain in C. testosteroni. Overall, we provide evidence that TeiR mediates steroid sensing and metabolism in C. testosteroni via its steroid binding and kinase activity.

Comamonas testosteroni (formerly termed Pseudomonas testosteroni (1)) is a Gram-negative bacterium that is able to utilize a variety of steroids and aromatic compounds as the sole carbon and energy source (2–4). Steroids are widespread in the environment occurring as cholesterol, sex, and adrenal cortical hormones of mammals, molting hormones in insects, or phytosterols in plants (5–8). Accordingly, C. testosteroni inhabits a wide variety of environments, including soil and water as well as animal and plant tissues (9–11).

The complex degradation pathway for steroids in C. testosteroni has been studied earlier by simultaneous identification of the participating genes and by isolation of the main intermediate compounds that have accumulated in gene-disrupted mutants (12–19). The steroid catabolic pathway is initiated by oxidizing the hydroxyl group at the C3 position, thereby forming a ketone group. This oxidation is followed by isomerization, dehydrogenation, and hydroxylation of the steroid, which leads to the opening of the B-ring. Aromatization and further oxidation reactions result in meta cleavage of the A-ring, finally resulting in common intermediates that enter conventional central metabolism pathways (13). The ring cleavage procedure and the enzymes involved therein are similar to those of a common bacterial aromatic compound degradation pathway (20, 21). It has been estimated that the complete degradation of the steroid nucleus to CO2 and H2O requires more than 20 enzymatic reactions.

Interestingly, the catabolic enzymes for steroid degradation are usually not constitutively expressed but, rather, are induced by their respective substrates (20–25). However, the organization and regulation of their corresponding genes is largely unknown, although two steroid degradation gene clusters in C. testosteroni TA441 were identified (12, 14, 15, 26).

In previous investigations we identified 3α-hydroxysteroid dehydrogenase/carbonyl reductase (3α-HSD/CR)2 as an important enzyme in steroid degradation of C. testosteroni (21, 23, 25). By catalyzing the interconversion of hydroxyl and oxo groups at position 3 of the steroid ring structure, 3α-HSD/CR initiates steroid ring opening and is, therefore, of importance for complete steroid mineralization. Moreover, this enzyme is capable of catalyzing the carbonyl reduction of nonsteroidal xenobiotic carbonyl compounds (25, 27). It has been demonstrated that this substrate pluriopotency not only enhances the metabolic capacity of insecticide degradation but also increases the resistance of C. testosteroni towards the steroid antibiotic fusidic acid (24). Because the expression of the encoding gene, hsdA, is inducible by steroids such as testosterone and progesterone, elucidation of the hsdA-inducing mechanism may shed light on the regulation and genetics of the entire steroid degradation pathway in C. testosteroni.

Recently, we identified two genes involved in hsdA regulation and reported a two repressor model to control hsdA gene

2 The abbreviations used are: 3α-HSD/CR, 3α-hydroxysteroid dehydrogenase/carbonyl reductase; ELISA, enzyme-linked immunosorbent assay; RepA, repressor A; RepB, repressor B; TeiR, testosterone-inducible regulator; GFP, green fluorescence protein; SIN, standard I nutrition.
expression. Repressor A (RepA) was found to bind to two operon sequences upstream of hsdA, thereby preventing hsdA transcription (28). Repressor B (RepB) turned out to bind to the mRNA of 3α-HSD/CR, thereby interfering with hsdA translation (29). By transposon mutagenesis, teiR (testosterone-inducible regulator) was identified, a gene that was hypothesized to encode a positive regulator of steroid degradation in C. testosteroni ATCC11996 in the presence of testosterone (30). A similar positive regulator (TesR) was cloned and postulated to regulate a steroid degradation gene cluster in C. testosteroni TA441 (14).

However, the mechanism and mode of action by which these positive regulatory elements control the steroid degradation gene cluster remained obscure. On the other hand, detailed knowledge on hsdA regulation is of great importance and might give a general view on steroid dependent gene regulation in bacteria.

In the present investigation we could unravel the role of TeiR in steroid-dependent gene regulation in C. testosteroni and demonstrate that TeiR is a kinase that drives steroid sensing and metabolism in C. testosteroni. We cloned the teiR gene, produced recombinant TeiR protein as well as respective antibodies, and by constructing a TeiR-GFP fusion protein, proved that TeiR is a membrane protein with almost exclusive polar localization. Studies with wild type and teiR knock-out mutants revealed that TeiR induces hsdA expression and testosterone catabolism in C. testosteroni. These knock-out studies also showed that, in addition to testosterone, a variety of other steroids can bind to TeiR. Deletion mutants of the teiR gene disclosed that the protein consists of three main domains, an N-terminal membrane attachment site, a central hydrophobic steroid binding sequence, and a C-terminal part mediating its regulatory function. More detailed analysis showed that TeiR is required for twitching and swimming of C. testosteroni in the medium to the steroid source and that the mode of intracellular signal transduction occurs via its kinase activity.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—Host strains Escherichia coli HB101 (Promega) and C. testosteroni ATCC 11996 (Deutsche Sammlung von Mikroorganismen) were used for cloning and gene expression. Subcloning of fragments was carried out in plasmids pBBR1MCS-2 (containing the kanamycin resistance gene; a gift from Peterson and co-workers (31)) and pUC18 (containing the ampicillin resistance gene and obtained from Invitrogen). Plasmid pK18 containing the kanamycin resistance gene was a gift from Ciba Pharmaceuticals, Inc., Department of Biotechnology (Basel, Switzerland). The plasmid copy numbers determined were 80 copies of pK18 and pUC18 and 5 copies of pBBR1MCS-2 per cell in E. coli. For overexpression and purification of TeiR, E. coli strain BL21(DE3)pLysS together with plasmid pET15b from Novagen was used. The tac promoter (274 bp) was obtained by BamHI digestion from plasmid pHA10, which was a gift from H. Ariai (32). With plasmid pcDNA3.1/CT-GFP-TOPO (Invitrogen), a TeiR-GFP fusion protein was prepared, and plasmid pCR2.1-TOPO (Invitrogen) served for PCR cloning of teiR fragments and sequencing.

**Growth Media and Growth Conditions**—Bacterial cells were grown in a shaker (180 rpm) in Standard 1 Nutrient broth medium (Merck) or LB medium at 37 °C (E. coli) or 27 °C (C. testosteroni). Growth media contained 60 μg/ml ampicillin and 30 μg/ml kanamycin.

**Restriction Enzymes and Other Reagents**—Restriction enzymes, T4 ligase, S1 nuclease, and shrimp alkaline phosphatase were obtained from Roche Applied Science, New England Biolabs, MB1, Promega, and Amersham Biosciences, and used according to the manufacturers’ instructions. Sodium lauroyl sarcosinate was from Fluka. The steroid compounds were supplied by Sigma. Ampicillin and kanamycin were from AppliChem and Calbiochem, respectively. [γ-32P]ATP and [γ-32P]GTP was obtained from Amersham Biosciences.

**DNA Manipulations**—Recombinant DNA work was carried out following standard techniques according to Sambrook and Russell (33). The fragments cloned in this work are shown in Fig. 1. All of the primers were prepared by MWG (Ebersberg, Germany). Before further cloning, fragments prepared by PCR were cloned into pCR2.1-TOPO and then checked for correct sequence by MWG.

**Transformation of Bacteria**—All constructs were verified by restriction enzyme analysis of the resulting band patterns. Plasmids were purified with the Tip-100 kit from Qiagen. Ligated constructs were transferred into competent E. coli or C. testosteroni cells prepared by the calcium chloride or electroporation method. Double plasmid cotransformations were performed by exploiting the kanamycin resistance gene of pBBR1MCS-2 or pK18 and the ampicillin resistance gene of pUC18. In these cases, both antibiotics were added to the culture medium. Plasmid isolation and agarose gel electrophoresis were performed to prove successful double transformations.

**Cloning of the teiR Gene from C. testosteroni and Subcloning of teiR Gene Fragments**—The teiR gene was cloned from C. testosteroni (ATCC 11996) chromosomal DNA by using the following pair of primers: forward primer 5′-CGAGCTCAT-CGCTTGCGTG-3′ and reverse primer 5′-GGCGGCCGCTCTATGCCC-3′ (30). The full teiR gene was then cloned into pCR2.1-TOPO to yield plasmid pTOPO4, which after sequence confirmation (MWG) was used as template for further PCR reactions (Fig. 1A). To generate pKteiR10, forward primer 5′-GGAATTCATATGCTGCCCATATTTCGAC-3′ and reverse primer 5′-GGAATTCTCGAATCACTTGTTCCCCAGC-3′ were used in a PCR reaction with plasmid pTOPO4 as template, and the resulting fragment was digested with EcoRI and introduced into pK18 downstream from the lacZ promoter. After sequence confirmation, the same fragment was digested by NdeI and XhoI and introduced into pET-15b downstream from the N-terminal His tag coding sequence to yield pET-TeiR1. To obtain the TeiR-GFP fusion plasmid pBTac-teir-GFP1, forward primer 5′-CTAGCTAGCATGTGCCCATATTTCGAC-3′ and reverse primer 5′-TAGCTAGCCTGGCTTTCCCAAGC-3′ were used in a PCR reaction with plasmid pTOPO4 as template, and the resulting fragment was digested with HinfI and cloned into pcDNA3.1/CT-GFP-TOPO to yield pcGFP-teiR (not shown). After digestion with XbaI, pcGFP-teiR was introduced into pBRR1MCS-2 under the control of the tac promo.
TeiR Is a Kinase That Drives Steroid Sensing and Metabolism

FIGURE 1. Schematic illustration of the various deletion, reporter, and knock-out constructs of the teiR gene. The fragments of teiR are drawn as horizontal lines and were subcloned into vectors, pK18 (pK), pET-15b (pET), pBBR1MCS-2 (pBB), or pCR2.1-TOPO (pTOPO). A, plasmids used for E. coli and C. testosteroni transformation. B, strategy for the preparation of teiR knock-out mutants teiR<sub>okl</sub> and teiR<sub>okr</sub> in C. testosteroni. To generate a reading frameshift mutation, an additional cytosine was incorporated into the sequence at places indicated by ×. placZ, lacZ promoter; ptac, tac promoter; 6xHis, His tag; gfp, green fluorescence protein.

tor. Plasmids pKteiRA, pKteiRB, and pKteiRC, which harbor different fragments of the TeiR gene, were obtained by partial digestion with PstI and identification by restriction fragment analysis. Plasmid pBBtacIII was generated by producing a PCR fragment with forward primer 5'-TCCCCCCCCGATGACC-GAAGCATG-3' and reverse primer 5'-GGATGTGCCCATATTTCGAC-3'. The latter was used as forward primer for the second PCR. The latter was ligated into pBBR1MCS-2. Plasmid pBBtacIV was obtained by resulting fragment was digested with XmaI and HindIII and was used as forward primer for the second PCR. The latter was ligated into pBBR1MCS-2. Plasmid pBBtacIV was obtained by resulting fragment was digested with XmaI and HindIII and cloned into vectors pK18 and pKteiR5. Plasmid pBBtacIII was generated by producing a PCR fragment with forward primer 5'-GGATGTGCCCATATTTCGAC-3' and reverse primer 5'-GGATGTGCCCATATTTCGAC-3', and primers for yielding pTOPON5teiR (not shown) or pKteiR6 were forward primer 5'-TCCCCCCCCGATGACC-GAAGCATG-3' and reverse primer 5'-GGATGTGCCCATATTTCGAC-3'. Plasmids pKteiR627 and pKteiR567, bearing C-terminal-deleted fragments of teiR, were constructed by standard PCR and cloned into pK18 via XmaI and HindIII restriction sites. Primers for pKteiR627 were forward primer 5'-TCCCCCCCCGG-GATGTGCCCATATTTCGAC-3' and reverse primer 5'-TCCCCCCCCGG-GATGTGCCCATATTTCGAC-3', and primers for pKteiR567 were forward primer 5'-TCCCCCCCCGG-GATGTGCCCATATTTCGAC-3' and reverse primer 5'-TCCCCCCCCGG-GATGTGCCCATATTTCGAC-3'. Plasmids pKteiR627 and pKteiR567, bearing C-terminal-deleted fragments of teiR, were constructed by standard PCR and cloned into pK18 via XmaI and HindIII restriction sites. Primers for pKteiR627 were forward primer 5'-TCCCCCCCCGG-GATGTGCCCATATTTCGAC-3' and reverse primer 5'-TCCCCCCCCGG-GATGTGCCCATATTTCGAC-3', and primers for pKteiR567 were forward primer 5'-TCCCCCCCCGG-GATGTGCCCATATTTCGAC-3' and reverse primer 5'-TCCCCCCCCGG-GATGTGCCCATATTTCGAC-3'. An additional fragment between teiR sequence 627 bp and 1176 bp (including a HindIII restriction site) was received by PCR (forward primer 5'-GGATGTGCCCATATTTCGAC-3' and reverse primer 5'-GGATGTGCCCATATTTCGAC-3', both containing an additional cytosine (underlined), against pTOPO4. The resulting fragment harboring a frameshift mutation within the teiR gene was digested with EcoRI and cloned into pK18 to yield pKPCR. For knock-out mutant teiR<sub>okl</sub>, a PstI fragment (384–750 bp) was isolated after PstI digestion of pTOPO4 and cloned into pK18 to yield pKteiR5. Because of its sensitivity to kanamycin, C. testosteroni can only grow after homologous integration of the kanamycin resistance gene from pK18, which on the other hand, cannot replicate as a plasmid in C. testosteroni. Accordingly, 10 μg of the pK18 descendent pKPCR or pKteiR5 plasmids, which also contain teiR sequences homologous to C. testosteroni chromosomal DNA, were transformed into C. testosteroni by electroporation (1.8 kV, 1-mm cuvette, Bio-Rad). The cells were cultured in 0.4 ml of SIN medium at 27 °C for 3 h (110 rpm). The culture was spread on 30 μg/ml kanamycin SIN agar plates and cultured in a 27 °C incubator overnight. Only cells with pKPCR or pKteiR5 integrated into the chromosomal DNA could grow in the kanamycin-containing medium. Total DNA from the colonies was isolated and checked for knock-out mutations by Southern blot hybridization.
Subcloning of the 3α-HSD/CR Gene—As described previously, a 5.257-kilobase EcoRI fragment of C. testosteroni chromosomal DNA was cloned into pUC18 (ampicillin-resistant) to yield p6 (29). Plasmid p6 contains the 3α-HSD/CR gene, hsdA, together with its regulatory region and including the two repressor genes repA and repB (28, 29). The expression of hsdA served as a detection system for TeiR-dependent steroid regulation in cotransformation experiments with plasmid p6.

Preparation and Purification of Recombinant TeiR Protein—Overexpression of TeiR was performed in E. coli strain BL21(DE3)pLysS and plasmid pET15b (Novagen), and the recombinant protein was purified by its His-tag sequence. Cells transformed with plasmid pET-TeiR1 (Fig. 1A) were grown at 37 °C in a shaker (180 rpm), and maintenance of plasmids was ensured by adding 60 μg/ml ampicillin to the culture medium. 100 μl of a culture grown overnight was used to inoculate 3 ml of fresh medium. At an A<sub>600</sub> of 0.6, expression was induced by the addition of isopropyl-β-D-thiogalactoside to a final concentration of 1 mM. After 4 h cells were sedimented by centrifugation. The cell pellet was either stored at −80 °C for further usage or directly suspended in 200 μl of buffer B (100 mM sodium phosphate, 10 mM Tris-HCl, 8 M urea, pH 8.0) (Qiagen). Cells were lysed by freezing (−20 °C, 30 min) and thawing (room temperature, 30 min) 3 times, and the resulting mixture was centrifuged (20 min, 13,000 rpm, 4 °C). The supernatant was applied to a mini nickel-agarose affinity column (Qiagen). After washing 2 times with 600 μl of buffer C (100 mM sodium phosphate, 10 mM Tris-HCl, 8 M urea, pH 6.3) (Qiagen) and washing one time with washing buffer (50 mM sodium phosphate, 300 mM sodium chloride, 20 mM imidazole, 1% sodium lauroyl sarcosinate, pH 8.0), TeiR was eluted from the column by applying 100 μl of elution buffer 4 times (50 mM sodium phosphate, 300 mM sodium chloride, 250 mM imidazole, 1% sodium lauroyl sarcosinate, pH 8.0). Samples containing pure and soluble TeiR protein, as assessed by SDS-polyacrylamide gel electrophoresis (Fig. 2), were used to prepare antibodies, to determine steroid binding specificity, and to prove TeiR kinase activities.

Protein Determination—Protein concentration was determined by the method of Bradford (34). Protein analysis by SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli (35).

Western Blot Analysis—Electroblotting was performed in a semidry blotting system. After separation by SDS-polyacrylamide gel electrophoresis, proteins were transferred to a nitrocellulose membrane, and antigen-antibody complexes were visualized by chemiluminescence (ECL PLUS-detection system, Amersham Biosciences). For GFP detection, membranes were incubated with primary antisera against GFP (raised in mice) in a dilution of 1:10,000 for 1 h at room temperature. The secondary antibody (peroxidase conjugated goat anti-mouse immunoglobulin) was used in a 1:10,000 dilution for 1 h at room temperature. After 4 × TBST (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.5) (Roche Diagnostics) washing, 2 ml of fluorescence reagent was spread on the membranes, covered with plastic membranes, and exposed to x-ray films.

Laser-scanning Microscopy—C. testosteroni and E. coli cells from the late log-phase and expressing the GFP-TeiR fusion protein (after pBBtac-teiR-GFP1 transformation) were taken and fixed with 4% formaldehyde in phosphate-buffered saline. The samples were illuminated with an argon laser (488 nm for detection of GFP fluorescence) and recorded using a Leica TCS SP1 confocal laser-scanning microscope.

Swimming and Twitching Motility Assay—Swimming and twitching activities of C. testosteroni wild type and teiR knockout mutants were assayed by the agar stab method. For swimming, C. testosteroni wild type and teiR<sub>ko</sub> knock-out cells (5 μl) were inoculated onto 0.3% agar plates, and the sample was stab-spotted (5 μl of 1 mM each) into the agar surface in some distance to the bacterial inoculates. For twitching, bacterial cells (5 μl) were inoculated in a line onto 0.5% agar plates. Testosterone was stab-spotted (5 μl of 1 mM each) into the agar surface in increasing distances to the bacterial inoculates. After 2 days of incubation at 27 °C, the size of the swimming and twitching zones around the bacterial inoculation site at the interface to the testosterone spots was determined.

Testosterone Binding and Degradation—To test the [3H]testosterone binding activities, wild type and teiR knockout mutant cells of C. testosteroni were grown overnight and then diluted to an A<sub>600</sub> of 1.0 with water. An aliquot of 400 μl was taken and centrifuged at 13,000 rpm for 20 s and resuspended in 100 μl of water. For preparation of the bacterial membranes, 1 μl of lysozyme (10 mg/ml) was added and incubated at 37 °C for 30 min. The lysed cells were centrifuged at 13,000 rpm for 20 min, and the pellet containing the membranes was resuspended in 100 μl of water. In the testosterone binding assay, 10 μl of intact cells were mixed with 0.4 × TEN buffer (10 × TEN buffer: 1 mM NaCl, 0.01 M EDTA, 0.1 M Tris-HCl, pH 8.0) and 0.1 μl of [3H]testosterone (1 μCi/ml) to a final volume of 20 μl. After incubation times of 5 min or 30 min at 27 °C, the incubates were transferred onto 1-cm<sup>2</sup> pieces of Whatman No. 3MM paper. The pieces were put on Whatman No. 3MM strips by adhesion and continuously rinsed by gravity with a solvent containing...
TeiR Is a Kinase That Drives Steroid Sensing and Metabolism

0.2 × TEN and 0.3% Tween. After 3 h of washing, the 1-cm² pieces were dried at 37 °C for 1 h and mixed with 3 ml of scintillation solution, and [³H]testosterone measured as cpm in a liquid scintillation counter (Wallac 1409; machine efficiency = 35%). The same procedure was performed to analyze the testosterone binding capabilities of teiR gene deletion mutants that were expressed in E. coli to identify the steroid binding domain within the TeiR protein.

For measuring the testosterone uptake activities, C. testosteroni wild-type cells and teiR knock-out mutants were cultured in 1 ml of SIN medium. After an A₉₀₀₅₀₀ of 0.6 was reached, 1 μCi (1 μl) of [³H]testosterone was added to the medium, and the cells allowed to grow for up to 3 h. Cells were harvested, and [³H]testosterone was measured in the remaining culture medium as described above.

Protein Extraction—The probes for 3α-HSD/CR ELISA detection were prepared from 3 ml of bacterial cell culture and subsequent centrifugation at 13,000 × g for 10 s. The pellet was washed 3 times with 1 ml of phosphate-buffered saline and resuspended in 200 μl of phosphate-buffered saline with 100 μg/ml lysozyme. To complete cell lysis, the suspension was frozen (at −20 °C, 3 times). Finally, the samples were centrifuged again at 13,000 × g for 20 min. The supernatant was diluted to 1 mg/ml protein and used for 3α-HSD/CR ELISA assays. To extract TeiR, as a membrane-bound protein, the addition of 0.5% Triton X-100 to the phosphate-buffered saline was necessary, which was then diluted to 5 × 10⁻⁴% for ELISA determination.

ELISA of 3α-HSD/CR and TeiR—To quantify 3α-HSD/CR protein expression, an ELISA was established, and respective antibodies directed against 3α-HSD/CR from C. testosteroni were prepared in rabbits according to standard methods (28). ELISA plates were coated with protein extracts containing 3α-HSD/CR in coating buffer. After washing, antibodies against 3α-HSD/CR were added in a 1:1000 dilution. The secondary antibodies (peroxidase-conjugated swine anti-rabbit immunoglobulin) were used in a 1:1000 dilution. A further procedure corresponded to that of the chloramphenicol acetyltransferase ELISA kit from Roche Diagnostics. Comparison of the signals detected by ELISA and 3α-HSD/CR activity measured by high pressure liquid chromatography (23, 28, 29) always showed a good correlation (not shown). For TeiR detection, antibodies against TeiR were prepared in rabbits by immunization 3 times with 200 ng of purified protein. The secondary antibodies (peroxidase-conjugated swine anti-rabbit immunoglobulin) were used in a 1:1000 dilution. To separate bacterial membranes and cytoplasm, 3 ml of cells were centrifuged at 13,000 rpm for 10 s, and the pellet was resuspended in 100 μl of water. After the addition of 1 μl of lysozyme (10 mg/ml) and incubation for 30 min at 37 °C, the cells were lysed by freezing and thawing 3 times. The lysed cells were centrifuged at 13,000 rpm for 20 min. The supernatant represented the cytoplasm, and the pellet containing the membranes was resuspended in 100 μl of water.

Response of C. testosteroni to Different Steroid Inducers—Different steroids were tested for their ability to induce gene expression in wild type and teiR knock-out mutants of C. testosteroni. The gene coding for 3α-HSD/CR, hsdA, which is inducible by testosterone and which is not constitutively expressed (21, 23, 25), was used as an indicator gene in an ELISA system. SIN medium (3 ml) was inoculated with 100 μl of C. testosteroni wild type and teiR knock-out mutants (100 μl) and cultured for 4 h at 27 °C and 180 rpm to an A₉₀₀₅₀₀ of 0.6. A 15-μl solution of steroids (0.1 mM dissolved in ethanol) was then added to a final steroid concentration of 0.5 mM. After overnight culture, the cells were lysed for protein extraction, and 3α-HSD/CR expression was determined by ELISA.

Kinase Assay—Overnight cultures of C. testosteroni cells (500 μl) were harvested and centrifuged (13,000 rpm for 1 min), and the pellet was resuspended in 200 μl of water (A₉₀₀₅₀₀ of 2.0). After the addition of 2 μl of lysozyme solution (10 mg/ml) and freezing and thawing 3 times, the solution was centrifuged at 13,000 rpm for 20 min. The supernatant, representing the cytoplasm, was tested for kinase activity either alone or after addition of purified TeiR protein. The incubation mixture (total volume of 10 μl) consisted of 4 μg of cytoplasmic protein and 0.01 μl of [γ⁻³²P]ATP (10 mCi/ml) or 0.01 μl of [γ⁻³²P]GTP (10 mCi/ml) (Amersham Biosciences in 5 mM Tris-HCl, pH 7.4). Where appropriate, purified TeiR (dissolved in 1% sodium lauryl sarcosinate) was added. For stability determination of phosphorylated amino acids, 5 μl of 2 M HCl or 5 μl of 2 M NaOH were added. Samples were incubated for 30 min at room temperature without shaking. After another incubation time of 30 min at 43 °C, the samples were transferred onto 1-cm² pieces of Whatman No. 3MM paper. The pieces were put on Whatman No. 3MM stripes by adhesion and continuously rinsed by gravity (with a solvent containing 0.2 × TEN and 0.2% SDS). After 3 h of washing, the 1-cm² pieces were mixed with 3 ml of scintillation solution, and [γ⁻³²P]ATP or [γ⁻³²P]GTP were measured as cpm in a liquid scintillation counter (Wallac 1409).

RESULTS

Overexpression and Purification of TeiR—Cloning of the teiR gene from C. testosteroni strain ATCC 11996 into the vector pET-15b and subsequent overexpression in E. coli BL21(DE3)pLysS cells resulted in a TeiR protein with an N-terminal His-tag sequence. After induction with isopropyl-β-D-thiogalactoside, recombinant TeiR could be purified in one step using nickel-chelate chromatography. Here, the addition of 1% sodium lauryl sarcosinate as a detergent was critically important to remove urea (8 M) and to keep TeiR soluble and active, thereby indicating its hydrophobic nature (see below). The molecular mass of the recombinant protein (43.1 kDa) plus the His-tag sequence (2.2 kDa) as seen on the SDS-polyacrylamide gel (45.3 kDa) was identical to that predicted from the genomic sequence (Fig. 2). The purified protein was used to produce polyclonal antibodies in rabbits, to determine steroid binding specificity of TeiR, and to prove TeiR kinase activities.

Subcellular Localization of TeiR—To determine the subcellular localization of TeiR, we used three distinct approaches, Western blotting, fluorescence scanning microscopy, and ELISA (see below). For Western blotting, E. coli and C. testosteroni cells were transformed with plasmid pBBtac-teiR-GFP1, and cells expressing the TeiR-GFP fusion protein were fractionated by ultracentrifugation. Membranous and cytoplasmic fractions were separated by SDS-polyacrylamide gel
TeiR Is a Kinase That Drives Steroid Sensing and Metabolism

To elucidate the biological function of TeiR, two different knock-out strains of C. testosteroni were prepared by homologous integration, teiRkoI and teiRkoII (Fig. 1B). Both knock-out mutants were generated by teiR gene interruption with plasmid insertions that carry either a reading frameshift mutation of teiR (teiRkoI) or a non-functional fragment (teiRkoII). Knock-out plasmid insertions were ensured due the sensitivity of C. testosteroni to kanamycin and due to the kanamycin resistance gene of plasmid pK18, which was inserted into the chromosomal DNA during growth of C. testosteroni in the kanamycin-containing medium. In addition, plasmid integration into the chromosomal DNA was proven by Southern blot hybridization and PCR. The knock-out strains of C. testosteroni were used in subsequent studies for steroid binding, steroid transport, and swimming and twitching experiments (see below).

Is TeiR a Chemotaxis-sensing Protein?—The specific localization to the cell pole in C. testosteroni (Fig. 4) led us to investigate if TeiR was involved in chemotaxis to steroid substrates. Swimming and twitching motility assays revealed that wild type cells moved to the steroid substrate, whereas teiR knock-out cells could not (Fig. 5). Obviously, TeiR is required for C. testosteroni to respond to steroid substrate signals, which stimulate chemotaxis by swimming or twitching motility.

Specificity of Testosterone Binding to TeiR—To test the specificity of testosterone binding to TeiR, we incubated whole cells of C. testosteroni wild type and the two teiR knock-out strains with labeled [3H]testosterone. It could be shown that wild type cells, expressing TeiR, did bind higher amounts of testosterone than did teiR knock-out cells both after 5 and 30 min of incubation at room temperature (Fig. 6). The specificity of testosterone binding to TeiR was confirmed by competition with non-labeled (cold) testosterone to wild type cells (Fig. 6).

A similar experiment was performed to test [3H]testosterone uptake. After incubation of C. testosteroni wild type and teiR knock-out strains of C. testosteroni were prepared by homologous integration, teiRkoI and teiRkoII (Fig. 1B). Both knock-out mutants were generated by teiR gene interruption with plasmid insertions that carry either a reading frameshift mutation of teiR (teiRkoI) or a non-functional fragment (teiRkoII). Knock-out plasmid insertions were ensured due the sensitivity of C. testosteroni to kanamycin and due to the kanamycin resistance gene of plasmid pK18, which was inserted into the chromosomal DNA during growth of C. testosteroni in the kanamycin-containing medium. In addition, plasmid integration into the chromosomal DNA was proven by Southern blot hybridization and PCR. The knock-out strains of C. testosteroni were used in subsequent studies for steroid binding, steroid transport, and swimming and twitching experiments (see below).

Is TeiR a Chemotaxis-sensing Protein?—The specific localization to the cell pole in C. testosteroni (Fig. 4) led us to investigate if TeiR was involved in chemotaxis to steroid substrates. Swimming and twitching motility assays revealed that wild type cells moved to the steroid substrate, whereas teiR knock-out cells could not (Fig. 5). Obviously, TeiR is required for C. testosteroni to respond to steroid substrate signals, which stimulate chemotaxis by swimming or twitching motility.

Specificity of Testosterone Binding to TeiR—To test the specificity of testosterone binding to TeiR, we incubated whole cells of C. testosteroni wild type and the two teiR knock-out strains with labeled [3H]testosterone. It could be shown that wild type cells, expressing TeiR, did bind higher amounts of testosterone than did teiR knock-out cells both after 5 and 30 min of incubation at room temperature (Fig. 6). The specificity of testosterone binding to TeiR was confirmed by competition with non-labeled (cold) testosterone to wild type cells (Fig. 6).

A similar experiment was performed to test [3H]testosterone uptake. After incubation of C. testosteroni wild type and teiR
TeiR Is a Kinase That Drives Steroid Sensing and Metabolism

TeiR Is Necessary for 3α-HSD/CR Gene Expression in C. testosteroni—A variety of steroid compounds was tested for their ability to induce the expression of 3α-HSD/CR, which serves as an indicator of an enhanced steroid metabolism and an adaptation to steroids as carbon source of C. testosteroni. To prove the specific role of TeiR for 3α-HSD/CR induction, C. testosteroni wild type cells were compared with the teiR knockout strains teiRkoI and teiRkoII (Fig. 8). 17α-Hydroxyprogesterone, 11α-hydroxyprogesterone, 21α-hydroxyprogesterone, pregnenolone, androstendione, 1-dehydrotestosterone, 5-androsten-3β-17β-diol, deoxycorticosterone, and testosterone were shown to increase the expression of 3α-HSD/CR in C. testosteroni wild type cells. However, the same steroids were not able to induce hsdA expression in the teiR knockout mutants teiRkoI and teiRkoII (Fig. 8). These differences indicate some specific role of TeiR in C. testosteroni to respond to steroid substrates.

Solubilization of TeiR from the Plasma Membranes—In our studies it turned out that TeiR is a membrane-bound protein that can only be isolated in the presence of detergents. On the other hand, detergents are known to interfere with the ELISA assay. To find a suitable detergent and to determine the critical detergent concentration for TeiR solubilization and ELISA detection, Triton X-100, Tween 80, Tween 20, and SDS were used in concentrations between 3.125 × 10⁻⁴ and 0.01%. From all detergents tested, Triton X-100 at 3.125 × 10⁻⁴ and 6.25 × 10⁻⁴ % was best to solubilize and detect highest amounts of TeiR. Higher detergent concentrations led to a wash out of TeiR from the 96-well plates, and lower concentrations did not solubilize TeiR from the plasma membranes (not shown). Consequently, we used 5 × 10⁻⁴ % Triton X-100 in ELISA assays (see "Experimental Procedures").

TeiR Consists of Different Functional Domains—The E. coli genome does not contain the gene coding for TeiR, a fact that has been proven both by PCR and southern blotting (not shown). Subcloning of a variety of deletion fragments of the teiR gene (Fig. 1A) into E. coli followed by functional analyses of the resulting constructs revealed TeiR to consist of different domains for membrane association (Fig. 9), steroid binding (Fig. 10), and hsdA gene-inducing activity (Fig. 11). For example, TeiR constructs bearing the entire N terminus do appear in the cytoplasmic fractions only at basal levels (less than 1 µg of TeiR per mg of protein), whereas N-terminal-deleted constructs such as from plasmids pBBtacIII, pKN5teiR, pKN8teiR, pKPCR, and pKteiR5 concentrate at 2-fold higher levels in the cytoplasm as determined by ELISA (Fig. 9). On the other hand,
fragments lacking at least 20 amino acids in the central region of TeiR could not bind significant amounts of labeled testosterone, whereas fragments derived from pKteiR10, pKteiRBB, and pKteiR627 (bearing this region) bind considerable amounts of testosterone (Fig. 10). Finally, constructs with the complete C terminus led to an enhanced expression of the reporter gene hsdA (pKteiR10, pK5teiR, pK8teiR, and pKteiRdY), which was not the case with constructs lacking this region. It should be noted here that fragments derived from plasmids pKteiRC, pBBtacIII, and pBBtacIV did not show functionality despite the presence of the final C terminus, which might be due to the fact that these constructs lack the amino acids 209–250 upstream of the C terminus.

A synopsis of the specific functional domains is given in Fig. 12. Whereas the N-terminal part with amino acid residues 1–83 (corresponding to 1–249 bp) provides membrane localization, the central part with amino acid residues 189–209 (corresponding to 567–627 bp) is responsible for steroid binding, and the C-terminal part with amino acid residues 209–392 (corresponding to 627–1176 bp) ensures hsdA induction and, thus, functionality of TeiR. A hydrophobicity blot (36) of the TeiR primary structure was done in parallel (not shown) and confirms the presence of a hydrophobic region (amino acid residues 189–209), which obviously serves as the steroid binding pocket that was already identified with the TeiR deletion mutants.

TeiR Has Histidine Kinase Activity—Because signal perception in bacteria often occurs via intracellular phosphorylation cascades, it seemed reasonable to test if TeiR works as a kinase in the steroid signaling network in C. testosteroni. Phosphorylation of cytosolic proteins by TeiR was tested with 32P-labeled ATP or GTP. In addition, acid or alkali resistance of the phosphorylated proteins was tested by the addition of HCl or NaOH, respectively. As shown in Fig. 13, purified TeiR mediated protein phosphorylation in the presence of C. testosteroni cytoplasm when incubated with 32P-labeled GTP. TeiR was not able to use 32P-labeled ATP for its kinase activity (not shown). The specificity for 32P-labeled GTP was further proven with cold GTP in competition experiments. Interestingly, protein phosphorylation...
FIGURE 12. Synopsis of results proving three main functional domains of TeiR. Different plasmids harboring deleted mutant forms of the teiR gene (Fig. 1A) were transformed into E. coli HB101. Membrane association of TeiR (cf. Fig. 9), testosterone binding to TeiR (Fig. 10), and hsdA expression induced via TeiR (Fig. 11) revealed the presence of an N-terminal membrane binding domain, a central steroid binding site, and a C-terminal region important for activity of TeiR. Nd, not determined.

FIGURE 13. Histidine kinase activity of TeiR. Histidine kinase activity of TeiR was determined with purified TeiR (first column) and cytoplasm (second column) either alone or in combination (third column) by a filter-based method allowing detection of acid-labile phosphorylation of histidine residues (cf. “Experimental Procedures”). It appears that purified TeiR is able to catalyze the phosphorylation of cytosolic protein(s) in the presence of γ-[32P]ATP. The specificity for γ-[32P]GTP was proven by the lack of phosphorylation with [γ-32P]ATP (not shown) and by competition with non-labeled GTP (fourth column). Acid lability and base stability of the reaction was demonstrated by the addition of HCl (fifth column) or NaOH (sixth column), respectively.

was acid-labile but alkali-resistant (Fig. 13), consistent with phosphorylation at histidine residues. Combined, these results indicate that TeiR is a histidine kinase and uses GTP for protein phosphorylation.

DISCUSSION

In contrast to eucaryotic systems, the mechanism of steroid regulation (37) was largely unknown in bacteria until today. It has long been postulated that before their degradation, steroids require a signaling system that mediates their recognition and transportation through the bacterial plasma membrane as well as ensuring accumulation and catabolism of the steroid substrates in the cytoplasm of C. testosteroni (38). With two-dimensional electrophoresis we previously found that testosterone induced the expression of several steroid catabolizing enzymes in C. testosteroni ATCC11996 (21). We then focused on the regulation of the gene (hsdA) encoding 3α-HSD/CR, one of the enzymes being considered at the top of the steroid degradation pathway (21, 23, 39). We identified two genes coding for negative regulators of hsdA expression, repA and repB. Whereas RepA was found to block hsdA transcription, RepB was proven to interfere with hsdA translation (28, 29). Later, the teiR gene encoding a positive regulator of steroid-degrading enzymes, including 3α-HSD/CR, was identified in C. testosteroni ATCC11996 by transposon mutagenesis (30). Furthermore, Horinouchi et al. (14) reported on a bacterial steroid degradation cluster in C. testosteroni TA441, which is regulated by TesR, the latter being 98% identical to TeiR. Disrupted mutants of TeiR and TesR did not show induction of several steroid catabolizing enzymes, indicating that both proteins are positive regulators of steroid degradation. However, the mechanism by which TeiR or TesR regulate steroid catabolism in C. testosteroni remained obscure. In the present work we cloned the teiR gene and have identified the TeiR protein to mediate steroid sensing and signaling in C. testosteroni ATCC11996 via a kinase mechanism.

Cloning and overexpression of the teiR gene in E. coli resulted in a recombinant protein (Fig. 2) that was hardly soluble without detergents, thus giving a first indication on its hydrophobic nature. Purification and solubilization experiments with different subcellular fractions and different detergents revealed that TeiR might be localized in or attached to the plasma membrane of C. testosteroni. To conclusively infer its subcellular distribution, we produced a TeiR-GFP fusion protein and analyzed the localization of the fusion protein by Western blot and fluorescence microscopy. To our surprise, we found TeiR not only occurring within the plasma membrane of C. testosteroni (Fig. 3). Rather, it turned out that TeiR is asymmetrically concentrated at one of the poles of the bacterial cell (Fig. 4).

Asymmetrical cellular localization of proteins is often critical for their proper function (40), and polar localization is one striking example for proteins involved in chemotaxis (41). For example, in E. coli it appears that the chemotaxis proteins work cooperatively to generate a unified signal that coordinates the direction of flagellar rotation (41). To determine whether TeiR was involved in chemotaxis, teiR knock-out mutants of C. tes-
TeiR Is a Kinase That Drives Steroid Sensing and Metabolism

tosteroni were generated and compared with teiR wild type cells for their chemotactic ability to migrate in soft agar in the presence of testosterone. It turned out that teiR wild type cells, but not teiR knock-out mutants, migrated to the steroid source by means of their swimming and twitching motilities (Fig. 5). Swimming and twitching motility in teiR knock-out mutants could not be stimulated by testosterone. These data strongly suggest that TeiR is involved in chemotaxis of C. testosteroni in response to the steroid substrate.

Despite their relative lipophilicity, steroids require a transport system across bacterial membranes and to accumulate in the cytoplasm (38). We found that teiR wild type cells specifically bind $[^3H]$testosterone compared with teiR knock-out mutants (Fig. 6). Moreover, teiR knock-out mutants of C. testosteroni were not able to transport and degrade testosterone. Rather, whereas $[^3H]$testosterone accumulated in the culture medium of teiR knock-out cells, this labeled steroid almost completely disappeared in the culture medium of wild type cells (Fig. 7). Hence, TeiR is involved in steroid uptake and degradation in C. testosteroni.

Because 3α-HSD/CR plays a central role in the steroid degradation pathway in C. testosteroni, we used the expression of its gene, hsdA, as an indicator for an enhanced steroid metabolism and adaptation to steroids as carbon source via TeiR. To prove the specific role of TeiR for hsdA induction, C. testosteroni wild type cells were compared with two teiR knock-out strains. We found a severalfold increase in hsdA gene expression upon testosterone induction with teiR wild type cells compared with the respective null mutants (Fig. 8). Moreover, a variety of steroids other than testosterone was found to positively regulate hsdA expression via TeiR, namely 17α-hydroxyprogesterone, 11α-hydroxyprogesterone, 21α-hydroxyprogesterone, pregnenolone, androstanolone, 1-dehydrotestosterone, 5-androsten-3β,17β-diol, and deoxycorticosterone (Fig. 8). The same steroids were not able to induce hsdA expression in the teiR knock-out mutants. The fact that both of the C. testosteroni knock-out strains did not respond to any of the steroid compounds underlines the specific role of TeiR in steroid-dependent gene regulation in C. testosteroni.

In studies by Horinouchi et al. (14), TesR was described to function as a positive regulator of steroid degradation in C. testosteroni TA441. Northern blot analysis showed that TesR is necessary for induction of the steroid degradation gene cluster identified in C. testosteroni TA441. In addition, the TesR-disrupted mutant when incubated with androsta-1,4-diene-3,17-dione (ADD) exhibited little conversion of ADD, indicating that expression of ADD degradation genes is inhibited in the TesR-disrupted mutant.

According to its primary structure, the teiR gene product has a molecular mass of 43.1 kDa and consists of 391 amino acids. To characterize TeiR in more detail, we produced a variety of teiR gene deletion mutants (Fig. 1A) and performed functional analyses with these constructs in E. coli. We could show that the N terminus is important for membrane association of TeiR, since all N-terminal-deleted forms appeared to a considerably higher concentration in the cytosol (Figs. 9 and 12). Furthermore, we found that the central part of TeiR (amino acids 189–209) is responsible for steroid recognition and binding, since mutants deleted by these amino acids are not able to bind and to respond to testosterone (Figs. 10 and 12). This result was supported by a Kyte-Doolittle hydropathy plot, which predicted a hydrophobic steroid binding cleft between residues 189 and 209 of TeiR and which is identical to the positions of amino acids responsible for steroid binding identified by the deletion mutant analyses. Finally, by using the same constructs, it turned out that the C terminus is important for TeiR function, since all C-terminal-deleted constructs of TeiR did not lead to a steroid response within the bacterial cells (Figs. 11 and 12). Combined, TeiR can be subdivided into three sections, an N-terminal membrane attachment region, a central steroid recognition site, and a C-terminal domain for its gene regulatory function.

Adaptation to environmental changes is essential for cell survival in all organisms. Proteins that sense C. testosteroni to such environmental stimuli and transfer the external steroid signal to an intracellular switch of gene activation are, therefore, of great interest. Obviously, C. testosteroni utilizes a membrane-bound steroid receptor, TeiR, that transfers the signal into the cytoplasm via its histidine kinase activity. Histidine kinases catalyze the phosphorylation of either the 1- or 3-nitrogen of the imidazole ring of the histidine side chain (42).

It is believed that signal detection by the membranous sensor domain is propagated to the catalytic kinase core, which then undergoes conformational changes required for activity (43). In addition, the central part of TeiR (amino acids 192–258) contains a PAS (Per-Arnt-Sim) domain. PAS domains are commonly found in signaling proteins and appear to allow conversion of external stimuli into signals that propagate to downstream components by altering intra- and intermolecular protein-protein interactions (44). Interestingly, PAS domains are found in serine/threonine kinases and histidine kinases but also in photoreceptors and chemoreceptors for taxis and tropism. Many PAS domains bind cofactors or ligands, which are required for the detection of sensory signals (45). This conforms to findings with TeiR, in which the steroid binding region (amino acids 189–209) overlaps with the PAS region (amino acids 192–258).

Furthermore, downstream from the PAS domain a helix-turn-helix motif was identified in the TeiR sequence (amino acids 313–381). This motif is also present in multiple signaling contexts and is accordingly also referred to as HAMP domain (domain present in histidine kinases, adenyl cyclases, methyl accepting proteins, and phosphatases) (46). Fifteen of the 30 known E. coli histidine kinases and all 5 methyl-accepting chemotaxis proteins contain a single HAMP domain. Based on the requirement of the amphipathic nature of the HAMP domain helices, models have been postulated that predict that ligand binding alters the confirmation of histidine kinases finally leading to protein-protein interactions thereby modulating histidine kinase function (43).

Based on our results we hypothesize that the mechanism of TeiR-dependent gene regulation occurs via its kinase activity, which kicks off a cascade of further phosphorylation events that results in induced expression of steroid catabolizing enzymes. The identification of the phosphorylated target protein(s) or components of the kinase signaling cascade that trigger hsdA induction is subject of our further investigation.
TeiR Is a Kinase That Drives Steroid Sensing and Metabolism

Overall, we propose the following model of TeiR function in C. testosteroni. TeiR, which is assymmetrically localized in the membrane at the cell pole of C. testosteroni, undergoes a conformational change upon binding of appropriate steroids such that its kinase moiety is activated. This kinase activation leads to the enhanced expression of steroid catabolic genes, which enables this bacterium to use steroids as carbon and energy source. The same sensing mechanism may trigger chemotaxis of C. testosteroni to the steroid substrate. Hence, TeiR can be considered as a major player in the steroid dependent signaling network in C. testosteroni.

Acknowledgments—We thank Dr. C. Plieth for kindly providing anti-GFP antibodies and the Centre of Molecular Biology and K. Thierbach for helping with confocal laser scanning microscopy.

REFERENCES
1. Tamaoka, J., Ha, D. M., and Komagata, K. (1987) *Int. J. Syst. Bacteriol.* **37**, 52–59
2. Marcus, P. I., and Talalay, P. (1956) *J. Biol. Chem.* **218**, 661–674
3. Ahmad, D., Masse, R., and Sylvestre, M. (1990) *Can. J. Microbiol.* **36**, 5275–5281
4. Kieslich, K. (1985)
5. Horinouchi, M., Kurita, T., Yamamoto, T., and Kudo, T. (2003) *J. Bacteriol.* **175**, 551–559
6. Busse, H. J., el Banna, T., Oyaizu, H., and Auling, G. (1992) *Int. J. Syst. Bacteriol.* **42**, 19–26
7. Arda, B., Aydemir, S., Yamazhan, T., Hassan, A., Tunger, A., and Serter, D. (2003) *APMS* **111**, 474–476
8. Koolman, J. (1990) *Prog. Clin. Biol. Res.* **342**, 704–709
9. Arai, H., Igarashi, Y., and Kodama, T. (1991) *Biochem. Biophys. Res. Commun.* **183**, 395–400
10. Flotin, C., Kohler, T., Grandgirault, M., and Plesiat, P. (1996) *J. Bacteriol.* **178**, 3322–3330
11. Khorchid, A., and Ikura, M. (2006)
12. Oppermann, U. C. T., Belai, I., and Maser, E. (1996) *J. Steroid Biochem. Mol. Biol.* **58**, 217–223
13. Oppermann, U. C. T., and Maser, E. (1996) *Eur. J. Biochem.* **241**, 744–749
14. Hefti, M. H., Francoijs, K. J., de Vries, S. C., Dixon, R., and Vervoort, J. (1997) *Trends Biochem. Sci.* **22**, 331–333
15. Hefti, M. H., Francoijs, K. J., de Vries, S. C., Dixon, R., and Vervoort, J. (2004) *Eur. J. Biochem.* **271**, 1198–1208
16. Gibson, D. T., Wang, K. C., Sih, C. J., and Whitlock, H., Jr. (1966) *J. Biol. Chem.* **241**, 551–559
17. Sih, C. J., Lee, S. S., Tsong, Y. Y., and Wang, K. C. (1966) *J. Biol. Chem.* **241**, 540–550
18. Sih, C. J., Wang, K. C., and Tai, H. H. (1968) *Biochemistry* **7**, 796–807
19. Coulter, A. W., and Talalay, P. (1968) *J. Biol. Chem.* **243**, 3238–3247
20. Skowasch, D., Möbus, E., and Maser, E. (2002) *Biochem. Biophys. Res. Commun.* **294**, 560–566
21. Möbus, E., Jahn, M., Schmid, R., Jahn, D., and Maser, E. (1997) *J. Bacteriol.* **179**, 5951–5955
22. Florin, C., Kohler, T., Grandgirault, M., and Plesiat, P. (1996) *J. Bacteriol.* **178**, 3322–3330
23. Möbus, E., and Maser, E. (1998) *J. Biol. Chem.* **273**, 30888–30896
24. Oppermann, U. C. T., Belai, I., and Maser, E. (1996) *J. Steroid Biochem. Mol. Biol.* **58**, 217–223
25. Oppermann, U. C. T., and Maser, E. (1996) *Eur. J. Biochem.* **241**, 744–749
26. Horinouchi, M., Hayashi, T., Koshino, H., Yamamoto, T., and Kudo, T. (2003) *Appl. Environ. Microbiol.* **69**, 2139–2152
27. Oppermann, C. T., Netter, K. J., and Maser, E. (1993) *Adv. Exp. Med. Biol.* **328**, 379–390
28. Xiong, G., and Maser, E. (2001) *J. Biol. Chem.* **276**, 9961–9970
29. Xiong, G., Martin, H. J., and Maser, E. (2003) *J. Biol. Chem.* **278**, 47400–47407
30. Pruneda-Paz, J. L., Linares, M., Cabrera, J. E., and Genti-Raimondi, S. (2004) *J. Biol. Chem.* **281**, 13430–13437
31. Kovach, M. E., Elzer, D. P., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., and Peterson, K. M. (1995) *Gene (Amst.)* **166**, 175–176
32. Ara, H., Igarashi, Y., and Kodama, T. (1991) *Agric. Biol. Chem.* **55**, 2431–2432
33. Sambrook, J., and Russel, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor Laboratory, New York
34. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
35. Laemmli, U. K. (1970) *Nature* **227**, 680–685
36. Kye, J., and Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132
37. Beato, M. (1989) *Cell* **56**, 335–344
38. Watanabe, M., and Po Sy, L. (1974) *Biochim. Biophys. Acta* **391**, 419–429
39. Boyer, J., Baron, D. N., and Talalay, P. (1965) *Biochemistry* **4**, 1825–1833
40. Lybarger, S. R., and Maddock, J. R. (2001) *J. Bacteriol.* **183**, 3261–3267
41. Maddock, J. R., and Shapiro, L. (1993) *Science* **259**, 1717–1723
42. Stock, J. B., Stock, A. M., and Mottonen, J. M. (1990) *Nature* **344**, 395–400
43. Zhulin, I. B., Taylor, B. L., and Dixon, R. (1997) *Trends Biochem. Sci.* **22**, 331–333
44. Horinouchi, M., Hayashi, T., Hatori, E., Hayashi, T., and Kudo, T. (2004) *Biochem. Biophys. Res. Commun.* **324**, 597–604
45. Oppermann, U. C. T., Belai, I., and Maser, E. (2003) *J. Steroid Biochem. Mol. Biol.* **92**, 143–154
46. Gibson, D. T., Wang, K. C., Sih, C. J., and Whitlock, H., Jr. (1966) *J. Biol. Chem.* **241**, 551–559
47. Boyer, J., Baron, D. N., and Talalay, P. (1965) *Biochemistry* **4**, 1825–1833
48. Lybarger, S. R., and Maddock, J. R. (2001) *J. Bacteriol.* **183**, 3261–3267
49. Maddock, J. R., and Shapiro, L. (1993) *Science* **259**, 1717–1723
50. Stock, J. B., Stock, A. M., and Mottonen, J. M. (1990) *Nature* **344**, 395–400
51. Khorchid, A., and Ikura, M. (2006) *Int. J. Biochem. Cell Biol.* **38**, 307–312
52. Zhulin, I. B., Taylor, B. L., and Dixon, R. (1997) *Trends Biochem. Sci.* **22**, 331–333
53. Hefti, M. H., Francoijs, K. J., de Vries, S. C., Dixon, R., and Vervoort, J. (2004) *Eur. J. Biochem.* **271**, 1198–1208
54. Aravind, L., and Ponting, C. P. (1999) *FEMS Microbiol. Lett.* **176**, 111–116